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Advances and challenges in studying noncoding RNA regulation of drug metabolism and development of RNA therapeutics

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Abstract

Accumulating evidence has demonstrated that genome-derived noncoding RNAs (ncRNAs) play important roles in modulating inter-individual variations observed in drug metabolism and disposition by controlling the expression of genes coding drug metabolizing enzymes and transporters (DMETs) and relevant nuclear receptors (NRs). With the understanding of novel ncRNA regulatory mechanisms and significance in the control of disease initiation and progression, RNA-based therapies are under active investigation that may expand the druggable targets from conventional proteins to RNAs and the genome for the treatment of human diseases. Herein we provide an overview of research strategies, approaches and their limitations in biochemical and pharmacological studies pertaining to ncRNA functions in the regulation of drug and nutrient metabolism and disposition, and discussion on the promise and challenges in developing RNA therapeutics.

Keywords

Noncoding RNA; Pharmacoepigenetics; Drug metabolizing enzymes and transporters; RNA therapeutics; Bioengineered RNA; Drug development

1. From hype to reality: noncoding RNAs and oligonucleotide therapeutics

Noncoding RNAs (ncRNAs) are a group of ribonucleic acid (RNA) molecules that are transcribed from DNA but are not translated into proteins. Different classes of ncRNAs are involved in a great variety of cellular processes. For example, miRNAs, piRNAs and lncRNAs participate in the regulation of target gene expression; snRNAs and snoRNAs are

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involved in mRNA maturation; and rRNAs and tRNAs are important components in the process of protein translation. A survey of publications on the PubMed database conducted on 7/31/2019 using “non-coding RNA” as a search term resulted in 182,514 articles, of which 133,505 (73%) articles were published during the last 10 years, indicating that ncRNA has been the subject of intense scientific interest, particularly in the last decade. The results of numerous published studies showed that ncRNAs participate in almost all cellular functions, including gene regulation, metabolism, signaling and cell growth or death [1–3], and are involved in the pathogenesis of many diseases, such as cancer, Alzheimer’s disease, and cardiovascular disease [3–5].

High-throughput technologies have generated large amounts of genomic and transcriptomic data that provide valuable information concerning the expression of ncRNAs and the mRNA transcripts they might regulate. Integrated with bioinformatics tools, experimental approaches have been designed to investigate the biological functions of ncRNAs [6–8]. Notably, the role of ncRNAs in regulating the expression of drug metabolizing enzymes and transporters (DMETs) has drawn much attention; this research has shown that miRNAs and lncRNAs are important modulators of DMET expression [9–11]. Since 1) ncRNAs may influence the levels of proteins responsible for drug metabolism and drug toxicity and 2) drugs can affect the expression of certain ncRNAs in some cases, ncRNA levels have been studied for use as potential biomarkers for the evaluation of drug efficacy and safety. For example, certain ncRNAs mediate intrinsic and acquired resistance to therapies and the levels of these species could be indicative of drug response. Furthermore, altered ncRNA expression has been associated with hepatotoxicity, cardiotoxicity, and nephrotoxicity. Therefore, expanded research on the pharmacogenomic roles of ncRNAs should enhance our understanding of diverse mechanisms underlying inter-individual variability in drug efficacy and safety [12,13].

Findings on the importance of ncRNAs in the control of diseases can offer clues to the development of new therapeutic strategies. Some ncRNAs, such as tumor promotive miRNAs overexpressed in carcinoma cells, may be blocked with inhibitory oligonucleotides while tumor suppressive miRNAs lost in carcinoma cells may be restored for the treatment of lethal diseases, like cancers. In addition to aptamers for the inhibition of target proteins, small interfering RNAs (siRNAs) have been employed to suppress target mRNA transcripts, and small guide RNAs (gRNAs) have been studied to edit target gene sequences. Although many challenges remain, these developments indicate the promise of RNA therapeutics, which may not just be limited to acting on traditional proteins, but can be expanded to target functional RNAs and the genome [14,15]. The utility of chemically-engineered oligonucleotides has proven useful for improving pharmacokinetic properties and several examples have been approved by FDA for clinical practice [15]. Meanwhile, novel bioengineered or recombinant RNA molecules that are produced and folded in living cells are under investigation and should become more relevant to genome-derived cellular RNAs for research and drug development.

2. Ubiquitous regulation of DMETs by ncRNAs

A large portion (98%) of the human genome comprises noncoding genes that are transcribed to produce large amounts of ncRNAs with varying lengths [16]; in general, ncRNAs that are at least 200 nucleotides in length are classified as lncRNAs while the shorter ones are described as small ncRNAs [17]. It has been proposed that approximately 60% of protein-coding transcripts in the human genome are regulated post-transcriptionally by ncRNAs, particularly by miRNAs [18]. As many as 2,300 miRNAs have been identified in humans by *in silico* or wet-lab experimental approaches [19], while multi-omics data from experiments have identified more than 270,000 lncRNAs from different types of human tissues and cells [20]. Owing to their ubiquitous presence and high abundance, miRNAs and lncRNAs have been described as the most important types of regulatory ncRNAs to modulate gene expression epigenetically.

2.1. ncRNAs influence the expression of most DMET genes

A common preliminary screening tool to investigate the validity of a ncRNA/mRNA regulatory relationship predicted using *in silico* methods is to ascertain whether stable interactions are thermodynamically favorable between the ncRNA and the target mRNA using wet-lab experiments; many studies have shown that the utilization of integrated bioinformatics and multi-omics data is key to successful identification of functional ncRNA/target gene regulatory relationships [9,10,12,13,19–21]. Theoretically, every gene encoding a DMET could be regulated by ncRNAs because any DMET gene has the potential for at least one, and often several, potential target domains to interact with ncRNAs. Based on results from two of the most commonly used prediction algorithms, the publicly available [microRNA.org](http://www.microrna.org) (<http://www.microrna.org/>) [22] and TargetScan [23] (Release 7.1, <http://www.targetscan.org>), every DMET gene transcript is expected to possess at least one potential target for particular miRNAs; however, these predictions may not be true in reality, and wet-lab experiments are required to prove if *in silico* predictions are valid. We think that several factors determine if a miRNA actually regulates a particular cognate mRNA: 1) at least one functional miRNA binding site is present in the cognate transcript; 2) a miRNA and its cognate mRNA are present in the same cellular compartment; 3) the level of the miRNA is sufficient to favor the formation of a stable complex with its cognate mRNA; and 4) the interaction between the miRNA and the cognate mRNA is sufficiently robust to facilitate mRNA degradation and/or inhibition of translation. Up to date, wet-lab experiments have validated that more than 60 different miRNAs regulate the expression of 40 key DMETs directly or indirectly through the mediation of nuclear receptors (NRs). These DMETs include CYPs (such as CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4), GSTs (such as GSTP)[24], UGTs (such as UGT1A, UGT2B4, UGT2B7, UGT2B10 and UGT2B15) [9,10,12,25], SULTs (such as SULT1A1, SULT1A4 and SULT2A1) [26–28], transporters (such as ABCA1, ABCB9, ABCC1 and SLC2A3) [11–13], and NRs (such as HNF1A, HNF4A and NR1I2) [26]. We believe that lncRNAs also contribute to the regulation of different DMETs, since human cells contain more lncRNA species and they are able to utilize a variety of diverse mechanisms for gene regulation.

2.2. LncRNAs are involved in drug metabolism

Enhanced interest in the role of lncRNAs in the regulation of drug metabolism has been increasing. Recent studies demonstrated that lncRNAs are involved in gene repression or activation with different mechanisms of action, such as acting as “sponges” or “decoys” to sequester miRNAs, recruiting a suppressor or activator to transcriptional machinery, and functioning as scaffolds in ribonucleoprotein (RNP) complexes [29].

Li et al. [30] reported that the hepatic lncRNA species lncLSTR modulates a lipid metabolic pathway through the regulation of Cyp8b1 in mice. The depletion of lncLSTR by gene knockdown suppressed the expression of Cyp8b1 and affected the metabolism of two bile acids, muricholic acid and cholic acid. The alteration of bile acid composition in turn increased plasma triglyceride clearance in mice. This study showed that the interaction between a lncRNA and a Cyp resulted in the modulation of lipid metabolism. Cholesterol metabolism was also modulated by a lncRNA in another study, in which the authors demonstrated the involvement of RNP in lncRNA-mediated Cyp mRNA degradation [31]. First, the nuclear localization of hnRNPA2B1 was enhanced by the interaction between lnc-HC and hnRNPA2B1, and then the RNA/RNP complex facilitated the degradation of Cyp7a1 and decreased the production of Cyp7a1 protein, resulting in cholesterol accumulation in animals [31]. In another study, lnc5998 was found to interact with a DNA fragment of the enhancer region of the Cyp2b10 gene, by which lnc5998 prevented the nuclear receptor CAR from activating the transcription of Cyp2b10 in mice. Therefore, lnc5998 was able to decrease the potency of TCPOBOP (a CAR agonist)-induced tumorigenesis in female mouse liver [32]. In human liver cells, HNF1AAS1 and HNF4A-AS1 (both are anti-sense lncRNAs) were found to regulate the expression of the liver transcription factors HNF1A and HNF4A, which are critical for the expression of their downstream genes encoding NRs and CYPs. Positive correlations between the expression of HNF1A, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, PXR, and CAR, and the expression of HNF1A-AS1 lncRNA were observed in human liver tissue samples [33]. Further, it was experimentally demonstrated [34] that the knockdown of HNF1A-AS1 decreased the expression of HNF1A, PXR, CAR, AhR, CYP2B6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4, and increased the expression of CYP1A2 [34]. In an unpublished study, we recently found that the inhibitory effects of hsa-miR-495-3p or hsa-miR-486-5p on the expression of SULT2A1 were miRNA-mRNA interaction specific and the strength of the inhibition was dependent on concentrations of miRNAs. In addition, we observed the suppression of SULT2A1 by hsa-miR-486-5p interfered with the enhancing role of lncRNA linc00844. Further studies are highly warranted to investigate the crosstalk between miRNAs and lncRNAs for the modulation of DMETs.

3. The biochemical mechanisms involving ncRNAs might be more complicated than we thought

Canonically, miRNAs bind to their cognate targets on mRNAs by imperfect base pairing to miRNA response elements (MREs) in the 3'-ends of the untranslated regions (3'-UTR) of their target transcripts. The binding efficiency for miRNA/target interaction is largely dependent on a seed-sequence – a conserved heptamer located at positions 2–7 from the 5'-

end of a miRNA. The interaction between miRNAs and MREs induces post-transcriptional repression through mRNA degradation or inhibition of translation, in which a miRNA is an essential molecule in an RNA-induced silencing complex (RISC) comprised of multiple proteins, including Argonaut (AGO) proteins, Dicer and other protein components [35]. In contrast to miRNAs, lncRNAs exhibit numerous functions in diverse cellular pathways with complex mechanisms using different modes of action. Based on our limited knowledge, several primary functions of lncRNAs are summarized as: 1) decoys: to preclude the interaction of miRNAs, mRNAs or DNAs with regulatory proteins; 2) guides: to bring or lead regulatory proteins to their targets in a sequence specific fashion; and 3) scaffolds: to serve as adaptors to facilitate the formation of large macromolecular complexes [36]. LncRNAs may apply multiple features of their functions for the same purpose; therefore, above mechanisms are not mutually exclusive, but multidimensionally cooperative. In general, a common phenomenon is that lncRNAs interact with RNP complexes, which in turn regulates gene expression [36].

3.1. Interactions between miRNAs and their target genes in mammalian cells may involve 5'-UTRs or protein coding regions

It is well recognized that miRNAs bind to the specific sequences of MREs within the 3'-UTRs of their target transcripts to suppress the gene expression through translation inhibition and/or RNA degradation. Although the details of mechanisms are unclear, miRNA binding sites also have been found within promoter regions, 5'-UTRs, and the protein coding regions of target genes, using *in silico* prediction and wet-lab experimental validation. In most cases, the binding of miRNAs to the 3'-UTRs or protein coding regions resulted in suppression of gene expression, whereas the binding of miRNAs to the 5'-UTRs or promoters of the target genes could either enhance or inhibit gene transcription by interacting with different components of the transcriptional machinery [37].

Several studies showed that miRNAs suppress the expression of CYP2E1 by different mechanisms. MiR-378a-5p targeted the 3'-UTR of CYP2E1 resulting in decreased CYP2E1 protein production and enzymatic activity without down-regulation of its mRNA level, indicating that this inhibitory effect is achieved by translational repression [38]. Additional translational inhibition of CYP2E1 was achieved by miR-552. Mir-552 has dual effects on the suppression of CYP2E1. Firstly, it was able to bind to its MRE in the 3'-UTR of CYP2E1 for translational inhibition in the cytosol [39]. Secondly, miR-552 in the nucleus can bind specifically to a DNA fragment located within the promoter of CYP2E1 via a non-seed sequence interaction, resulting in an inhibition of RNA Pol II-dependent CYP2E1 transcription. Therefore, the double negative regulatory effects of miR-552 on the suppression of CYP2E1 demonstrated that 1) the binding of a miRNA can involve both the 3'-end and 5'-end of the gene; 2) the counterpart molecule of a miRNA can be either DNA or RNA; and 3) the regulation can occur at both transcriptional and translational levels.

In mammalian cells, miRNAs can also bind to their MREs located in the coding regions of their target transcripts to function similarly as in plant cells for translation inhibition or mRNA degradation. For example, miR-602 and miR-608 target the coding regions of the SHH (Sonic Hedgehog) mRNA transcript [40], and miR-34a has an inhibitory role for the

expression of MDM4 by targeting its coding sequence [41]. In the case of miR-29 mediated regulation of CYP2C19, *in silico* analysis predicted that hsa-miR-29a-3p could efficiently target the coding region of CYP2C19, and wet-lab experimental data further validated the inhibitory effect of hsa-miR-29a-3p on the expression of CYP2C19 in human liver cells [42]. In contrast to well-recognized mechanisms of miRNA action through targeting the 3'-UTRs of transcripts, the detailed inhibitory mechanisms underlying the interaction between miRNAs and the coding regions of target genes remain unclear.

3.2. The involvement of ribonucleoproteins in the interactions between miRNAs and target genes

The miRNA-induced silencing complex (miRISC), a multiple protein complex, contains one guide strand sequence (of the miRNA) that interacts with the complementary sequence of the target transcript and proteins. Among the proteins present in the RISC, AGO proteins are involved in activation or cleavage of the target mRNA. Canonically, a perfect complementary hybridization of miRNA-MRE activates the endonuclease activity of AGO2 to induce a cleavage in the mRNA target, while an imperfect pairing between a miRNA and a target transcript prevents the target transcript from being cut by AGO2, and AGO2 functions similarly as AGO1, AGO3 or AGO4 with features analogous to a mediator of an RNA interference (RNAi). The formation of the miRISC complex involves the recruitment of many proteins, including the GW182 family of proteins (functions as scaffolding proteins), PAN3 and CCR4-NOT (de-adenylase complexes), exoribonucleases and others [37].

AGO protein family members are critical components existing in the majority of RISCs. However, in some studies to investigate the modulation of DMETs by miRNAs, we observed the phenomenon in which the known AGO proteins were not detectable. For example, miRNA/mRNA/protein complexes were identified from the interaction between the miRNA hsa-miR-214-3p and CYP2E1 transcript in the presence of HepaRG cell extracts, but no AGO protein was found by Western blots. When the miRNA/mRNA/protein complexes were subjected to mass spectrometry analyses, a group of ribonucleoproteins were revealed; however, AGO proteins were still not found [43]. In another study, proteomics approaches were used to characterize protein components presented in the hsa-miR-370-3p/CYP2D6-mRNA/protein complex. We found that FUS and EIF4A3 interacted with hsa-miR-370-3p/CYP2D6-mRNA duplex, in addition to other proteins. It was explained that the primary role of EIF4A3 is facilitating mRNA decay and FUS is also involved in the regulation of gene expression by affecting RNA stability and decay [21]. Our work suggests the possibility of AGO-independent mechanisms for at least some cases of miRNA-mediated gene regulation [21]; however, better understanding on how these ribonucleoproteins participate in the suppression of CYP2E1 or CYP2D6 requires more detailed biochemical and cell biological studies to elucidate precise mechanisms. On the other hand, we cannot rule out the possibility that our inability to detect AGO proteins in some miRNA complexes could be due to the limitations of our experimental conditions.

3.3. Cross-talking: miRNAs, lncRNAs, NRs and DMETs work as a network

The interaction among miRNAs, lncRNAs, NRs and DMETs constitutes a multi-dimensional network that responds to endogenous (such as hormones) and exogenous (such as environmental chemicals) stimuli to affect drug efficacy and safety. Fig. 1 can be interpreted as: 1) Endogenous or exogenous stimuli influence the expression of miRNAs, lncRNAs, NRs and DMETs [9,10,12,13]; 2) The expression of NRs and DMETs may also be modulated indirectly by these stimuli via the mediation of lncRNAs and miRNAs [9,10,12,13]; 3) NRs can regulate the expression of miRNAs and lncRNAs [44]; 4) MiRNAs can directly interact with lncRNAs as a sponge to suppress the expression of lncRNAs [45]; and 5) lncRNAs can also directly function as sponges or decoys for miRNAs to affect miRNAs' activities [45]. In addition to genetic polymorphisms or other types of epigenetic regulatory mechanisms, such as DNA methylation and histone acetylation/deacetylation, this network introduces extra dimensions of complexity in the regulation of DMETs, which has a substantial impact on inter-individual variability in drug efficacy and drug safety.

Exogenous and endogenous stimuli, genetic makeup, and epigenetic components interact together to determine the inter-individual variability of the final expression and enzyme activity of DMETs. It is typically asked which the most potent regulator of drug metabolism and efficacy among stimuli, genetic polymorphisms, ncRNAs, DNA methylation or histone modification is. We believe that this question must be answered gene-by-gene or case-by-case. Several circumstances can be discussed. 1) Genetic polymorphisms dominantly control gene expression or enzyme activity. For example, null-genotype polymorphisms of GSTM1 and GSTT1 in humans result in variants of which carriers do not have any expression of GSTM1 and GSTT1 genes in their cells [46]. 2) DNA methylation and/or histone modification status are typically the most potent epigenetic regulators of DMET expression. As we know, unlike in primary human hepatocytes, the majority of DMETs, including CYPs, are not expressed in many established human hepatic cell lines due to epigenetic modifications; however, the inhibition of DNA methylation and histone modification can restore the expression of many DMETs [47]. 3) The induction of DMETs by chemicals could be the most potent inducible regulatory mechanism. For example, the expression of CYP1A1 was increased 480-fold by 3-methylcholanthrene, and was increased 280-fold by omeprazole in human primary hepatocytes [48]. 4) The potency of epigenetic regulation of DMET expression by ncRNAs may be relatively moderate by comparison. Several studies conducted in our laboratory suggested that miRNA mediated regulation of CYPs (including CYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4) suppresses expression by approximately 15%–60% [9,21,26,42,43,49]; however, less-potent regulation of DMETs by ncRNAs may be important in drug safety and efficacy, particularly in the case of drug-drug interaction, in which one drug may increase the expression of ncRNAs that in turn modulate the expression of DMETs for metabolizing a different drug. Furthermore, while genetic variations encoded within the genome are permanent and DNA methylation and histone modifications are relatively stable, altered expression of regulatory ncRNAs is able to develop rapidly and relax quickly, providing a more dynamic mechanism to allow fine control of DMET gene expression. Considering that inter-individual variability in the expression of DMETs is an overall consequence of the interaction among genetic variations, chemical induction/

inhibition, DNA methylation and histone modification, and ncRNA modulation, the impact of ncRNA-mediated gene regulation should be neither overlooked nor overstated.

3.4. Limitations and challenges in the characterization of ncRNA-mediated regulation of DMET expression

Many studies have been conducted to characterize mechanisms involved in ncRNA-mediated regulation of DMETs, using integrated approaches including *in silico*, *in vitro* and *in vivo* [9,10,12,13]. The results of these studies demonstrate that ncRNAs indeed participate in the modulation of drug metabolism; however, limitations and challenges have also emerged. For example, the consensus opinion of DMET downregulation by miRNAs and upregulation by lncRNAs may have overlooked many important aspects of ncRNA functions; *in silico* prediction may mis-lead experimental validation; animal studies were rarely carried out to assess the regulation of DMETs, and “off-target” effects were rarely evaluated.

Typically, *in silico* approaches are always used before wet-lab validations. Many publicly available miRNA prediction tools can be used to predict the interaction between miRNAs and mRNAs. It is common for a prediction algorithm to generate many false positive candidates, since the prediction theory is primarily based on a base-pairing between the seed of a miRNA and its cognate MREs on target genes with different stringencies in utilizing predictive parameters. Therefore, the confidence in the predicted result can be improved by comparing predictions using different algorithms. The calculation of minimum free energy of hybridization (ΔG) between an ncRNA and its cognate mRNA using *in silico* tools is also based on base-pairing in a cell-free condition. The stability of ncRNA/MRE interaction is more complicated, since many cellular components including proteins involved in the process could facilitate ncRNA-mRNA interactions. To confirm the hybridization between an ncRNA and an mRNA, the FREMSA, a fluorescence-based RNA electrophoretic mobility shift assay, was created in our laboratory to visualize the interaction between an ncRNA and its target mRNA with or without the presence of cellular components, which provides direct evidence of ncRNA-mRNA interactions [42]. Previously, we concluded that a minimum free energy of hybridization smaller than -20.0 kcal/mol is necessary to visualize the ncRNA/mRNA interaction by FREMSA; however, in a recent study we confirmed that, in the presence of proteins, a minimum free energy of hybridization smaller than -10.0 kcal/mol was sufficient to detect the interaction of a specific ncRNA/mRNA complex by FREMSA. Given the fact that *in silico* prediction is a suggestive indicator, experimental data from *in vivo* and *in vitro* approaches can provide more solid evidence to confirm the interaction between an ncRNA and its target mRNA.

Using the expression patterns of ncRNAs and mRNAs to elucidate the relationships between ncRNAs and mRNAs is another powerful approach. Many databases contribute a wealth of resources for *in silico* analyses of expression patterns to examine the correlations between ncRNA candidates and their target mRNA molecules. For example, the TCGA (The Cancer Genome Atlas) database provides the biggest collection of genomic data to all researchers [6], which enables us to analyze associations between different expression profiles. However, the generally accepted consensus that miRNAs suppress the expression of target

genes while lncRNAs always enhance the expression of target genes may not be correct in all cases. Notably, this consensus opinion does not consider all other potential functions and mechanisms involving ncRNAs. For example, the knockdown of lncRNA HNF1A-AS1 decreased the expression of CYP2B6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4, but increased the expression of CYP1A2 [34]. In another example, miR-374 increased the expression of E-cadherin and CSDC2 [50]. Further, it is worthwhile to note that the gene expression profile in the human population is extremely heterogeneous due to different genetic backgrounds, environmental and dietary exposures, and health status of tissue donors. A weak association or a non-significant association does not necessarily mean that the expressions of an ncRNA and an mRNA are not related.

In addition to *in silico* prediction and correlation analysis of the expression relationship between the expression of a miRNA and the expression of its target mRNA, *in vitro* experiments applying transfection of ectopic miRNA mimics or inhibitors into cells is a commonly used strategy to validate the regulatory function of a miRNA. However, in some circumstances, the ectopic miRNA mimics or inhibitors in cells are “overexpressed” to a level that is usually much higher than the endogenous level of the miRNA, suggesting a possibility of introduction of an “exaggerated” effect in the study. On the other hand, the concentration of a miRNA may not reach the level that is necessary within an intracellular compartment to allow its function to suppress target genes (in reality, subcellular concentrations of specific miRNAs are very difficult to measure), which could introduce a “minimized” or “ignored” effect of the miRNA *in vitro*. Further, almost all *in vitro* studies demonstrated the “direct” regulations of DMETs by miRNAs; however, potential “indirect” regulations could not be ruled out, since “off-target” effects of miRNAs have been overlooked. These “artificial” results from *in vitro* studies may explain partially *in vitro/in vivo* discrepancies.

A few studies [51–53] have provided *in vivo* evidence to show the functions of miRNAs on drug metabolism at the systemic level. For example, miR-34a was analyzed to understand its impact on the pharmacokinetics (PK) of CYP probes including midazolam, dextromethorphan, phenacetin, diclofenac and chlorzoxazone using a practical single-mouse PK model [52]. MiR-34a was known to regulate NRs and several DMETs; however, when human miR-34a was intravenously administered into mice as a cancer therapeutic, no or just minor effects were observed on the pharmacokinetics of the probe drugs. Species differences for miRNA targets and/or contributions from other regulatory factors may explain partially why the pharmacokinetics of these probe drugs was not affected in mice; but it is clear that more extensive studies should be pursued to fill the gaps between *in vitro* experiments and *in vivo* models with regard to the importance of miRNAs in drug metabolism.

4. Roles of ncRNAs in the regulation of nutrient transport and metabolism

The transport and metabolism of nutrients such as glucose and amino acids (AAs) provide cells with essential components for survival and growth. Carcinoma cells are reprogrammed to become dependent on a continuous supply and metabolism of key nutrients, as well as disposition of end metabolites, for the production of energy and synthesis of nucleotides, proteins and lipids required for cancer cell proliferation, tumorigenesis and metastasis

[54,55]. Such changes include the overexpression of many solute carrier (SLC) family transporters, such as glucose transporter 1 (GLUT1 or SLC2A1) and large neutral amino acids transporter small subunit 1 (LAT1 or SLC7A5), and metabolic enzymes, such as hexokinase II (HK2) and glutaminase (GLS), involved in glucose and AA transport and metabolism (Fig. 2).

Many ncRNAs including miRNAs are unsurprisingly dysregulated in carcinoma cells [56–58], among which some have been demonstrated to control nutrient metabolism and transport through direct or indirect modulation of the expression of metabolic enzymes and SLC transporters, respectively (Fig. 2). A number of tumor suppressive miRNAs are notably involved in the regulation of glycolysis [59–62] and glutaminolysis [62–65]. For instance, there is an inverse correlation between the expression levels of miR-22 and GLUT1 in patient breast cancer tissue samples, and miR-22 directly targets GLUT1, contributing to the inhibition of breast cancer cell proliferation and invasiveness [59]. Besides the actions in the control of efflux transporter ABCC1/MRP1 expression, sensitization of drug-resistant cells, and suppression of xenograft tumor growth [66–70], miR-1291–5p directly modulates the expression of GLUT1 in human renal cell carcinoma (RCC) cells [61]. Meanwhile, SLC2A1/GLUT1 and miR-1291 expression levels are inversely correlated in RCC clinical specimens, where SLC2A1/GLUT1 mRNA levels are much higher and miR-1291 levels are significantly lower in cancer tissues than non-cancerous tissues [61]. In addition, studies on let-7a-1/let-7d/let-7f-1 cluster (let-7adf) knockout mouse models revealed that let-7adf greatly reduced the supply and metabolism of glucose and glutamine through direct and indirect targeting of murine Hk2 and Slc1a5 [62]. Nevertheless, many questions remain to be addressed concerning how important ncRNAs are in the control of cell metabolism and whether any particular ncRNAs could be utilized to interfere with metabolic pathways towards the control of specific diseases such as tumor progression.

5. Translating ncRNA biology into novel therapies

Many ncRNAs including miRNAs and lncRNAs have been shown to control target gene expression behind the initiation and progression of human diseases, especially lethal cancers [71–73]. Any ncRNA enhancing tumorigenesis and growth represent tumor promoters, and ncRNAs inhibiting tumor development act as tumor suppressors. This is due to the mechanistic actions of ncRNAs in modulating the expression of tumor-related genes critical for cancer cellular processes, including nutrient transport and metabolism discussed above. Interestingly, uncontrolled carcinoma cell growth and tumor development is not only associated with the alterations of proteins and signaling pathways but also dysregulation of many functional ncRNAs, for which the latter may be caused by various mechanisms such as gene deletion or methylation, and/or changes in transcription factors, enzymes or binding proteins involved in the biogenesis of ncRNAs. Among them many tumor suppressive ncRNAs (e.g., let-7–5p, miR-34–5p, miR-124–3p, miR-1291–5p, and lncRNA HOXD-AS1, etc.) are generally downregulated in carcinoma cells, while some tumor promotive ncRNAs (e.g., miR-21–5p, miR-155–5p, miR-221–3p, and lncRNA LINK-A, etc.) are commonly upregulated. With the improved understanding of ncRNA biogenesis, mechanistic actions, and importance in human diseases, two major strategies have been established for the development of novel ncRNA-based therapies. Specifically, one may restore some critical

disease-suppressive ncRNAs lost or reduced in diseased cells, or inhibit other disease-promotive ncRNAs overexpressed or activated in diseased cells to achieve therapeutic efficacy (see recent reviews [14,15,72,74]).

5.1. Opportunities and challenges in developing RNA-based therapies

RNA-based therapies represent the next generation of therapeutics as they are different from traditional drugs, namely small-molecule and protein agents, which are also noted to predominantly act on protein targets [14,15] (Fig. 3). Firstly, RNA aptamers are able to block the activity of a specific protein target to exert pharmacological effects. Secondly, antisense RNAs (asRNAs), miRNAs, and siRNAs can be developed to directly target mRNAs or functional ncRNAs for the control of a particular disease. Thirdly, gRNAs may be utilized to specifically edit target sequences of a given gene for the treatment of monogenic disease. As such, RNA therapeutics hold the promise to expand drug-gable targets.

Indeed, a number of RNA therapeutics, including the first siRNA drug Patisiran, have been already approved for clinical practice while many others are under preclinical and clinical development [14,15,75–77]. For example, with the understanding of the critical role of miR-122–5p in the amplification of hepatitis C virus (HCV), an oligonucleotide, namely, Miravarsen, has been developed to target miR- 122–5p and it was shown to effectively reduce HCV RNA levels in a dose-dependent manner among patients with chronic HCV genotype 1 infection [78,79]. Since miR-34a-5p is firmly established as a tumor suppressor while it is commonly downregulated in various tumors, a Phase I clinical trial was performed to investigate the safety and anti-tumor efficacy of liposomal miR-34a mimic, namely MRX34, among patients with advanced solid tumors [80]. While the effectiveness of MRX34 was established in some patients, severe adverse effects were found among most patients, including the occurrence of multiple deaths with complex and uncertain causes. Therefore, this trial was terminated due to safety concerns, which reiterates the importance of both safety and efficacy in drug development.

The development of RNA therapeutics does pose a few unprecedented challenges, as RNA drugs differ from small-molecule and protein drugs in many ways, such as chemistry, pharmacology and toxicology (Fig. 3). Conventional macromolecule protein drugs usually act on cell surface or extracellular protein targets, and small-molecule drugs are generally able to pass through cellular membranes to act on cytoplasmic protein targets to exert pharmacological effects. In contrast, RNA molecules are readily degraded by many serum RNases and RNA is generally unable to cross cellular membrane barriers to access their target transcripts; these obstacles represent major challenges for the development of RNA therapeutics [14,15,81,82]. It is even more challenging for CRISPR-based gene editing therapy because both the gRNA and the Cas9 protein which has a high molecular mass, or their corresponding DNA materials, are required to cross the additional nuclear membrane and then act on specific sequences within the highly-structured genome to explicitly change genetic codes. Furthermore, introduction of exogenous biologics into cells may trigger an inappropriate immune response or cytokine release syndrome. Certain chemical modifications and particular formulations with biocompatible lipids or polymers are

effective improvements for stability and achieving the delivery of RNA agents [15,83,84], which are still the primary strategies to make RNAs druggable. Nevertheless, the question is raised whether chemically-engineered RNA mimics are the best or optimal means for RNA research and drug development [14,15] because chemo-engineered RNA molecules made by chemical synthesis and natural RNAs derived from genome and folded within living cells are different molecules, and extensive chemical modifications undoubtedly lead to distinct higher-order structures, physicochemical properties, biologic activities, and safety profiles.

5.2. En route to a novel class of bioengineered RNA molecules

The very recent development of bioengineered or recombinant RNA molecules that are produced and folded in living cells, similar to endogenous types of cellular RNAs, offers a novel and alternative class of agents for RNA research and drug development [14,15]. This notion is also in line with protein research and therapy that relies mainly on bioengineered or recombinant proteins folded in living cells rather than synthetic polypeptides or proteins, leading to ultimate success over the past decades, including the elucidation of thousands of protein structures [85] and more than 200 FDA-approved protein drugs [86]. A number of approaches have been developed for the production of recombinant or biologic RNA agents (see recent reviews [14,15]). The tRNA scaffold [87,88] has been successfully utilized for the production of many recombinant RNAs, among which some are employed for structural studies [87,88] and others were found to be biologically active [89,90]. The tRNA/pre-miRNA carrier established most recently [91,92] is revealed to be more versatile and robust in achieving high-yield and large-scale production of target ncRNA molecules at high success rates, which convey warhead miRNAs, siRNAs, aptamers, or other forms of small RNAs. Comprehensive studies have further demonstrated that warhead miRNAs and siRNAs are selectively released from bioengineered ncRNA agents in human cells to regulate target gene expression and modulate cancer cellular processes including drug and nutrient disposition [52,69,91–98]. Most importantly, a number of bioengineered miRNA prodrugs are revealed to be effective in suppressing xenograft tumor growth and metastasis in mouse models, while all RNA agents are well tolerated in animals [69,92,93,96–98]. These findings demonstrate the promise of bioengineered ncRNA agents, whereas more extensive studies are warranted to assess their applications to drug development.

6. Conclusions and perspectives

The rapid growth in the ncRNA field indicates that ncRNAs are important modulators for the gene expression of DMETs critical for drug development and clinical pharmacotherapy. It is amazing that the superfamily of genome-derived ncRNA molecules can have significant impacts on a broad variety of biological functions. The interactions among ncRNAs, NRs and DMETs exhibit a complex network in the regulation of drug metabolism and disposition, in which ncRNAs modulate the expression of DMETs and NRs through various mechanisms, although a clear picture of ncRNA-mediated DMET gene expression and the precise mechanisms have not yet been fully elucidated. Further studies with integrated *in silico*, *in vitro* and *in vivo* approaches may help to fill many gaps in understanding specific regulatory mechanisms, which shall provide new insights into roles of ncRNAs in drug metabolism and distribution.

Understanding ncRNA functions in disease progression and pharmacotherapy opens a new avenue for the development of oligonucleotides as therapeutics for the treatment of human diseases via the intervention of mRNA targets. RNA therapies hold great promise to expand the druggable targets from common proteins to RNAs and the genome, while lower stability and difficulty in crossing membrane barriers remain big challenges for the development of RNA drugs. As current ncRNA research and drug development relies mainly on chemically-synthesized oligonucleotides with extensive and various types of chemical modifications, the development of bioengineered or recombinant RNA molecules produced and folded in living cells offers a novel and alternative class of agents for basic research and drug development, which warrants more extensive evaluations. An improved understanding of the roles of ncRNAs in various biological processes, contributions of ncRNAs to individual variations in response to drug or xenobiotic exposure, and influence of ncRNAs on pathogenesis and disease progression, through critical studies using proper tools more relevant to genome-derived RNAs processed and folded in living cells, will certainly underpin the development and implementation of RNA therapeutics.

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Fig. 1. Cross-talking: miRNAs, lncRNAs, NRs and DMETs work as a network. The interaction among miRNAs, lncRNAs, NRs and DMETs constitutes a network that works together to respond to endogenous and exogenous stimuli. The network is a key modulator contributing to drug efficacy and safety.

Fig. 2.

Functional miRNAs contribute to the regulation of nutrient transport and metabolism essential for cancer cell survival and proliferation. Glycolysis and aminolysis are interconnected by supplying TCA cycle intermediates to generate energy and drive the synthesis of nucleotides, proteins and lipids for cancer cell proliferation. Some miRNAs (e.g., miR-1291-5p and let-7-5p) have been shown to regulate the expression of a number of transporters (e.g., GLUT1 and LAT1) and enzymes (e.g., HK2 and GLS) involved in glucose and AA transport and metabolism. G6P, glucose-6-phosphate; AA, amino acids; Gln, glutamine; Glu, glutamic acid; SLC2A1/GLUT1, glucose transporter 1; SLC7A5/LAT1, large neutral amino acids transporter small subunit 1; HK2, hexokinase II; GLS, glutaminase.

Fig. 3.

RNAs may be translated into novel therapeutics and hold the promise to expand druggable targets. Proteins are common therapeutic targets for the development of small-molecule and protein/antibody drugs, which can also be attacked by RNA aptamers to exert pharmacological effects. In addition, asRNAs, miRNAs and siRNAs can be utilized to directly target mRNAs or functional ncRNAs, and gRNAs may be employed to edit gene sequences for the control of diseases.