Published in final edited form as:

Wiley Interdiscip Rev RNA. 2016 March ; 7(2): 186–197. doi:10.1002/wrna.1324.

Bioengineering of noncoding RNAs for research agents and therapeutics

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Abstract

The discovery of functional small noncoding RNAs (ncRNAs), such as microRNAs and small interfering RNAs, in the control of human cellular processes has opened new avenues to develop RNA-based therapies for various diseases including viral infections and cancers. However, studying ncRNA functions and developing RNA-based therapeutics relies on access to large quantities of affordable ncRNA agents. Currently, synthetic RNAs account for the major source of agents for RNA research and development, yet carry artificial modifications on the ribose ring and phosphate backbone in sharp contrast to posttranscriptional modifications present on the nucleobases or unmodified natural RNA molecules produced within cells. Therefore, large efforts have been made in recent years to develop recombinant RNA techniques to cost-effectively produce biological RNA agents that may better capture the structure, function, and safety properties of natural RNAs. In this article, we summarize and compare current *in vitro* and *in vivo* methods for the production of RNA agents including chemical synthesis, *in vitro* transcription, and bioengineering approaches. We highlight the latest recombinant RNA approaches using transfer RNA (tRNA), ribosomal RNA (rRNA), and optimal ncRNA scaffold (OnRS), and discuss the applications of bioengineered ncRNA agents (BERAs) that should facilitate RNA research and development.

INTRODUCTION

Small noncoding RNAs (ncRNAs) have emerged as a big class of key epigenetic factors in the regulation of gene expression over the past two decades. A broad spectrum of ncRNAs have been uncovered, namely, microRNAs (miRNAs or miRs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). These small RNAs (sRNAs) are mainly involved in gene silencing in the cell through RNAi interference (RNAi) mechanisms after incorporation into the miRNA- or siRNA-induced silencing complex (RISC)^{1–4}. The discovery of RNAi has enabled researchers to exploit various sRNA tools to control target gene expression, delineate gene function, and investigate cellular signaling and networks. In addition, many sRNAs control transcriptome dynamics and proteome outcomes underlying vital cellular processes, which are often distorted in cancer or other diseases. Therefore, restoration of sRNA expression/function or direct silencing of a druggable target with

antisense RNAs or RNA aptamers may be developed as novel RNA-based therapies (Figure 1)^{4–7}.

Several nucleic acid-based drugs have been approved by the United States Food and Drug Administration (FDA) for the treatment of various types of diseases (Table 1). Fomivirsen, a 21-nt phosphorothioate oligonucleotide complementary to human cytomegalovirus (CMV) mRNA to inhibit CMV amplification, is the first-in-class antisense RNA therapy that was approved by the FDA in 1998 for the treatment of CMV retinitis⁸. Alicaforfen is another antisense oligonucleotide approved as an orphan drug for the treatment of pouchitis by mechanistically acting on intercellular adhesion molecule 1 (ICAM-1) mRNA to block the production of ICAM-1 protein⁹. Mipomersen is the most recent antisense drug approved by the FDA for the treatment of familial hypercholesterolemia, which is a 20-nt oligonucleotide targeting the mRNA of apolipoprotein B¹⁰. In addition, RNA aptamers may directly bind to protein targets (Figure 1) to produce pharmacological effects. Pegaptanib is the first-in-class RNA aptamer drug approved by the FDA in 2004 for the treatment of age-related macular degeneration (AMD), which directly acts on the vascular endothelial growth factor (VEGF) protein¹¹ (Table 1). The outlined number of approved RNA drugs demonstrates the feasibility of RNA agents as a new class of rapidly developing therapeutics.

With the development of new carriers and platforms for the delivery of nucleic acids, many RNA-based drug candidates are currently under clinical trials (Table 1). Patisiran is an intravenously administered, lipid nanoparticle (LNP)-formulated, 25-bp siRNA agent targeting Transthyretin (TTR) mRNA, and is in Phase 3 clinical trials for the treatment of TTR-mediated amyloidosis¹². ALN-PCS is another siRNA agent designed to block the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) to treat hypercholesterolemia¹³. A PEGylated, 40-nt L-RNA aptamer, NOX-E36 (Emapticap pegol), is able to bind and inhibit chemokine C-C motif ligand 2 (CCL2 or monocyte chemoattractant protein-1 MCP-1) and relieve complications arising from diabetic nephropathy for diabetic patients with proteinuria¹⁴. In addition, there are also several miRNA-based therapies under clinical investigations. Miravarsen is a 15-nt phosphorothioate locked nucleic acid (LNA) oligo complementary to hepatic miR-122-5p to inhibit hepatitis C virus (HCV) amplification, which was shown to reduce HCV RNA levels in a dose-dependent manner among patients with chronic HCV genotype 1 infection¹⁵. MRX34, a liposome formulated miR-34a mimic showing anticancer activity against various types of tumors through acting on multiple oncogenes, has entered into Phase 1 clinical trials to treat unresectable primary liver cancer⁷. Despite the efficacy of RNA drug candidates detailed above, delivery of sRNAs remains as a major challenge in clinical investigation^{16, 17}. However, the discovery of new candidates and progression towards therapy are also hindered by current RNA production systems, highlighted in detail in this review.

Research on sRNA functions and development of RNA-based therapies rely on the access to large quantities of highly homogeneous RNA agents or recombinant DNA agents. The use of DNA materials (e.g., viral or non-viral vector-based ncRNA expression plasmids) may complicate the RNA-based processes because this approach is not only dependent upon the efficiency of gene delivery but also the host cells or organisms to transcribe the target

sequences to ncRNAs before exercising ncRNA actions. To date, RNA agents are commonly produced *in vitro* through chemical synthesis¹⁸ or transcription with recombinant T7 RNA polymerase^{19, 20}. However, such RNA agents either consist of excessive artificial modifications (Figure 2) or do not carry necessary posttranscriptional modifications found in naturally occurring RNAs, which may alter folding properties, biological activity, and safety profiles. Therefore, there are increasing interests in developing *in vivo* fermentation-based approaches that would allow large-scale production of biological ncRNA agents (e.g., multi-milligram quantities of pure recombinant ncRNA from one liter bacterial culture) properly folded in the cells and consisting of natural modifications (Figure 2) critical for its stability, activity, and safety. Herein we provide an overview of *in vitro* and *in vivo* RNA production methods, and highlight the latest recombinant RNA approaches^{21–25} and the applications of bioengineered ncRNA agents (BERAs) to research and development.

CHEMICAL SYNTHESIS OF RNA AGENTS

Standard phosphoramidite chemistry is the most commonly used and has been automated for the production of oligonucleotides¹⁸. Different from the production of DNA oligonucleotides, chemical synthesis of RNA agents requires additional protection for the 2'-hydroxy group, e.g., with t-butyldimethylsilyl (TBDMS) or tri-iso-propylsilyloxymethyl (TOM) group that may be removed following the treatment with fluoride. While the success of chemical production depends upon the yields of sequential deprotection-coupling-oxidation reactions, target RNAs are often purified by high performance liquid chromatography (HPLC) to the desired purity and thus widely commercially available. These synthetic RNAs may contain additive features (e.g., an improved stability or selective fluorescence property), and thus have shown a broader range of applications (e.g., as therapeutic or imaging agents).

Currently, many artificial modifications are introduced into the RNA molecule to enhance metabolic stability and/or increase binding affinity²⁶. Common chemical modifications include phosphorothioate (PS) backbone and a series of ribose substitutions (e.g., 2'-O-methyl, 2'-fluoro, 2'-O-methoxyethyl, and LNA; Figure 2). The PS backbone modification replaces the nonbridged oxygen atom with sulfur, which improves resistance to nucleolytic degradation and increases affinity of the oligonucleotide for plasma proteins to hinder renal clearance^{27, 28}. As PS backbone modification is relatively simple and inexpensive, this chemical modification is widely found in antisense therapeutics as well as siRNA, miRNA and RNA aptamer agents²⁹. Many substitutions are also found at the 2'-hydroxyl position such as 2'-O-methyl, 2'-fluoro, 2'-O-methoxyethyl groups to increase RNA stability and potency. LNAs are introduced to form a bridge between the 2' oxygen and 4' carbonyl, which "locks" the ribose in A-form position to enhance base stacking interactions and hybridization properties³⁰. Although synthetic oligonucleotides with chemical modifications exhibit some enhanced pharmacokinetic properties, the extent of off-target effects and/or alteration of structural and functional properties remain obscure.

In this regard, natural modifications are most often identified on the nucleobases while chemical modifications are introduced on the sugar or phosphate backbone (Figure 2). With more than 100 different natural RNA modifications presently known, the diversity of these

modifications may have a profound effect on RNA dynamics and functions^{31–34}. Our knowledge of the most common posttranscriptionally modified nucleosides such as pseudouridine, 1-methyladenosine, 7-methylguanosine, and 5-methylcytidine (Figure 2) stem from altering folding patterns on tRNA^{35, 36}. Despite the influence of posttranscriptional modifications of other RNA species is relatively less understood, it has been speculated that nucleoside modifications may allow the host immune system to differentiate between self and pathogenic RNA³⁷. It has been revealed that a variety of toll-like receptors (TLRs) are responsible for the response to nucleic acids. For example, double stranded RNA triggers TLR3 in response to viral intermediates³⁸. Subtle differences in sequence and structure activate distinct families of TLRs^{39–41}, which again hint at the importance of RNA modifications. This defense mechanism likely has a more profound effect in mammals, as mammalian RNAs are modified to a greater extent than bacterial RNAs, although there are both unique and shared modifications between the two.

While chemical synthesis provides the capability to add an array of modifications to target RNAs, natural RNA modifications may not be placed at the desired positions with satisfactory high yields. Furthermore, the cost of synthetic RNA increases quickly with a parallel increase in RNA length and number of modifications. In addition, synthetic RNA agents that surpass these multiple requirements are usually provided in micromolar scale, which is far from milligram quantities needed for animal studies and clinical investigations. In short, although chemical synthesis is a practical approach and suitable for the production of sRNA agents, the artificial modifications might alter RNA structural dynamics, stability, biological activity and safety profiles, and a high cost may be tagged to chemical synthesis of milligram quantities of longer RNA agents.

IN VITRO TRANSCRIPTION AND ENZYMATIC PRODUCTION OF RNA AGENTS

In vitro transcription is a widely used biochemical approach to produce single stranded RNA agents^{19, 20}. This approach is based on the capacity of RNA polymerase (RNAP) to specifically construct RNA molecules using DNA sequences under a particular promoter. Therefore, *in vitro* transcription relies on the construction of a DNA template, while efficiency depends on the appropriate promoter driving the coding sequence of target RNA (e.g., T7 promoter) and the fidelity of purified RNAP (e.g., T7 polymerase) to transcribe the template *in vitro*. A linearized plasmid DNA is commonly used as the template to allow run off transcription, however synthetic oligonucleotides and PCR products may also be utilized. In addition, various methods such as preparative polyacrylamide gel electrophoresis and anion exchange fast protein liquid chromatography (FPLC) may be employed to purify the RNA products from transcription reactions.

The greatest advantage of *in vitro* transcription by T7 polymerase is in the simplicity of this method, as well as the versatility to generate RNA molecules at variable lengths for different applications including *in vitro* translation, detection assays, structural and functional studies (Table 2). Despite *in vitro* transcription has been well established and many commercialized kits are available for the production of RNA agents, certain RNA products display heterogeneity at 3' and 5' ends⁴². The reliability of T7 RNA polymerase regresses as

transcript length increases, usually resulting in the addition of untemplated nucleotides to the 3' end⁴³. Next Generation Sequencing study on a T7 RNAP-generated mRNA pool has also revealed a relatively high level of template-dependent transcriptional infidelity⁴⁴. In addition, RNA species generated from *in vitro* transcription do not consist of necessary natural modifications because of the absence of posttranscriptional modification machineries. Nevertheless, *in vitro* transcribed single stranded RNAs may be annealed to give double stranded RNAs and thus directly used for RNAi studies^{45, 46}, and the double stranded RNAs may be further processed into siRNAs by recombinant Dicer to knock down target genes⁴⁷. Dicer-mediated cleavage may be combined with *in vitro* transcription as a one-pot enzymatic reaction to generate functional siRNA agents⁴⁸. Such enzymatic approaches have also been demonstrated for successful production of siRNAs from synthetic RNAs using recombinant bacterial RNase III⁴⁵.

IN VIVO PRODUCTION OF RNA AGENTS

There are growing interests in developing new, fermentation-based strategies to cost-effectively produce ready-to-use ncRNA agents on a large scale. The resulting biological ncRNA may carry necessary posttranscriptional modifications critical for its secondary structure, folding, and stability. However, *in vivo* expression of recombinant RNAs is generally known to be inefficient because of a high susceptibility of heterogeneous RNA molecules to various RNases within the host cells. Therefore, recombinant RNAs should be presented as stable molecules, “masked” as endogenous RNA entities or “protected” within a stable complex to achieve efficient production. Indeed, a variety of target RNAs have been successfully produced by fermentation when “protected” by siRNA-binding protein⁴⁹ or “carried” by stable RNA scaffolds^{21–25}, and genetically engineered RNAs represent a new class of biological RNA agents for research and development.

Use of siRNA-Binding Protein

One approach reported for *in vivo* production of fully-processed, ready-to-use siRNA agents is based on ectopic expression of a 19 kD siRNA-binding protein, p19, in *E. coli*⁴⁹. The p19, encoded by a plant virus *tombusvirus*, is known to selectively bind to double stranded siRNAs with high affinity and suppress gene silencing^{50, 51}. It was also shown that recombinant p19 protein could be utilized for the isolation/detection of siRNAs/miRNAs⁵². When p19 was expressed in *E. coli*, this siRNA-binding protein stabilized those ~21-nt double stranded siRNA species produced by bacterial RNase III⁴⁹. Therefore, a plasmid was constructed for the co-expression of p19 protein and long hairpin RNA in bacteria. The long hairpin RNA transcript was processed by bacterial RNases to target siRNA, which was able to bind to co-expressed p19 protein. The resulting siRNA-p19 complex consisting of a histidine tag was then purified by nickel affinity chromatography. Following the isolation of siRNA-p19 complex, target siRNA species were purified by anion exchange HPLC and were able to efficiently and selectively knock down target gene expression in mammalian cells⁴⁹. Nevertheless, the yield was found to be low (e.g., 10–80 µg per liter bacterial culture), which may be related to the expression yield of p19 and capacity of p19 to load target siRNAs, as well as multiple steps for the purification of both siRNA-protein complex and siRNAs.

Transfer RNA Scaffold

Transfer RNA (tRNA) is the simplest stable RNA in cells, ranging from 76 to 90 nt in length. Although small, tRNAs contain an extensive number of posttranscriptional modifications totaling around 50% of all bases in the entire tRNA molecule^{53–55}. Nucleobases found near the TΨC and D arms and anticodon loop are heavily modified as these modifications designate secondary structure folding patterns, leading to stable stem loop structures. The stable three-dimensional four-leaf clover structure of tRNA enables resistance to heat-denaturation and nuclease cleavage⁵⁶. Indeed, successful overexpression of elongator methionine-tRNA (tRNA^{met}) in *E. coli* using a strong lipoprotein (*lpp*) gene promoter and a ribosomal RNA operon transcription terminator (*rrnC*) was reported in 1988, and the recombinant tRNA^{met} was purified by HPLC method to give 1–2 mg tRNA^{met} from 1 liter bacterial culture⁵⁷. This strategy was further extended to transfer-messenger RNA (tmRNA, 10Sa RNA or ssrA) with tRNA and mRNA properties⁵⁸. It was estimated that recombinant tmRNA tRNA-like domain (TLD) accounted for approximately 5% of total bacterial tRNAs, and 1–3 mg of tmRNA-TLD may be isolated from 1 liter bacterial culture by gel-filtration and ion-exchange chromatography methods to 85–90% purity. These recombinant tmRNA-TLD agents were thus used for structural analyses.

Use of tRNA as a scaffold (Figure 3) for *in vivo* production of large quantities of RNA molecules (e.g., milligrams from 1 liter bacterial culture) was first reported in 2007²². The RNA of interest was substituted at the anticodon region, while the acceptor, TΨC and D stems were retained to maintain the tertiary structure of tRNA. The recombinant RNA chimera are presumably recognized by the cells as tRNA species and modified by ribonuclease P to add -CCA at the 3' ends. As a result, the recombinant RNAs are able to escape RNase degradation and accumulate in *E. coli* to significantly high levels. The tRNA-based recombinant RNA approach has been employed to the production of many target RNAs including an epsilon sequence of human hepatitis B virus (HBV), *E. coli* 23S rRNA, RNA aptamers, hammerhead riboswitch RNAs, and pre-miRNAs^{21, 22, 25, 59–63}, as well as co-expression of RNA-protein complexes⁶⁴. The recombinant RNA chimeras are thus purified, and the target RNA species may be released on demand by corresponding RNase or ribozyme for structural and biophysical analyses or directly utilized for imaging studies. While the tRNA-scaffold approach shows great promise, the success and efficiency in producing chimeric RNAs largely relies on the structure and metabolic stability of target RNAs in bacteria or other expression systems. It is obvious that any target RNAs labile to bacterial RNase digestion is undoubtedly subject to nucleolytic cleavage and thus there will be limited to no accumulation of recombinant RNAs^{24, 63}. In addition, lower levels of recombinant RNA expression have been consistently associated with longer chimeric RNA species^{21, 22, 61–63}. This is presumably due to an increase of unstructured regions that are misfolded and/or cleaved by bacterial RNases.

Ribosomal RNA Scaffold

Ribosomal RNAs (rRNAs) are the most abundant RNA species in all cells and comprise 80% of total RNAs in rapidly growing cells. Bacteria possess three distinct rRNA components: the 16S small ribosomal subunit, and the 5S and 23S components, which together form the large subunit. Due to the abundance and stability of ribosomal RNAs in *E.*

coli, the 5S rRNA was shown to be strongly expressed under the control of *E. coli* *rrnB* promoter and *rrnB* T1 and T2 terminators^{65–67}. Thus the 5S rRNA was exploited to serve as a scaffold for the production of recombinant ncRNA species (Figure 3)^{23, 68}. Specifically, a 71 nt artificial 3 $\times$ *pen* RNA was inserted into stem II and completely replaced the stem III loop B and C regions of 5S rRNA, which maintained the highly structured rRNA intact, and cleavable by DNazymes. Using a similar plasmid-based template, the 5S rRNA chimera was placed under two rRNA gene promoters, *rrnB* P1 and P2 followed by *rrnB* T1 and T2 transcription terminators. The plasmid was transformed in *E. coli*, and the chimeric RNAs (160 nt) were purified by preparative polyacrylamide gel electrophoresis and cleaved with DNazymes to excise desired RNA fragments (71 nt) from the 5S rRNA scaffold.

A novel aspect incorporated into this rRNA-based approach is sequence specific excision of target RNA using DNazymes. DNazymes or deoxyribozymes can be designed to cleave specific dinucleotides between the 5S rRNA scaffold and target RNA. Furthermore, 5'-biotinylated DNazymes may be recovered through affinity capture for renewable use. The overall yield of this method can reach up to 90% of desired RNA product after processing, resulting in an average of 2 mg target RNA per gram of wet bacterial cells. In addition, the 5S rRNA scaffold not only accommodates RNA inserts under 80 nt at stem II²³ but also adopts various ssRNAs to stem II and III⁶⁸. While other regions of the 5S rRNA such as stems IV and V may be exploited, the stability of this insertion site has not been tested. Furthermore, it has not been reported if recombinant rRNA chimeras consist of any posttranscriptional modifications.

Optimal Noncoding RNA Scaffold (OnRS)

Motivated by the idea to use biological RNAs to perform RNA actions, we sought to produce pre-miRNA agents for research and development through genetic engineering. It was unsurprising to find out that bare pre-miRNAs were either not expressed in bacteria or not accumulated to substantial levels (e.g., recombinant pre-miR-34a accounted only about 1–3% of total RNAs in bacteria; unpublished data). The addition of a tRNA scaffold to pre-miRNAs generally increased the yields, whereas most target tRNA/pre-miRNA chimeras were rather surprisingly not or minimally expressed^{24, 63}. Nevertheless, several tRNA/pre-miRNA chimeras (e.g., tRNA/mir-34a and tRNA/mir-1291) were able to accumulate in bacteria to significantly high levels (e.g., 10–20% of total RNAs)^{24, 25, 62}. Most importantly, our studies demonstrated that chimeric tRNA/pre-miRNAs, while showing a favorable cellular stability, were selectively processed to mature miRNAs in various types of human carcinoma cells, which consequently modulated target gene expression and cellular processes. Based on these high-yield expressing tRNA fusion pre-miRNAs (e.g., tRNA/mir-34a), we thus developed an optimal ncRNA scaffold (OnRS) approach for the production of recombinant ncRNAs, in which the miRNA-5p/miRNA-3p duplex within the OnRS cargo may be replaced by 17- to 25-nt double-stranded RNA (dsRNA) of interest cleavable by Dicer (Figure 3)²⁴. The OnRS also offers the flexibility to carry variable lengths of single-stranded RNA (ssRNA) molecules at various sites, e.g., 5' or 3' of pre-miRNA (Figure 3).

The coding sequences of target OnRS/sRNAs are thus cloned into a target plasmid and transformed in a common *E. coli* strain (e.g., HST08) (Figure 4). After fermentation at 37°C for an optimal period, total RNAs are isolated and expression of recombinant ncRNAs is verified by denaturing urea polyacrylamide gel electrophoresis. Target BERAs are then purified to a high degree of homogeneity (> 95%) by anion exchange FPLC method, which may be subjected to structural characterization including posttranscriptional modifications, as well as preclinical and clinical investigation of biological fate, activity, efficacy and safety properties (Figure 4).

The OnRS strategy has allowed us to achieve a consistent high-yield (e.g., >10% of total RNAs) and large-scale (multi to tens milligrams of ncRNAs per liter of bacterial culture) production of BERAs bearing various types of sRNAs including miRNAs (e.g., miR-124, miR-27b and miR-22, etc.), siRNAs (e.g., siRNA against green fluorescent protein (GFP) and scrambled siRNA control, etc.), and RNA aptamers (e.g. malachite green aptamer (MGA), and vascular endothelial growth factor (VEGF) aptamer, etc.)²⁴. Mass spectrometry-based analyses revealed that the tRNA scaffold indeed comprised a number of posttranscriptionally modified nucleosides that are critical for tRNA stability. It is also noteworthy that these miRNAs/siRNAs differs much in length (e.g., 20, 21 and 22 nt) and arm of origin (both 5' and 3') and ssRNAs can be assembled to both 5' and 3' of pre-miRNA (Figure 3), indicating the robustness and versatility of OnRS to accommodate various miRNAs/siRNAs and ssRNAs of interest.

APPLICATIONS OF BIOENGINEERED RNA AGENTS

BERAs produced in cells through fermentation are expected to be more relevant to natural RNAs for biological investigations. Extensive studies have been conducted to elucidate the structural features of BERA species using various techniques including nuclear magnetic resonance^{22, 58, 61}, mass spectrometry^{25, 62}, and X-ray crystallography⁵⁸, which have revealed the presence of substantial posttranscriptional modifications on the tRNA scaffold. These findings not only confirm the structural difference between natural and synthetic RNAs but also enhance our knowledge of RNA structures. Furthermore, fully-processed siRNAs isolated from p19 complex formed in the bacterial systems are biologically active in silencing target gene expression in mammalian cells⁴⁹. In addition, chimeric RNA aptamers/sensors isolated from *E. coli* systems are also functional and have been successfully applied to imaging, diagnostic and therapeutic investigations^{24, 25, 59, 60, 65}.

Acting as a “prodrug”, the OnRS/sRNA agent may be directly utilized for various preclinical investigations (Figure 4). In this regard, the OnRS not only offers a high-yield and large-scale production of target sRNA in *E. coli* but also serves as a carrier for the delivery of sRNA to mammalian cells. Indeed, both unbiased RNA sequencing study and targeted quantitative real-time PCR analysis have demonstrated a selective production of target sRNA agents (e.g., miR-34a, miR-124, GFP-siRNA, and scrambled sRNA) from the chimeric ncRNA (e.g., OnRS/miR-34a, OnRS/miR-124, OnRS/GFP-siRNA, and OnRS/scrambled-sRNA, respectively) in human cells and animals^{24, 25}. Meanwhile, the tRNA segments within OnRS/siRNAs and OnRS/miRNAs as well as the control tRNA scaffold and OnRS/scrambled-sRNA were processed to the same tRNA fragments to similar levels.

Consequently, OnRS-carried siRNAs/miRNAs were able to selectively reduce target gene expression (e.g., Signal transducer and activator of transcription 3 (STAT3) by OnRS/miR-124, and GFP by OnRS/GFP-siRNA) *in vitro* and *in vivo*. While it is debatable whether there is any “golden” standard or reference RNAi agent/method to define the efficiency and/or selectivity for other RNAi agents, our data showed that the same concentrations of biological OnRS- or tRNA-carried miRNAs/siRNAs were more effective than synthetic miRNA/siRNA agents in the regulation of target gene expression and cellular processes^{24, 25}. In addition, BERAs were well tolerated in mouse models, as manifested by the absence or minimal change of mouse blood chemistries and chemokine IL-6 levels²⁵. With this robust OnRS approach available, a library of functional sRNA agents may be produced and fed into high-throughput screening for the desired phenotype (Figure 5) and identification of more effective and safe BERAs for further development.

CONCLUSIONS AND PERSPECTIVES

Small ncRNAs are gaining ground in advancing our understanding of gene functions, and there are also a significant number of RNA-based drugs currently in clinical use or under clinical development. While sRNA research and development is mainly based on the use of synthetic sRNA agents with excessive artificial modifications, the full physicochemical, functional and safety profiles of genome-derived sRNAs may not be represented. In addition, there is a limited access to large quantities of sRNAs that hampers rather much needed investigations in animal models and humans. Therefore, the development of large-scale and cost-effective fermentation-based recombinant RNA approaches may offer a more suitable platform, and the resulting BERAs should better represent biological ncRNAs for RNA research and development⁶⁹. Further research is necessary to challenge and refine available expression scaffolds to accommodate more diverse RNA structures. Nonetheless, bioengineering RNAs may follow the paths of recombinant DNA and recombinant protein technologies, which shall not only benefit the whole basic biomedical research community but also permit the development of novel RNA therapeutics.

Acknowledgments

A.-M.Y is supported by NIH grants numbers U01CA175315 and R41AA024029.

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Figure 1. RNA-based drugs act mechanistically on the target transcript derived from the genome, which are different from small-molecule and protein therapeutics that interact with target proteins. In addition, RNA aptamers may directly bind to target protein and exert pharmacological effects.

Natural versus synthetic RNA modifications

Natural

Synthetic

Figure 2.

Comparison of some common posttranscriptional modifications of natural RNAs and artificial modifications of synthetic RNAs. It is noteworthy that natural RNAs are usually modified on the nucleobases (e.g., pseudouridine, 1-methyladenosine, 7-methylguanosine, 5-methylcytidine, etc.), whereas synthetic RNAs display modifications on the phosphate linkage (e.g., phosphorothioate) or sugar ring (e.g., 2'-fluoro, locked nucleic acid, 2'-O-methoxy-ethyl RNA, etc.).

Figure 3. Recombinant RNA approaches using tRNA, 5S rRNA, and chimeric tRNA/pre-miRNA as a scaffold. The tRNA scaffold allows the replacement of anticodon sequence by RNA species of interest, and the 5S rRNA scaffold permits the insertion of desired RNAs into Stem II or substitution of Stems II and III. The optimal noncoding RNA scaffold (OnRS) approach encompassing a stable tRNA/pre-miR-34a structure offers the flexibility to accommodate various types of small RNAs (sRNAs) and most importantly, retain a high-yield, large-scale production of biological ncRNA chimeras.

Figure 4.

Bioengineering ncRNA agents for research and development. The ncRNA coding sequence is cloned into a target vector and transformed in *E. coli*. Total RNAs are extracted from bacterial cultures and verified for the overexpression of target ncRNA (red arrow). BERAs are then purified by anion exchange FPLC method. Pure BERAs are subjected to structural characterization including posttranscriptional modifications, as well as preclinical and clinical investigation of biological fate, activity, effectiveness and safety properties.

Figure 5.

The robust optimal noncoding RNA scaffold (OnRS) approach may be employed to construct OnRS/sRNA library for high-throughput screening of more effective OnRS/sRNA agents and examination of desired phenotype.

Table 1

List of FDA-approved nucleic acid drugs and some RNA-based therapeutics currently under clinical trials.

Name	Agent	Target	Clinical Application	Status
Fomivirsen (Vitravene)	Antisense 21 nt oligo	Cytomegalovirus (CMV) mRNA	Cytomegalovirus Retinitis	FDA approved in 1998
Pegaptanib (Macugen)	RNA aptamer 27 nt oligo	VEGF protein	Age-related macular degeneration (AMD)	FDA approved in 2004
Alicaforsen	Antisense 20 nt oligo	ICAM-1 mRNA	Pouchitis	FDA approved in 2008
Mipomersen (Kynamro)	Antisense 20 nt oligo	Apolipoprotein B mRNA	Familial hypercholesterolemia	FDA approved in 2013
Aganirsen	Antisense 25 nt oligo	IRS-1 mRNA	Ocular neovascularization	Phase 3 clinical trial
Custirsen	Antisense 21 nt oligo	Clusterin mRNA	Prostate, lung, breast cancer	Phase 3 clinical trial
Patisiran	siRNA 25 bp oligo	Transthyretin mRNA	TTR-mediated amyloidosis (ATTR)	Phase 3 clinical trial
QPI-1002 (I5NP)	siRNA 21 bp oligo	p53 mRNA	Acute kidney injury Delayed graft function	Phase 2 clinical trial
AS1411	DNA aptamer 26 nt	Nucleolin protein	Metastatic renal cell carcinoma	Phase 2 clinical trial
NOX-E36 (Emapticap pegol)	RNA aptamer 40 nt	CCL2 protein	Type II diabetes	Phase 2 clinical trial
Miraversen	Antisense 15 nt oligo	miR-122	Chronic Hepatitis C	Phase 2 clinical trial
ALN-PCS	siRNA < 25 bp oligo	PCSK9 mRNA	Hypercholesterolemia	Phase 1 clinical trial
ALN-AT3	siRNA	Antithrombin mRNA	Hemophilia and rare bleeding disorders	Phase 1 clinical trial
MRX34	miRNA mimics	Many oncogene mRNAs	Unresectable primary liver cancer	Phase 1 clinical trial

Table 2
Comparison of current techniques to produce ncRNA agents for research and therapy.

Method	Mechanism	Product	Comments	Reference
<i>In vitro</i>	Chemical synthesis			
	Chemical reaction	<1,000 nt	<ul style="list-style-type: none">Automated, large scaleArtificial modificationsExpensive (with the increase of length)	See review ¹⁸
<i>In vitro</i> transcription	Transcribed from DNA template	Various	<ul style="list-style-type: none">Median scaleCleavable by RNase to sRNAsModifications unavailableHeterogeneity at 5' and 3' ends	See reviews ^{19, 20}
<i>In vivo</i>	Fermentation	Stabilization by p19, a siRNA-binding protein	<ul style="list-style-type: none">Median scaleFull processed siRNAs	49
Fermentation	rRNA as scaffold	<100 nt	<ul style="list-style-type: none">Large (to median) scaleCleavable on demandCost effectiveModifications not assessed yet	23, 68
Fermentation	tRNA as scaffold	<400 nt	<ul style="list-style-type: none">Large scale and high yield (RNA dependent)Natural modificationsExcisable on demandCost effectiveApplicable to some ncRNAs	21, 22
Fermentation	Optimal ncRNA (tRNA/pre-miRNA) scaffold	100–400 nt	<ul style="list-style-type: none">Large scale and high yieldNatural modificationsCleavable on demandCost effectiveApplicable to various types of small RNAs	24, 62