Pharmacology & Therapeutics xxx (2018) xxx

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

RNA therapy: Are we using the right molecules?

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ARTICLE INFO

Keywords:
Therapy
RNAi
miRNA
ncRNA
Biotechnology
Cancer

ABSTRACT

Small-molecule and protein/antibody drugs mainly act on genome-derived proteins to exert pharmacological effects. RNA based therapies hold the promise to expand the range of druggable targets from proteins to RNAs and the genome, as evidenced by several RNA drugs approved for clinical practice and many others under active trials. While chemo-engineered RNA mimics have found their success in marketed drugs and continue dominating basic research and drug development, these molecules are usually conjugated with extensive and various modifications. This makes them completely different from cellular RNAs transcribed from the genome that usually consist of unmodified ribonucleotides or just contain a few posttranscriptional modifications. The use of synthetic RNA mimics for RNA research and drug development is also in contrast with the ultimate success of protein research and therapy utilizing biologic or recombinant proteins produced and folded in living cells instead of polypeptides or proteins synthesized in vitro. Indeed, efforts have been made recently to develop RNA bioengineering technologies for cost-effective and large-scale production of biologic RNA molecules that may better capture the structures, functions, and safety profiles of natural RNAs. In this article, we provide an overview on RNA therapeutics for the treatment of human diseases via RNA interference mechanisms. By illustrating the structural differences between natural RNAs and chemo-engineered RNA mimics, we focus on discussion of a novel class of bioengineered/biologic RNA agents produced through fermentation and their potential applications to RNA research and drug development.

Contents

Abbreviations: A, adenine; ASO, antisense oligonucleotide; asRNA, antisense RNA; BERA, bioengineered/biologic RNA agents; C, cytosine; CMV, cytomegalovirus; dsRNA, double-stranded RNA; FPLC, fast protein liquid chromatography; FDA, Food and Drug Administration; G, guanine; GalNAc, *N*-acetylgalactosamine; GFP, green fluorescent protein; gRNA, guide RNA; HPLC, high performance liquid chromatography; lncRNA, long noncoding RNA; MGA, malachite green aptamer; miRNA, microRNA; ncRNA, noncoding RNA; NSCLC, non-small cell lung cancer; Ψ, pseudouridine; PMO, phosphorodiamidate morpholine oligonucleotide; PNA, peptide nucleic acid; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; PS, phosphorothioate; RNAi, RNA interference; RNase, ribonuclease; rRNA, ribosomal RNA; shRNA, short-hairpin RNA; siRNA, small interfering RNA; sRNA, small RNA; T, thymine; tRNA, transfer RNA; U, uracil; VEGF, vascular endothelial growth factor.

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https://doi.org/10.1016/j.pharmthera.2018.11.011

Please cite this article as: A.-M. Yu, C. Jian, A.H. Yu, et al., RNA therapy: Are we using the right molecules?, Pharmacology & Therapeutics, https://doi.org/10.1016/j.pharmthera.2018.11.011

1. Introduction

Pharmacotherapy utilizes pharmaceutical compounds for the treatment of human diseases, different from other means such as surgery, radiation, and physical therapy. Pharmaceutical drugs usually interact with particular biologic molecules to exert pharmacological actions for the control of disease. Proteins translated from mRNAs are common targets of current pharmaceutical drugs (Gashaw, Ellinghaus, Sommer, & Asadullah, 2012; Overington, Al-Lazikani, & Hopkins, 2006; Santos et al., 2017) which are predominantly small molecules (Fig. 1). Although protein therapeutics such as antibodies have revolutionized pharmacotherapy and drug development (Dimitrov, 2012; Secher et al., 2018; Sliwkowski & Mellman, 2013; Trail, Dubowchik, & Lowinger, 2018), their targets are still mainly protein macromolecules (Fig. 1) while the majority of proteins encoded by the human genome are actually undruggable or non-druggable by conventional modes of therapeutics. Furthermore, genetic or epigenetic changes of an existing protein target can escape from current medications or acquire drug resistance (Brown, Curry, Magnani, Wilhelm-Benartzi, & Borley, 2014; Choi & Yu, 2014). In addition, over 95% of DNA sequences in the human genome are non-protein coding sequences (Mattick, 2004), which many could be transcribed in cells and may be further processed to enormous numbers of functional noncoding RNAs (ncRNAs) (Fig. 1), including transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs or miRs), and long noncoding RNAs (lncRNAs) (Esteller, 2011; Liz & Esteller, 2016; Matsui & Corey, 2017). However, such massive families of functional ncRNAs remain as undrugged or unexplored targets for pharmacotherapy, which thus offers new opportunities for drug development.

RNA molecules have emerged as a new class of therapeutics that may permit the re-targeting of mutated targets, which holds great promise to expand the range of druggable targets from proteins to RNAs as well as the genome (Fig. 1). First, the present protein targets as well as previously-undruggable proteins may be inhibited by appropriate RNA molecules, namely aptamers, to elicit the desired pharmacological effects (Chen et al., 2018; Nimjee, White, Becker, & Sullenger, 2017; Zhou & Rossi, 2017). Second, mRNAs and ncRNAs could be directly targeted by a variety of RNA entities such as antisense RNAs (asRNAs), miRNAs, small interfering RNAs (siRNAs), and other forms of small RNAs (sRNAs) to silence target gene expression or function towards the control of disease (Chen et al., 2018; Khorkova & Wahlestedt, 2017; Moschos, Usher, & Lindsay, 2017; Rupaimoole & Slack, 2017). Third, the sequence of a gene dictating disease initiation or progression may be directly altered by using a proper guide RNA (gRNA) and other necessary components to achieve a complete eradication of the disease for a patient (Doudna & Charpentier, 2014; Komor, Badran, & Liu, 2017; O'Day et al., 2018; Wang et al., 2017; Zhang, Wen, & Guo, 2014). Indeed, the promise of RNA therapeutics was revealed by the approval of first-of-its-kind mRNA-targeting patisiran (ONPATTRO™) for clinical practice by the United States Food and Drug Administration (FDA) in August 2018 (Wood, 2018) (https://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugInnovation/ucm592464.htm, as well as other forms of nucleic acid drugs (Table 1).

RNA therapeutics not only exhibit different mechanisms of actions but also distinct chemistry and pharmacokinetics properties, when compared to conventional small-molecule and protein therapeutics. As such, the development of novel RNA therapeutics has proven to be highly challenging and the past two decades have witnessed only a limited number of nucleic acid drugs approved for clinical use (Table 1). Since delivery is a well-recognized challenge for RNA research and drug development (Crooke, Wang, Vickers, Shen, & Liang, 2017; Dowdy, 2017; Kanasty, Dorkin, Vegas, & Anderson, 2013), utilization of the right RNA molecules has been overlooked for decades (Duan & Yu, 2016; Ho & Yu, 2016). Currently, chemically engineered/synthesized oligonucleotides or RNA "mimics" dominate RNA research and drug development, and some have been successfully approved by the FDA for clinical practices (Table 1). However, these "mimics" are decorated with various and extensive chemical modifications (Bramsen & Kjems, 2012; Khvorova & Watts, 2017; Lundin et al., 2013; Winkler, 2013), making them totally different from natural RNAs transcribed from the genome and folded in living cells that carry no or minimal posttranscriptional modifications (Cantara et al., 2011; Limbach, Crain, & McCloskey, 1994; Morena, Argentati, Bazzucchi, Emiliani, & Martino, 2018). The use of chemo-engineered RNA mimics for research and development is also in sharp contrast to protein research and therapy (Leader, Baca, & Golan, 2008; Secher et al., 2018), and the latter has

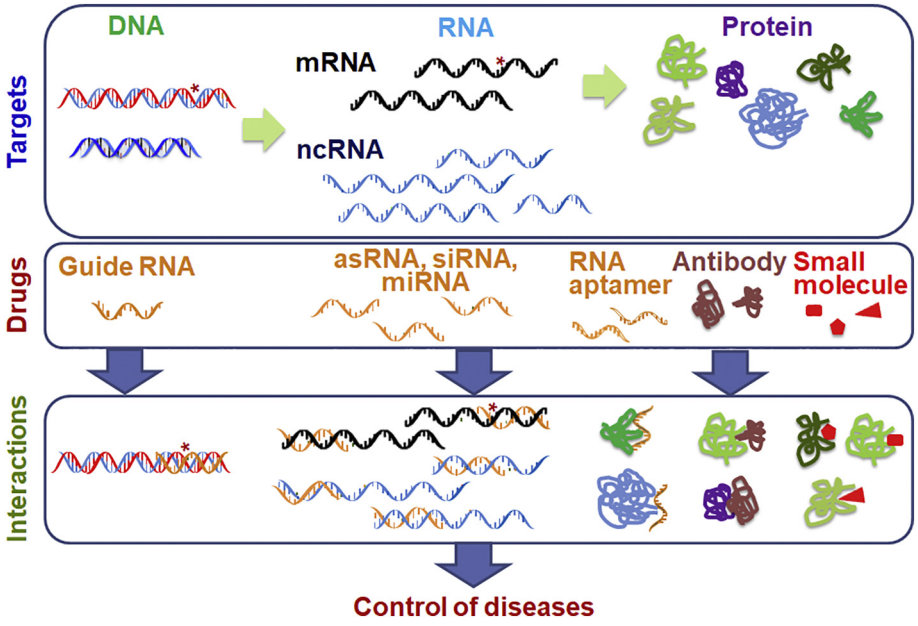

Fig. 1. Expanding the range of druggable targets with RNA therapeutics. Proteins derived from the genome remain as favorable targets for pharmacotherapy, whereas the majority of DNA sequences in the human genome are transcribed as non-protein coding transcripts. As current medications are mainly small molecules and proteins (e.g., antibodies) that act on proteins, RNA therapeutics hold great promise to expand druggable genome for the treatment of human diseases, including (1) RNA aptamers that block protein targets, (2) RNAs such as asRNAs, miRNAs, and siRNAs that target mRNAs or various forms of ncRNAs, and (3) gRNAs that directly edit gene sequences.

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Table 1
Nucleic acid drugs approved by the United States Food and Drug Administration.

Drug name	Agent	Target	Clinical application	Year approved by FDA	Reference
Fomivirsen (Vitravene)	21mer ASO (phosphorothioate linkages)	Cytomegalovirus (CMV) mRNA	Cytomegalovirus retinitis	1998	(Roehr, 1998)
Pegaptanib (Macugen)	27-nt aptamer (pegylated; 2′-fluoro or methoxyl)	Vascular endothelial growth factor (VEGF) protein	Age-related macular degeneration (AMD)	2004	(Gryziewicz, 2005)
Mipomersen (Kynamro)	20mer ASO (all phosphorothioate linkages; 2′-*O*-(2-methoxyethyl); 5-methylcytosine; 5-methyluridine)	Apolipoprotein B mRNA	Familial hyper-cholesterolemia	2013	(Robinson, 2013)
Eteplirsen (Exondys 51)	30mer ASO (phosphorodiamidate morpholino oligomer (PMO))	Exon 51 of dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	2016	(Aartsma-Rus & Krieg, 2017)
Nusinersen (Spinraza)	18mer ASO (all phosphorothioate linkages plus 2′-*O*-(2-methoxyethyl)	Survival of motor neuron 2 (SMN2) mRNA	Spinal muscular atrophy (SMA)	2016	(Ottesen, 2017)
Patisiran (Onpattro)	21-bp siRNA duplexes (2′-*O*-methyl; lipid complex)	Transthyretin (TTR) mRNA	Hereditary transthyretin-mediated amyloidosis (hATTR)	2018	(Wood, 2018)
Inotersen (Tegsedi)	20mer ASO (all phosphorothioate linkages; 2′-*O*-(2-methoxyethyl); 5-methyluridine; 5-methylcytosine; sodium salt)	Transthyretin (TTR) mRNA	hATTR	2018	https://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugInnovation/ucm592464.htm (Keam, 2018)

proven to be highly successful by preferentially using biologic or recombinant proteins produced and folded in living cells rather than polypeptides or proteins synthesized in vitro via peptide chemistry. Therefore, large efforts have been made recently to develop novel biotechnologies for the production of biological/bioengineered RNA agents (BERAs) in living cells (Chen, Wang, Zeng, Urayama, & Yu, 2015; Ho et al., 2018; Huang et al., 2013; Li et al., 2015; Li, Wang, Wu, Huang, & Yu, 2014; Li et al., 2018; Pereira et al., 2016; Pereira, Tomas, Queiroz, Figueiras, & Sousa, 2016), which should represent a new class of tools for RNA research and drug development (Duan & Yu, 2016; Ho & Yu, 2016; Pereira, Pedro, Queiroz, Figueiras, & Sousa, 2017).

Herein we provide an overview on the promise of RNA therapeutics for the treatment of human diseases via RNA interference (RNAi) mechanisms. By comparing the structural differences between natural RNAs transcribed within cells and chemo-engineered RNAs or mimics made in vitro, we focus on novel biologic RNA agents or BERAs produced via RNA biotechnology and potential applications to research and RNA drug development.

2. RNA interference and relevant RNA agents

2.1. RNA interference

RNAi is an evolutionarily-conserved mechanism among eukaryotes in which ncRNAs control target gene expression at the post-transcriptional level. These RNAi molecules include miRNAs derived from the genome and siRNAs generated from exogenously-introduced double-stranded RNAs (dsRNAs) (Ambros, 2004; Bartel, 2009; Cech & Steitz, 2014; Fire et al., 1998) (Fig. 2). The miRNA coding genes are initially transcribed by RNA polymerase II within the nucleus as primary miRNA transcripts, namely pri-miRNAs, which are subsequently processed to shorter precursor miRNAs (pre-miRNAs) by the ribonuclease (RNase) III termed Drosha (Fig. 2). On the other hand, some pre-miRNAs are directly excised from introns of protein coding genes. After being exported from nucleus into the cytoplasm by Exportin-5, pre-miRNAs are converted to double-stranded miRNA molecules by the cytoplasmic endoribonuclease Dicer. After being unwound from the miRNA duplexes or siRNAs derived from dsRNAs, the single-stranded guide miRNAs or siRNAs are loaded into the miRNA- or siRNA-induced silencing complexes (RISC or miRISC), and then selectively act on target mRNAs via perfect or imperfect base-pairing interactions, leading to target RNA degradation or translation repression (Fig. 2).

Through silencing or modulating target gene expression, RNAi is involved in almost all cellular processes including the defense against viral infection as well as cell transformation and disease progression (Ambros, 2004; Bartel, 2009; Cech & Steitz, 2014; Mendell & Olson, 2012; Rupaimoole & Slack, 2017). Compared to normal cells, some miRNAs or the whole miRNome profiles are apparently dysregulated in diseased cells to a dramatic degree, which may serve as helpful biomarkers for diagnosis or prognosis (Chen et al., 2008; Landgraf et al., 2007; Lu et al., 2005). Furthermore, functional miRNAs critical for disease progression could be utilized for the development of novel therapeutic strategies (Bader, Brown, & Winkler, 2010; Rupaimoole & Slack, 2017; Yu, Tian, Tu, Ho, & Jilek, 2016). On one hand, miRNAs that are overexpressed in the diseased cells and promote disease initiation and development may be inhibited or silenced to achieve the control of disease. On the other hand, miRNAs that are depleted in diseased cells, which are actually able to suppress disease initiation and deterioration, may be re-introduced into the cells to manage disease progression.

2.2. Relevant RNAi agents

The discovery of the RNAi mechanism, genome-derived regulatory miRNAs and their importance in human diseases has allowed investigators to develop various forms of RNA molecules to manipulate the RNAi process or mimic miRNA action towards the intervention of target gene expression, understanding of gene function, and control of cellular processes and disease (Bader et al., 2010; Lares, Rossi, & Ouellet, 2010; Rupaimoole & Slack, 2017; Yu et al., 2016). Traditional RNAi modulators include synthetic asRNAs, dsRNAs, siRNAs, and miRNA mimics (Fig. 2), all of which carry various types and degrees of chemical modifications expected to improve overall RNA metabolic stabilities (Bramsen & Kjems, 2012; Khvorova & Watts, 2017; Winkler, 2013). Most recently, "natural" RNAi molecules or BERAs have been introduced into human cells for the control of target gene expression (Fig. 2), which are produced and folded in bacteria through newly-developed RNA bioengineering technologies (Duan & Yu, 2016; Ho & Yu, 2016).

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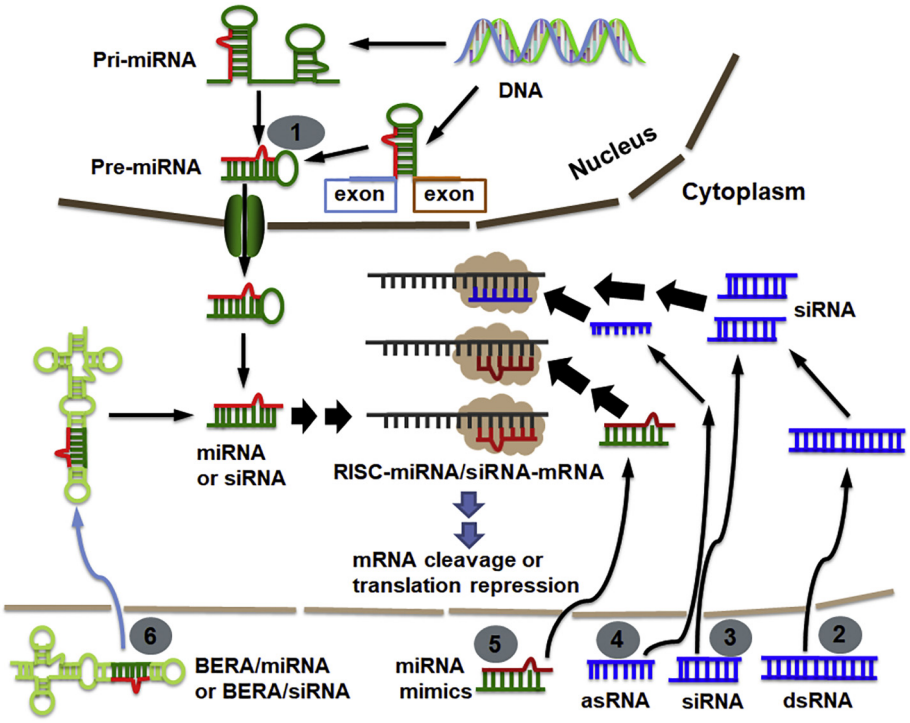

Fig. 2. Cellular RNA interference pathway and manipulation with various types of RNA agents. Endogenous RNAi pathway involves the initial production of pre-miRNA molecules (1) from longer pri-miRNAs transcribed from miRNA coding genes or directly excised out of introns in the nucleus. Pre-miRNAs are then exported into the cytoplasm and subsequently processed to miRNA duplexes. After unwinding from the duplexes and incorporation into the RISC complex, miRNAs and siRNAs recognize target transcripts via imperfect or perfect base-pairing, leading to RNA cleavage or translation inhibition. The use of synthetic dsRNAs (2), siRNAs (3), asRNAs (4), and miRNA mimics (5), which all consist of various types and degrees of chemical modifications, may exert target gene knockdown via cellular RNAi pathway. Bioengineered, single-stranded miRNA or siRNA or sRNA "prodrugs" (6) produced from bacteria fermentation represent a novel family of "natural" RNAi molecules.

BERAs are also distinguished from non-viral or viral-based miRNA or short-hairpin RNA (shRNA) expression systems (Fig. 3) that are widely used for in vitro and in vivo RNAi research and drug development (Brake et al., 2008; Czauderna et al., 2003; Liu & Berkhout, 2011). Those plasmids and vectors are really DNA molecules. Most importantly, use of such vectors/plasmids may complicate the RNA-based mechanisms because they are dependent upon many other factors as well, including the efficiency in the delivery of DNA materials into the nucleus, the integration of target shRNA- or pre-miRNA-coding sequences into the host cell's genome, and the transcription of coding sequences by host cell into target shRNA or pre-miRNA agents before shRNAs or pre-miRNAs are processed to target RNAi molecules to exert target gene silencing (Fig. 3). Additionally, it is unknown whether and to what degree the DNA materials might cause any side effects.

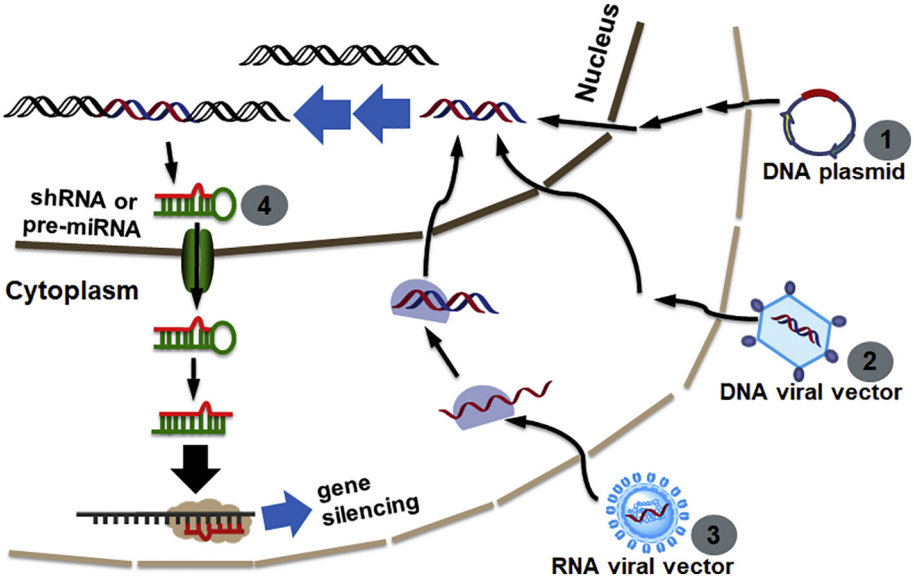

Fig. 3. Use of viral vectors or DNA plasmids for RNA interference. DNA plasmids (1) and genetically-modified DNA viral vectors (e.g., adenovirus) (2) or RNA viral vectors (e.g., lentivirus) (3) may be employed for the production of target pre-miRNA or shRNA molecule (1) within the nucleus of host cell, before which the target coding sequence need be integrated into the host cell's genome. Thus, transcribed shRNAs or pre-miRNAs (1) enter the cellular RNAi pathway; and the resultant siRNAs or miRNAs act on specific transcripts to exert target gene silencing.

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3. RNA therapeutics in clinical practice and trials

3.1. RNA therapeutics currently in clinical practice

A number of RNA-based drugs have been approved by the FDA for the treatment of various types of human diseases (Table 1). Fomivirsen is the first antisense oligonucleotide (ASO) drug (literally a DNA molecule; different from other ASO drugs), which was approved by FDA in 1998 for the treatment of cytomegalovirus (CMV) retinitis among immunocompromised patients including those with acquired immune deficiency syndrome (AIDS) (Roehr, 1998), whose antiviral effects are produced via direct targeting of the CMV mRNA (Orr, 2001). Since 2013, a number of other ASO drugs have been successfully marketed in the United States (Aartsma-Rus, 2017; Aartsma-Rus & Krieg, 2017; Keam, 2018; Ottesen, 2017; Robinson, 2013; Stein & Castanotto, 2017; Syed, 2016) (https://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugInnovation/ucm592464.htm) which all target mRNA molecules in cells. Furthermore, the approval of pegaptanib for the management of neovascular age-related macular degeneration (AMD) (Gryziewicz, 2005) supports the potential of using RNA aptamer to inhibit protein macromolecule for the control of disease. In addition, the most recent approval of first-of-its-kind double-stranded siRNA patisiran by the FDA (Wood, 2018), which was shown to improve multiple clinical manifestations of hereditary transthyretin amyloidosis (Adams et al., 2018), provides incentives to encourage the development of RNAi based therapeutics.

3.2. RNA therapeutics under clinical investigations

Many RNAi based therapeutics, including ten siRNAs, two ASOs, one aptamer and one siRNA-transfected peripheral blood mononuclear cells, are currently under active recruiting clinical trials for patients with cancers, infectious diseases, genetic or other disorders (Table 2), beyond those trials completed already. For instance, intravenously administered AZD4785, an ASO targeting the mRNA of oncogene KRAS (Ross et al., 2017), has entered into Phase I trial for the treatment of non-small cell lung cancer (NSCLC) and other advanced solid tumors. On the other hand, siG12D-LODER is a siRNA that specifically targets the mutant KRAS G12D mRNA (Golan et al., 2015) which is quite common in pancreatic cancers. After the Phase I clinical trial showing that combination of siG12D-LODE and gemcitabine was well tolerated and exhibited a potential efficacy as targeted therapy for locally advanced pancreatic cancer patients (Golan et al., 2015), siG12D-LODER is currently under Phase II clinical trial for patients with unresectable pancreatic cancers (Table 2). QPI-1002, a synthetic double-stranded siRNA designed to temporarily inhibit the expression of proapototic gene P53 showing no dose-limiting toxicities or safety signals in early trials (Demirjian et al., 2017), is presently under Phase III clinical trials for the treatment of acute kidney injury and delayed graft function (Table 2). In addition, an antithrombin-targeting siRNA fitusiran, which, administered once-monthly, showed a dose-dependent lowering of the antithrombin level and increased thrombin generation in participants with hemophilia A or B who did not have inhibitory alloantibodies (Pasi et al., 2017). As such, fitusiran has entered into a Phase III clinical trial for the treatment of hemophilia A and B (Table 2).

Clinical investigations also include the evaluation of miRNAs as therapeutics. As an example, numerous preclinical studies have demonstrated miR-34a as a tumor suppressor against various types of cancers through targeting of many oncogenic pathways (see review (Bader, 2012)). A Phase I clinical study on the safety, pharmacokinetics and effectiveness of a liposome-encapsulated synthetic miR-34a mimic, namely MRX34, was conducted in patients with advanced solid tumors (Beg et al., 2017). While the maximum tolerated dose was 110 mg/m^2 in non-hepatocarcinoma patients, that study revealed a rather high incidence of adverse events (e.g., 100% all grades and 38% Grade 3 among all patients) such as fever, fatigue, back pain, nausea, diarrhea, anorexia, and vomiting among patients receiving MRX34 treatment (10–50 mg/m^2, i.v., biweekly), which required palliative management with dexamethasone premedication (Beg et al., 2017). Nevertheless, a blood half-life of over 24 h and dose-dependent exposure (e.g., C_{max} and AUC) as well as evidence of antitumor activity among patients with refractory advanced solid tumors was identified for MRX34, which would provide valuable insights into developing miRNA therapeutics (Beg et al., 2019).

3.3. Challenges in developing RNA therapeutics

RNA therapeutics differs fundamentally from small-molecule and protein drugs in many aspects including the nature of molecules, targets and mechanisms (Fig. 1), while they all need to address the ultimate common questions regarding the efficacy and safety to control disease. Therefore, there are indeed some unique challenges for the development of RNA based therapies. The druggability of a therapeutic target, either protein, RNA or DNA molecule (Fig. 1), as well as the effectiveness, selectivity, and safety of RNA based interventions often require extensive evaluations. Targeting of a transcript within cells with an RNA molecule has proved to be a major challenge in developing RNA therapeutics (Crooke et al., 2017; Dowdy, 2017; Kanasty et al., 2013) because that RNA agent has to cross the cellular barriers, in addition to necessary blood stability. By contrast, current macromolecule protein therapeutics mainly act on cell membrane or surface protein targets, and many small-molecule drugs readily cross cell membrane barriers to access cytoplasmic protein targets. Rather, chemical modifications and liposomal formulations (Table 1) are able to improve the stability of RNAs in blood and deliver sufficient RNA molecules to cross cell membranes to elicit the desired pharmacological effects, and thus both approaches remain as popular strategies (Bramsen & Kjems, 2012; Chen et al., 2018; Ho, Zhang, & Xu, 2016; Khvorova & Watts, 2017).

Given multiple mechanisms occurring in cellular defense against xenobiotic RNAs (Dalpke & Helm, 2012; Robbins, Judge, & MacLachlan, 2009; Tanji et al., 2015), pharmacological RNA agents may induce an immunogenic response or cytokine release syndrome, which is unsurprisingly dependent upon the doses and structures (e.g., sizes, sequences, etc.) of RNA molecules. Interestingly, the types of chemical modifications have significant influence on RNA immunogenicity and thus can determine the overall safety profiles (Bramsen & Kjems, 2012; Robbins et al., 2009). By contrast, some RNA posttranscriptional modifications may suppress immune response (Gehrig et al., 2012; Kariko & Weissman, 2007; Nallagatla, Toroney, & Bevilacqua, 2008), in addition to their importance in RNA folding, stability and biologic functions. In addition, the complexity of RNA therapy is also increased by the safety of RNA delivery system even if the delivery vehicle itself is not toxic.

4. Natural RNAs versus chemo-engineered RNA mimics

4.1. Natural RNAs carrying no or minimal posttranscriptional modifications

RNAs are polymeric molecules comprised of different numbers and combinations of four major forms of ribonucleotide monomers distinguished by their corresponding nucleobases, adenine (A), guanine (G), cytosine (C), and uracil (U) (Fig. 4). An RNA molecule structurally differs from DNA in the presence of a hydroxyl group at the 2′ position of each ribose, besides the uracil base rather than thymine (T) (Fig. 4). While the primary assemblage of monomeric nucleotides or primary sequence of a particular RNA is undoubtedly critical for the function of that RNA molecule, folding into proper elaborate secondary (e.g., helices or stems, loops, bulges, etc.), tertiary (e.g., junctions, pseudoknot, motifs, etc.), and quaternary (complexes, etc.) structure via Watson-Crick complementary base pairs or other types of physicochemical interactions (Bai, Dai, Harrison, Johnston, & Chen, 2016; Butcher & Pyle, 2011;

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Table 2
Some RNAi based therapeutics currently under active recruiting clinical trials. Data were collected from ClinicalTrials.gov database (https://clinicaltrials.gov/ct2/home) by August 16, 2018. IV, intravenous infusion/injection; SC, subcutaneous; ID, intradermal; EUS, endoscopic ultrasound; PBMC, peripheral blood mononuclear cells; MiHA, minor histocompatibility antigens; CEP290, centrosomal protein of 290 kDa; EphA2, ephrin type-A receptor 2; CBL—B, Casitas B-lineage lymphoma proto-oncogene-b; CTGF, connective tissue growth factor; TRPV1, The transient receptor potential cation channel subfamily V member 1; TGF-β1, *Transforming growth factor* beta *1; Cox-2, Cyclooxygenase-2;* KRAS-G12D, K-ras G12D mutant; NSCLC, non-small cell lung cancer.

Drug name	Agent/formulation	Target(s)	Condition or disease	Route of administration	Phase	Status	ClinicalTrials.gov identifier	Reference
QR-110	Antisense oligonucleotide	CEP290 c.2991 + 1655A > G Mutation (p.Cys998X)	Leber's Congenital Amaurosis (LCA)	Intravitreal	I/II	Recruiting	NCT03140969	(Jacobson et al., 2017)
AZD4785	Antisense oligonucleotide	KRAS	NSCLC; Advanced solid tumors	IV	I	Recruiting	NCT03101839	(Ross et al., 2017)
Zimura (ARC1905)	Aptamer	Complement 5 (C5) protein	Geographic atrophy secondary to dry age-related macular degeneration	Intravitreous	II	Recruiting	NCT02686658	(Leung & Landa, 2013)
EPHARNA	siRNA (neutral liposomal)	EphA2	Advanced recurrent solid tumors	IV	I	Recruiting	NCT01591356	(Wagner et al., 2017)
OLX100010	siRNA	CTGF	Cicatrix or hypertrophic scars (Safety and PK profiles in healthy subjects)	SC or ID	I	Recruiting	NCT03569267	(Hwang et al., 2016)
SYL1001	siRNA	TRPV1	Moderate to severe dry eye disease (DED)	Topical drop	III	Recruiting	NCT03108664	(Benitez-Del-Castillo et al., 2016)
DCR-PHXC	siRNA, conjugated to N-acetylgalactosamine (GalNAc)	Lactate dehydrogenase A (LDHA)	Primary hyperoxaluria	SC	I	Recruiting	NCT03392896	http://dicerna.com/pipeline/novel-investigational-drug-ph-dcr-phxc/
STP705	Two siRNAs (Histidine-Lysine *co*-Polymer (HKP) peptide)	TGF-β1 and Cox-2	Hypertrophic scar	ID	I/II	Recruiting	NCT02956317	(Zhou et al., 2017)
SiG12D LODER	siRNA	KRAS G12D	Unresectable pancreatic cancer	Intratumor implantation via EUS	II	Recruiting	NCT01676259	(Golan et al., 2015)
QPI-1002	Naked siRNA	P53	Acute kidney injury following cardiac surgery	IV	III	Recruiting	NCT03510897	(Demirjian et al., 2017)
QPI-1002	Naked siRNA	P53	Delayed graft function	IV	III	Recruiting	NCT02610296	(Demirjian et al., 2017)
QPI-1007	siRNA	Caspase-2	Acute nonarteritic anterior ischemic optic neuropathy (NAION)	Intravitreal	II/III	Recruiting	NCT02341560	(Solano et al., 2014)
Fitusiran	siRNA (lipid nanoparticle)	Antithrombin	Hemophilia A; Hemophilia B	SC	III	Recruiting	NCT03417245	(Pasi et al., 2017)

Jones & Ferre-D'Amare, 2015; Schlick, 2018) ultimately determines RNA global structure that governs its stability, plasticity, interactions with partners or ligands, and biological functions and safety profiles in cells. It is also noteworthy that the ribose and the unconjugated 2′-hydroxyl group are key elements for the higher-order structures of natural RNA molecules (Butcher & Pyle, 2011).

While RNA molecules produced in living cells are usually comprised of unmodified ribonucleotides, some RNAs (e.g., tRNAs, rRNAs, and mRNAs) do carry a small fraction (e.g., <3%) of modified nucleosides which exhibit rather a broad chemical diversity (Fig. 4) (Cantara et al., 2011; Limbach et al., 1994). Pseudouridine (Ψ), an isomer of uridine, is the first modified natural ribonucleotide discovered and has been recognized as the fifth ribonucleotide because of its ubiquitous prevalence within cellular RNAs (Li, Ma, & Yi, 2016; Spenkuch, Motorin, & Helm, 2014). Methylation is revealed as a major form of modification occurring at both ribose (e.g., 2′-*O*-methyladenosine or Am; 2′-*O*-methylguanosine or Gm; 2′-*O*-methylcytidine or Cm; 2′-*O*-methyluridine or Um; 2′-*O*-methylpsudouridine or Ψm) and nucleobases (e.g., 1-methylpsudouridine or $m^1\Psi$; 5-methyluridine or m^5U; 1-methyladenosine or m^1A; 1-methylguanosine or m^1G; 3-methylcytidine or m^3C; etc.; Fig. 4). There are also other types of modifications including acetylation and hydroxylation as well as complex modifications, which leads to a variety of minor bases including N^4-acetylcytidine (ac^4C), 5-hydroxymethylcytidine (hm^5C), dihydrouridine (D), N^2,7-dimethylguanosine ($m^{2,7}G$). Interestingly, substantial RNA posttranscriptional modifications occur at the nucleobases while 2′-*O*-methylation and other types of modifications are present at the ribose (Fig. 4). Following posttranscriptional modifications, these RNA molecules exhibit unique chemical structures and physicochemical properties as well as biological functions and interactions with innate immune receptors. Indeed, RNA modification has been recognized as another layer of epigenetic mechanism in biology beyond DNA and protein modifications (El Yacoubi, Bailly, & de Crecy-Lagard, 2012; Liu & Pan, 2015; Morena et al., 2018), although modified miRNAs (Alon et al., 2012; Kawahara, Zinshteyn, Chendrimada, Shiekhattar, & Nishikura, 2007; Luciano, Mirsky, Vendetti, & Maas, 2004; Shoshan et al., 2015) are less common in cells, and miRNAs are generally stable within cells and extracellular vesicles in systemic circulation (Mitchell et al., 2008; Sethi & Lukiw, 2009; Winter & Diederichs, 2011).

4.2. Chemo-engineered RNA agents bearing various types and extensive degrees of chemical modifications

Many types of chemical modifications (Fig. 5) have been developed to improve the diversity of RNAs and enhance the stability of synthetic

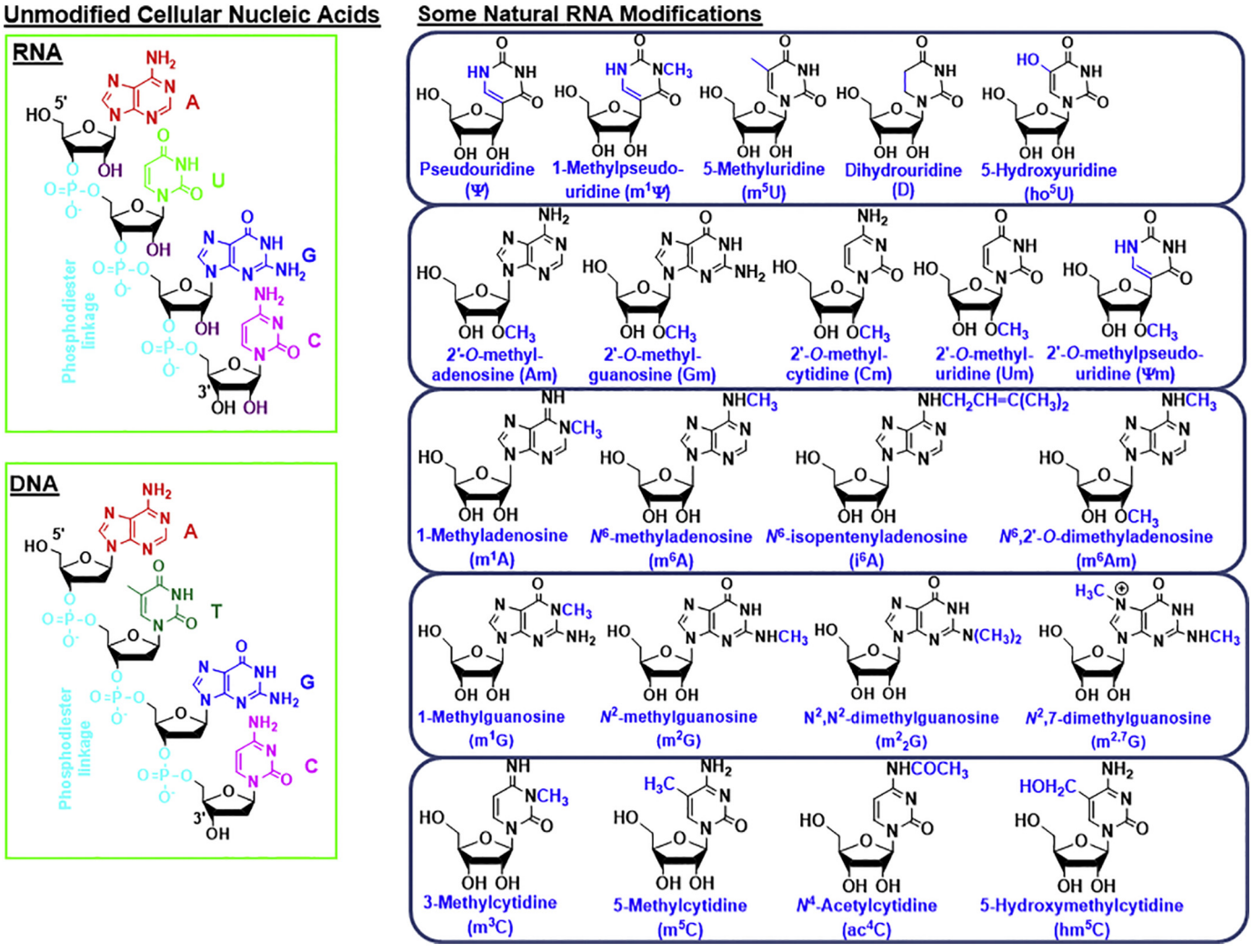

Fig. 4. Chemical structures of unmodified cellular nucleic acids and some posttranscriptionally-modified nucleosides. Different from DNA, an RNA molecule has a hydroxyl group at the 2′ position of each ribose, as well as the uracil base rather than thymine. Natural RNAs made in living cells are generally unmodified nucleic acids, and there is just a small fraction (e.g., <3%) of ribonucleosides carrying posttranscriptional modifications which actually show a broad chemical diversity. Among these modifications, methylation is the most common form which may occur at both ribose and nucleobases. It is also noteworthy that substantial posttranscriptional modifications are present at the nucleobases.

Please cite this article as: A.-M. Yu, C. Jian, A.H. Yu, et al., RNA therapy: Are we using the right molecules?, Pharmacology & Therapeutics, https://doi.org/10.1016/j.pharmthera.2018.11.011

RNA molecules (Bramsen & Kjems, 2012; Khvorova & Watts, 2017; Winkler, 2013), which have been dominating RNAi research and drug development. In contrast to natural RNA modifications that occur predominantly at the nucleobases (Fig. 4), as described above, synthetic RNA agents produced by chemical synthesis are usually altered at the phosphate linkage and ribose (Fig. 5) that are more susceptible to metabolic degradation. Change of phosphate backbone such as phosphorothioate (PS) (Kawasaki et al., 1993) may increase the metabolic stability of the resultant chemo-engineered nucleic acid molecule and thus a prolonged half-life. Phosphorothioate linkage modification has found its success in fomivirsen, the first antisense ASO drug approved by the FDA for clinical practice, as well as the other four ASOs approved since 2013 (Table 1). Masking or substituting the so-called vulnerable 2′-hydroxy group at the ribose is another popular strategy to improve RNA stability, which includes natural modification at the 2′-*O*-methyl group and many other types of unnatural alterations such as 2′-fluoro, 2′-*O*-methoxyethyl, 2′-*O*-4′-*C*-methylene bridged or locked nucleic acid (LNA) (Kurreck, Wyszko, Gillen, & Erdmann, 2002; Lundin et al., 2013) (Fig. 5). Indeed, FDA-approved aptamer (pegaptanib), ASO (mipomersen, nusinersen, and inotersen), and siRNA (patisiran) are all comprised of different types and degrees of sugar modifications (Table 1), supporting the utility of chemical modifications at the ribose.

Nucleic acids could also be stabilized through 5′-phosphate modifications such as 5′-(*E*)-vinylphosphoate (*E*-VP), 5′-methylphosphate and 5′-phosphorothioate (Elkayam et al., 2017; Haraszti et al., 2017; Kuimelis & McLaughlin, 1995; Parmar et al., 2016; Shumyatsky, Wright, & Reddy, 1993) (Fig. 5). There are also increasing interests in the development of 5′- or 3′-conjugates where a particular ligand or drug or targeting molecule (e.g. folate, *N*-acetylgalactosamine or GalNAc, etc.) is connected to the RNA molecule through a versatile linker (Dohmen et al., 2012; Foster et al., 2018; Matsuda et al., 2015; Nair et al., 2014; Winkler, 2013) (Fig. 5), which is expected to enhance RNA pharmacokinetics or pharmacodynamics properties. More advanced modifications include phosphorodiamidate morpholine oligonucleotide (PMO) (Iversen, 2001; Summerton & Weller, 1997) and peptide nucleic acid (PNA) (Nielsen, 2005; Nielsen, Egholm, Berg, & Buchardt, 1991; Wu et al., 2017) where natural bases are retained within oligomer for base pairing and the ribose 5-phosphate is completely replaced (Fig. 5). Eteplirsen is a successful PMO that was approved by FDA in 2016 for clinical management of Duchenne muscular dystrophy (Table 1).

While chemical modifications may lead to favorable pharmacokinetics properties (e.g., longer half-life) which have found their success in FDA-approved drugs (Table 1) and remain as major tools for RNAi research and drug development (Fig. 2), chemo-engineered "RNA mimics" are literally different molecules as compared to natural RNA molecules produced in living cells. With distinct functional groups or moieties, chemo-engineered oligomers undoubtedly have their own secondary and higher order structures as well as intrinsic chemical and biological properties. It is also inadvertently or inevitably overlooked that synthetic RNAi agents obtained from different manufacturers vary largely in their types (e.g., backbone versus sugar; 2′-methoxyethyl versus LNA; etc.), specific sites/positions (e.g., uridine at position 2 versus 3; nucleotides at positions 1–5 versus 11–15; etc.), and degrees (e.g., 100% or parts of the nucleotides; all or partial phosphate linkage; etc.) of chemical modifications. Variable modifications,

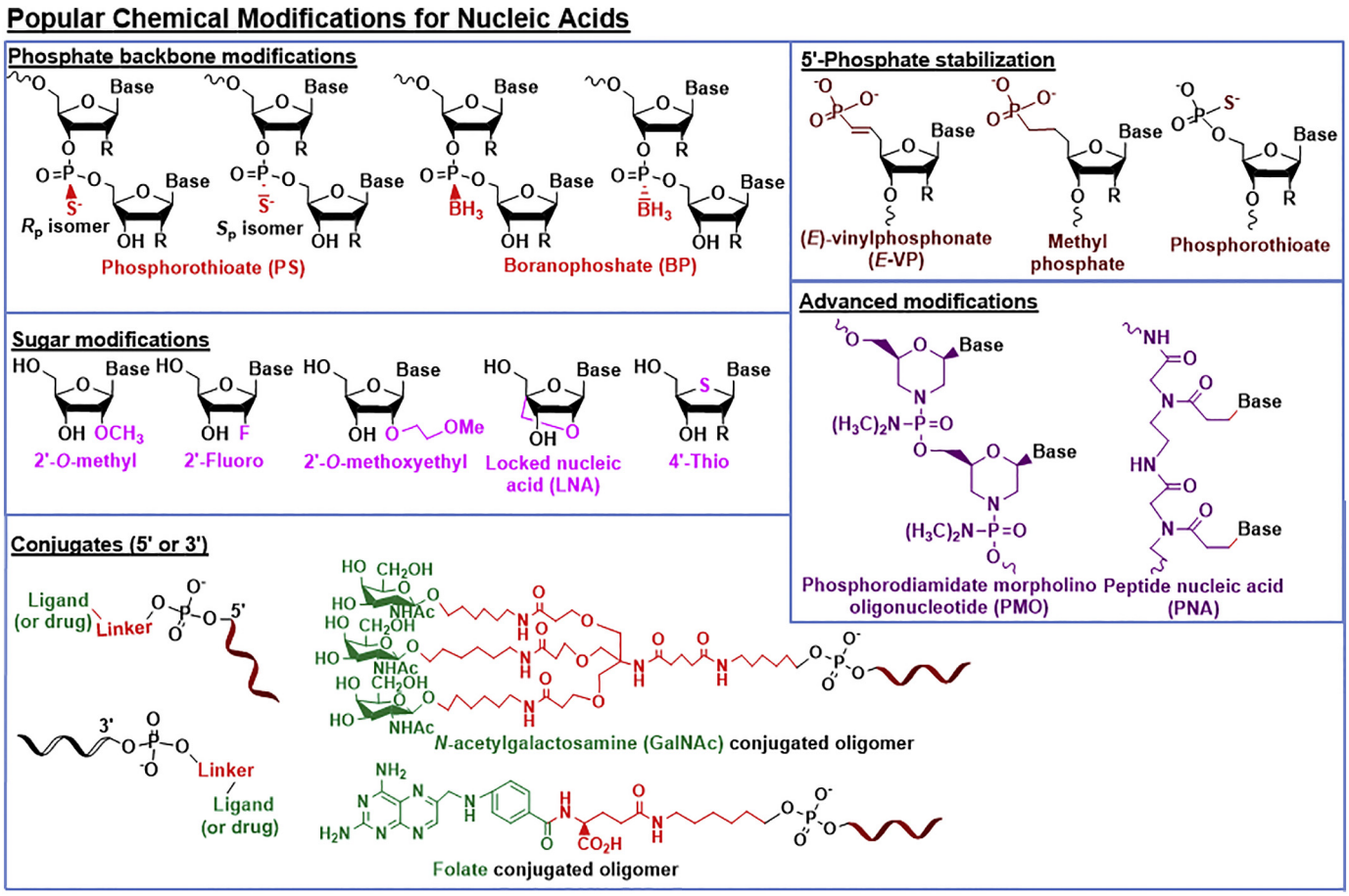

Fig. 5. Common chemical modifications used for the production of nucleic acid reagents including aptamers, ASOs, siRNAs, miRNA mimics, sgRNAs, tRNA fragments (tRFs), and other types of sRNAs. Chemical modifications are mainly aimed at improving the pharmacokinetics properties of RNA reagents. These synthetic RNA mimics are characterized by the changes predominantly at the phosphate linkage and ribose vulnerable to RNase-mediated degradation, which is in contrast to cellular RNA modifications mainly occurring at the nucleobases (Fig. 4). Many RNA conjugates are also developed to enhance targeting, and the advanced modifications completely change the linkage moieties and just retain nucleobases for pairing.

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which may or may not be disclosed, undoubtedly lead to distinct RNAi molecules with different structures and properties, and likely some controversial or variable results despite that they are assumed to have the same "primary sequence" with nitrogenous bases for pairing. Furthermore, depending upon the doses, some chemical modifications might induce immunogenicity (Bramsen & Kjems, 2012; Dalpke & Helm, 2012; Hornung et al., 2005; Judge et al., 2005; Robbins et al., 2009; Tanji et al., 2015), whereas natural posttranscriptional modifications may suppress immune response (Gehrig et al., 2012; Kariko & Weissman, 2007; Nallagatla et al., 2008). In addition, although automated synthesis has facilitated the production, milligrams of oligonucleotides necessary for animal and human trials remain less affordable for common laboratories or investigators, and RNAi drugs for patient care are among the most expensive drugs on the market (Burgart et al., 2018; Simoens & Huys, 2017; Stein, 2016). The involuntary use of synthetic RNAi agents is also in sharp contrast to protein research and development that preferably utilize biologic or recombinant proteins made and folded in living cells, rather than synthetic proteins, which has led to the ultimate success of protein therapeutics (Dimitrov, 2012; Leader et al., 2008; Secher et al., 2018; Sliwkowski & Mellman, 2013; Trail et al., 2018). Therefore, new approaches to producing biologic RNA agents in living cells may open new avenues for RNA research and drug development (P. Y. Ho & Yu, 2016).

5. Bioengineered RNA molecules

5.1. General features of RNA molecules bioengineered in living cells

RNAi agents may be produced in the same way as the production of various recombinant/bioengineered DNA and protein macromolecules in living cells or organisms (e.g., bacteria, yeast and mammalian cells) by a regular biomedical research laboratory. A high-yield and large-scale RNA bioengineering technology (e.g., mg pure RNA from 1 L fermentation) will make the resultant biologic RNA agents or BERAs more affordable (Table 3). Because recombinant RNAs are produced in living cells, BERAs carry no or minimal posttranscriptional modifications (Li et al., 2015; Wang et al., 2015), similar as natural RNAs but largely different from chemo-engineered RNA mimics with extensive unnatural modifications. Folded in cells, BERAs may exhibit intrinsic higher order structures necessary for their stabilities and biological functions within cells (Ponchon & Dardel, 2007; Ranaei-Siadat et al., 2014; Wang et al., 2015) despite that naked BERAs were revealed to be still susceptible to metabolism/degradation by serum RNases (Wang et al., 2015). In addition, although bioengineered RNA molecules derived from living cells were well tolerated in animal models (Ho et al., 2018; Huang et al., 2013; Jian et al., 2017; Tu et al., 2018; Wang et al., 2015; Zhao et al., 2015, 2016), more extensive studies are highly warranted to evaluate their utilities in RNA therapy, whereas a number of chemo-engineered RNA mimics are already in clinical practice and trials (Tables 1 and 2). Nevertheless, the barrier of RNA vulnerability in hosting cells has been overcome to achieve heterogeneous expression of significant levels of target RNA molecules, and BERAs have been successfully produced by novel RNA bioengineering technologies (Ho & Yu, 2016). Among these techniques, the tRNA/pre-miRNA-based approach (Chen et al., 2015; Ho et al., 2018) was revealed as a versatile and efficient means for consistent high-yield production of a variety of RNAi agents including miRNAs, siRNAs, aptamers and other types of sRNAs.

5.2. Fully-processed siRNAs

One way to produce BERAs is to employ RNA-binding protein to protect heterogeneously-expressed RNAs against RNase-mediated degradation. As opposed to the eukaryotic RNAi mechanism against viral infection aforementioned, counter-measures are also evolved among viruses to combat RNAi by producing molecules to repress RNA silencing (Ding & Voinnet, 2007; Li & Ding, 2006; Voinnet, Pinto, & Baulcombe, 1999). The p19 protein (Voinnet et al., 1999) was revealed as an RNAi "suppressor" expressed by the plant RNA virus *tombusviruses*, which exhibits high affinity and selective binding to double-stranded siRNAs (Silhavy et al., 2002). Therefore, viral p19 protein functions to sequester sRNAs and disrupt eukaryotic RNAi process. The p19 protein was successfully used for the isolation and detection of sRNAs (Jin, Cid, Poole, & McReynolds, 2010). Overexpression of recombinant p19 protein in *E. coli* was recently shown to protect double-stranded siRNA species against hydrolysis by RNases and thus allowed enrichment of siRNA molecules (Huang et al., 2013). Heterogeneous co-expression of p19 and target shRNA in bacteria offered p19 complexed with fully-processed siRNA of interest. Following the isolation of the p19-siRNA complex, the target siRNA was successfully purified by a high-performance liquid chromatography (HPLC) method (Huang et al., 2013). While p19-enriched, fully-processed siRNAs were active in silencing target gene expression in mammalian cells, the overall yield of target siRNAs was rather low (e.g., about 40 μg from 1 liter bacterial culture) (Huang et al., 2013), likely related to the limited levels of p19 protein and shRNA expressed in bacteria and/or low capacity of p19 in loading target siRNA molecules.

Table 3
Comparison of biologic RNA molecules produced in living cells and synthetic RNA agents made by chemical synthesis. Compared to traditional RNA mimics made by chemical synthesis that are successfully applied to clinical practice, new bioengineered RNAs produced and folded in living cells may better represent the physicochemical and biological properties of natural RNA molecules whose utilities awaits more extensive investigations.

Biologic RNAs (made in living cells)	Synthetic RNAs (by chemical synthesis)
Large scale (mg pure RNA per L culture)	Large scale (automated)
More affordable	Less affordable; expensive with increased size
No or minimal posttranscriptional modifications	Extensive unnatural & some natural modifications
Variable lengths (e.g., 20–300 nt)	Variable, with desirable short length (e.g., <60 nt)
Folded in living cells	Under chemical environment
Tolerated by cells; safety need more studies	Size, sequence and modifications affect safety

5.3. Chimeric RNAs

Another strategy to produce recombinant RNAs is to integrate target sRNA molecules into proper ncRNA scaffolds such as tRNA (Ponchon, Beauvais, Nonin-Lecomte, & Dardel, 2009; Ponchon & Dardel, 2007) that are relatively more stable within cells. Overexpression of recombinant methionine-tRNA ($tRNA^{met}$) in *E. coli* was first reported in 1988 to produce a few mg of $tRNA^{met}$ from 1 liter bacterial culture after HPLC purification (Meinnel, Mechulam, & Fayat, 1988). The same levels of production were shown for the transfer-messenger RNA (tmRNA, 10Sa RNA, or ssrA) that exhibit both tRNA and mRNA properties (Gaudin et al., 2003). Thus, tRNA was established as a scaffold for large-scale production of recombinant RNA molecules (e.g., multi-milligrams of RNAs from 1 liter bacterial culture) where the anticodon sequence was replaced by target RNA which may be further excised enzymatically from the chimeric RNA (Ponchon et al., 2009; Ponchon & Dardel, 2007). The tRNA-based strategy was used for the production of various target RNA molecules including a number of RNA aptamers, hammerhead riboswitch RNAs, pre-miRNAs, and RNA-protein complexes for structural and functional studies (Li et al., 2015; Li et al., 2014; Nelissen et al., 2012; Paige, Nguyen-Duc, Song, & Jaffrey, 2012; Paige, Wu, & Jaffrey, 2011; Ponchon et al., 2009; Ponchon et al., 2013; Ponchon & Dardel, 2007; Wang et al., 2015). It was also demonstrated that bioengineered tRNA molecules are consisted of some posttranscriptionally-modified nucleosides (Li et al., 2015; Wang et al., 2015). Nevertheless, the expression levels of chimeric RNAs were revealed to be highly variable (ranging from tens milligrams to micrograms recombinant RNAs per liter bacterial culture), and the majority

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of target chimeras were not even expressed (Chen et al., 2015; Li et al., 2015; Li et al., 2014; Nelissen et al., 2012; Ponchon et al., 2009; Ponchon & Dardel, 2007), indicating that the structure and stability of the tRNA-bearing chimera determines the expression level (Ho et al., 2018).

Another usable scaffold is 5S rRNA, a member of highly abundant rRNA molecules in cells and relatively larger in size than tRNA, which may accommodate target RNA sequences at different sites (e.g., stem II and III) (Liu et al., 2010; Zhang et al., 2009). An RNA-based carrier was initially derived from the 5S rRNA harboring an "identifier" nucleic acid that might be utilized as a "biomarker" (Pitulle, Hedenstierna, & Fox, 1995b) due to the lack of specific sequences in rRNA for the monitoring of genetically engineered microorganisms. To further optimize the insert sequence composition and length, a collection of hybrid rRNA/insertion entities consisting of random 13- and 50-base oligonucleotides were created and then introduced into *E. coli* and several *Pseudomonas* strains. Almost all of the rRNA chimeras were expressed at detectable levels using Northern blots (D'Souza, Larios-Sanz, Setterquist, Willson, & Fox, 2003), despite that the exact expression levels were not reported. Thus, this rRNA-based system was successfully used for production of functional aptamers by in vivo fermentation (Zhang et al., 2009). The addition of DNAzyme-specific sequences further offered the option of on-demand release of target inserts (Liu et al., 2010). In particular, DNAzymes could be used to release the desired RNA fragments (e.g., 71 nt) from 5S rRNA chimera (e.g., 160 nt) purified by preparative polyacrylamide gel electrophoresis. This approach yielded 2 mg of target RNA per gram of wet bacterial cells (Liu et al., 2010). Although there are relatively less research reports, rRNA scaffold holds great promise for the bioengineering of new RNA molecules.

A novel RNA bioengineering strategy was recently developed to offer more consistent, high-yield and large-scale production of biologic RNAi molecules (Chen et al., 2015; Ho et al., 2018; Li et al., 2015; Wang et al., 2015), based upon stable tRNA/pre-miRNA chimeric carriers (Fig. 6). Although bare pre-miRNAs could be heterogeneously expressed in bacteria, the levels were usually low (e.g., <2% of recombinant pre-miR-34a among total RNAs or μg from 1 liter bacterial culture), which may hinder purification and overall yields (Ho et al., 2018; Ho & Yu, 2016; Pereira, Pedro, et al., 2016; Pereira et al., 2014; Pereira, Tomas, et al., 2016). On the other hand, tRNA alone could not be overexpressed in bacteria (Ho et al., 2018). As the assembly of tRNA with pre-miRNA was hoped to increase the expression level, the majority of tRNA/pre-miRNA chimeras were still expressed at low levels (e.g., <2% of total RNAs) or not expressed at all (Chen et al., 2015; Li et al., 2014). Following the identification of several hybrid tRNA/pre-miRNAs (e.g., tRNA/pre-miR-34a, tRNA/pre-miR-1291, etc.) that were able to accumulate in bacteria to significantly greater levels (e.g., 5–20% of total RNAs) (Chen et al., 2015; Li et al., 2015; Wang et al., 2015), these tRNA/pre-miRNA chimeras were established as novel carriers permitting consistent high-yield production of various RNAi agents, where the miRNA sequences may be substituted by target miRNA or siRNA duplexes (Fig. 6). In addition, a single stranded sRNA molecule such as RNA aptamer and asRNA may be directly docked into the stable tRNA/pre-miRNA carrier to achieve overexpression (Chen et al., 2015; Ho et al., 2018; Li et al., 2018), and optimization of a particular pre-miR-34a sequence sharply increased the expression yield (e.g., 40–80% of total RNAs).

The workflow of RNA bioengineering using the tRNA/pre-miRNA carrier (Fig. 6) is similar as protein bioengineering. Following the design of a target BERA, its corresponding coding sequence is cloned. Expression and accumulation of target BERA in transformed *E. coli* may be monitored through denaturing urea polyacrylamide gel electrophoresis analysis of total bacterial RNAs (Fig. 6). It was also demonstrated that a common *E. coli* strain (e.g., HST08) offered the greatest levels of heterogeneous expression (Chen et al., 2015; Wang et al., 2015), and thus BERAs are readily purified to a high degree of homogeneity (>97%) at a high yield (multiple to tens milligrams from 1 liter bacterial culture) using a variety of methods including affinity chromatography and anion exchange fast protein liquid chromatography (FPLC) (Chen et al., 2015; Ho et al., 2018; Li et al., 2015; Li et al., 2018; Wang et al., 2015). Pure BERAs may be processed for structural analyses as well as functional studies and translational research such as mechanistic actions in the regulation of target gene expression and potential for therapy.

The tRNA/pre-miRNA-based platform was proven to offer high-yield and large-scale production of many miRNAs (e.g., miR-124, miR-27b, and miR-22, etc.), siRNAs (e.g., siRNA against green fluorescent protein (GFP), etc.), and RNA aptamers (e.g., malachite green aptamer (MGA), and vascular endothelial growth factor (VEGF) aptamer, etc.) (Chen et al., 2015; Ho et al., 2018; Li et al., 2018). These miRNAs and siRNAs are very different in size (e.g., 20–22 nt) and arm of origin (5′ or 3′), while single-stranded RNA aptamers or sRNAs may be directly assembled to either 5′ or 3′ of pre-miRNA, demonstrating that the tRNA/pre-miRNA platform is robust and versatile to accommodate various forms RNAi molecules of interest. It is also noteworthy that, different from fully-processed biological siRNAs produced with RNA-binding protein p19 (Huang et al., 2013), chimeric RNAi agents act as "prodrugs" and utilize mammalian cellular RNAi machinery for processing and actions as discussed below.

6. Applications and promise of bioengineered RNAi molecules

6.1. Structural studies

Distinguished from synthetic RNAi agents bearing extensive chemical modifications that may have distinct physicochemical and biological properties, BERAs made and folded in living cells are "natural" RNA molecules without any chemical modifications or just carrying necessary posttranscriptional modifications critical for higher order structures (Li et al., 2015; Ponchon et al., 2009; Ponchon & Dardel, 2007; Wang et al., 2015). A variety of techniques such as mass spectrometry (Li et al., 2015; Wang et al., 2015), magnetic resonance (Gaudin et al., 2003; Nelissen et al., 2012; Ponchon & Dardel, 2007), and X-ray crystallography (Gaudin et al., 2003) were used to study their primary sequences, natural modifications and secondary and high-order structures. Posttranscriptional modifications learned from BERAs not only illustrate the unique structural characteristics of biologic RNA molecules but also provide insights into RNA epigenetics.

6.2. Functional studies

Bioengineered RNA aptamers and ribozymes have been shown to be biologically active (Chen et al., 2015; Nelissen et al., 2012; Paige et al., 2012; Paige et al., 2011; Pitulle, Hedenstierna, & Fox, 1995a; Wang et al., 2015; Zhang et al., 2009). For instance, rRNA-carried VEGF aptamer effectively suppressed angiogenesis, as demonstrated by Chick Chorioallantoic Membrane (CAM) Assay (Zhang et al., 2009). A number of chimeric RNA aptamers were also developed and produced using a tRNA scaffold, which bound to small-molecule fluorophores and then produced fluorescent signals for the detection or imaging of target molecules in the cells (Paige et al., 2011; Paige et al., 2012). In addition, upon binding to malachite green, the MGAs produced on a large scale using a tRNA/pre-miR-34a carrier exhibited specific and strong fluorescence (Chen et al., 2015). Because the fluorescence intensity is proportional to the level of MGA, which the latter is susceptible to serum RNases, bioengineered MGA was further developed as a sensor for the quantification of RNase activity that may be useful for diagnosis and prognosis (Chen et al., 2015).

As a new class of RNAs, BERAs are active in suppressing target gene expression and consequently modulating cellular processes. Fully-processed "bacterial" siRNAs produced with p19 RNA-binding protein were effective in repressing target gene expression in mammalian cells without immunogenicity or off-target effects (Huang et al., 2013). Recombinant pre-miR-29b was able to reduce mRNA and protein

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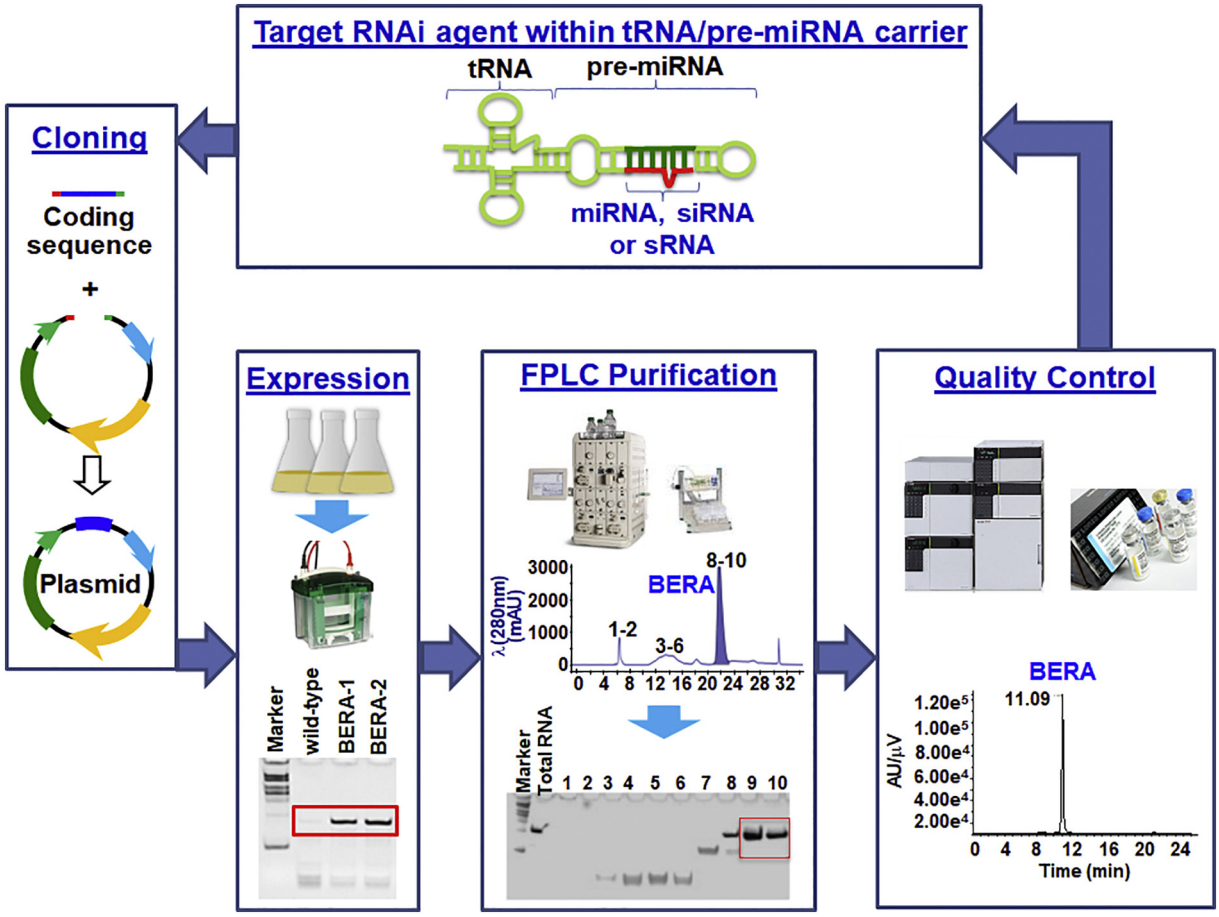

Fig. 6. The workflow for the production of biologic/bioengineered RNAi agent (BERA) where a target miRNA/siRNA/sRNA is assembled into a tRNA/pre-miRNA carrier. After a target BERA is designed, the corresponding coding sequence is cloned into a vector. Expression of target BERA in fermentation may be verified by RNA gel electrophoresis, and BERA can be purified to a high degree of homogeneity by using different methods (e.g., anion exchange FPLC). Purity of isolated BERA is determined by HPLC analysis and endotoxin pyrogen testing. These BERAs should better capture the properties of cellular RNAs and represent a novel class of RNAi agents for basic research (e.g., structural and functional studies) and drug development.

levels of its target gene human β-secretase (hBACE1) and then decrease the levels of amyloid-β (Aβ) peptide in N2a695 cells (Pereira, Tomas, et al., 2016). Bioengineered tRNA/pre-miR-27b significantly repressed its target gene expression (e.g., cytochrome P450 3A4 (CYP3A4)) and subsequently inhibited cellular drug metabolism capacity (e.g., midazolam 1′-hydroxylation) (Li et al., 2014). Recombinant tRNA/pre-miR-1291 produced at high yields from bacteria downregulated the expression of a number of target genes including efflux transporter and cancer related genes and then improved the sensitivity of human carcinoma cells to chemotherapeutics (Jian et al., 2017; Li et al., 2015; Zhao et al., 2015). In addition, tRNA/pre-miRNA-carried miRNAs/siRNAs were readily released to target miRNAs/siRNAs in human cells and animal models to control gene expression (Chen et al., 2015; Ho et al., 2018; Jian et al., 2017; Li et al., 2018; Wang et al., 2015; Zhao et al., 2015), which were demonstrated by untargeted (e.g., RNA sequencing) and targeted (e.g., qPCR and Western blots, etc.) analyses. Most importantly, bioengineered RNAi molecules were consistently shown to be equally or even more effective than synthetic miRNA/siRNA agents in the regulation of target gene expression and cell functions (Chen et al., 2015; Huang et al., 2013; Li et al., 2015; Li et al., 2014; Pereira, Tomas, et al., 2016; Wang et al., 2015). BERAs should be minimally an addition to current RNAi toolbox, serving as a novel class of RNAi agents for functional studies.

6.3. Therapeutic potential

BERAs as possible therapeutics were demonstrated to be of potential value in diseased animal models (Ho et al., 2018; Jian et al., 2017; Tu et al., 2018; Wang et al., 2015; Zhao et al., 2016, 2015). Intratumoral injection of bioengineered miR-34a prodrug showed dose dependent effects in the suppression of subcutaneous xenograft tumor growth in mice derived from human NSCLC A549 carcinoma cells (Wang et al., 2015). The efficacy of miR-34a for the treatment of NSCLC was further demonstrated with refined miR-34a prodrug molecule administered systemically into metastatic xenograft tumor mouse models (Ho et al., 2018). Meanwhile, intravenous administration of in vivo-jetPEI-formulated miR-34a prodrug dramatically inhibited the growth of orthotopic osteosarcomas in mice engrafted with human 143B cells (Zhao et al., 2016), as well as lung metastasis (Jian et al., 2017). Furthermore, the efficacy of chemotherapy for orthotopic 143B xenograft tumors was significantly improved when mice were co-administered with biological miR-34a prodrug (Jian et al., 2017; Zhao et al., 2015). In addition, BERAs did not show any severe hepatotoxicity, nephrotoxicity or immunogenicity in mouse models, as indicated by the lack of or minimal changes of mouse body weights, blood chemistries and chemokines (Ho et al., 2018; Jian et al., 2017; Tu et al., 2018; Wang et al., 2015; Zhao et al., 2015, 2016). These studies exemplify the utility of BERAs for translational research and demonstrate the potential of BERAs as therapeutics.

7. Conclusions and perspectives

The discovery of RNAi mechanisms and the development of RNAi agents not only enhance functional genomics research but also provoke the development of RNA therapeutics that holds the promise to expand the range of druggable targets essentially for all types of diseases. While

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conventional RNA agents made by chemical synthesis have found their success for clinical practice and continue dominating current RNAi research and drug development, caution should be exercised due to the fact that chemo-engineered RNA mimics are decorated with extensive and various types of chemical modifications, and different manufacturers might offer distinct molecules. By contrast, natural RNA transcribed from the genome in living cells is basically assembled by unmodified ribonucleotides. There are just a minimal fraction of nucleosides with posttranscriptional modifications proven to be critical for RNA folding into higher order structures as well as stability, biological functions and safety profiles. Therefore, bioengineering technologies have been developed very recently for the production of biologic RNAi molecules, which, made and folded in living cells, do exhibit intrinsic structural characteristics while carrying no or minimal posttranscriptional modifications.

The tRNA/pre-miRNA-based technology provides a robust platform for consistent, cost-effective, high-yield, and large-scale production of a variety of biologic RNAi agents including miRNAs, siRNAs and aptamers. The resultant BERAs are biologically active following cellular processing and pairing to their targets in human cells, which consequently modulates target gene expression and controls cellular processes such as metabolism, proliferation, and apoptosis. Furthermore, biologic RNAi molecules are well tolerated in mouse models and highly effective to suppress xenograft tumor growth, whose efficacy and safety (especially immunogenicity) awaits more extensive studies in different model systems. Therefore, bioengineered RNAi molecules represent a novel class of RNA agents that shall better capture the physicochemical, biological and safety properties of natural RNAs and hold great promise for basic and translational research. Nevertheless, while bioengineered RNAs showed favorable stability within human cells, naked BERAs remain vulnerable to large quantity of serum RNases. Proper formulation and delivery system are still needed to increase BERA stability in blood and overcome cellular barriers (Zhang et al., 2018) to access molecular targets, which is also essential for moving biologic RNAi molecules into clinical investigations and practice. In addition, further studies are warranted to refine current technologies or explore new approaches for RNA bioengineering that may lead to novel tools for RNA research and drug development.

Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgments

This work was supported in part by grants from The National Institute of General Medical Sciences [R01GM133888] and National Cancer Institute [U01CA175315], National Institutes of Health. C. Jian was supported by a fellowship from the Chinese Scholarship Council (No. 201506270112).

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Please cite this article as: A.-M. Yu, C. Jian, A.H. Yu, et al., RNA therapy: Are we using the right molecules?, Pharmacology & Therapeutics, https://doi.org/10.1016/j.pharmthera.2018.11.011

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Please cite this article as: A.-M. Yu, C. Jian, A.H. Yu, et al., RNA therapy: Are we using the right molecules?, Pharmacology & Therapeutics, https://doi.org/10.1016/j.pharmthera.2018.11.011

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Please cite this article as: A.-M. Yu, C. Jian, A.H. Yu, et al., RNA therapy: Are we using the right molecules?, Pharmacology & Therapeutics, https://doi.org/10.1016/j.pharmthera.2018.11.011