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SURVEY AND SUMMARY

Biological relevance and therapeutic potential of G-quadruplex structures in the human noncoding transcriptome

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ABSTRACT

Noncoding RNAs are functional transcripts that are not translated into proteins. They represent the largest portion of the human transcriptome and have been shown to regulate gene expression networks in both physiological and pathological cell conditions. Research in this field has made remarkable progress in the comprehension of how aberrations in noncoding RNA drive relevant disease-associated phenotypes; however, the biological role and mechanism of action of several noncoding RNAs still need full understanding. Besides fulfilling its function through sequence-based mechanisms, RNA can form complex secondary and tertiary structures which allow non-canonical interactions with proteins and/or other nucleic acids. In this context, the presence of G-quadruplexes in microRNAs and long noncoding RNAs is increasingly being reported. This evidence suggests a role for RNA G-quadruplexes in controlling microRNA biogenesis and mediating noncoding RNA interaction with biological partners, thus ultimately regulating gene expression. Here, we review the state of the art of G-quadruplexes in the noncoding transcriptome, with their structural and functional characterization. In light of the existence and further possible development of G-quadruplex binders that modulate G-quadruplex conformation and protein interactions, we also discuss the therapeutic potential of G-quadruplexes as targets to interfere with disease-associated noncoding RNAs.

INTRODUCTION

The vast majority of the human genome (up to 98%) does not code for proteins, though being transcribed into functional RNAs. Such RNAs, called noncoding RNAs (ncRNAs) have been recognized as master regulators of gene expression in a wide range of biological processes, operating at any step throughout the genetic expression process, from transcription to RNA maturation and translation (1–3). In the past 20 years, research in this field has made extraordinary progress in understanding how aberrations in ncRNA expression/function may affect normal levels of key regulatory genes, thus driving pathological phenotypes in a great number of human diseases (4), spanning from cancer (5–9) to viral infections (10), from neurodegenerative (11,12) to cardiovascular disorders (13,14). In this regard, full understanding of ncRNA biological roles and mechanisms of action will allow to better comprehend the molecular bases of disease and develop novel therapeutic strategies.

As a general rule, RNA exerts its function through sequence-based mechanisms (i.e. classical base pairing) or forming complex secondary and tertiary structures, including bulges, hairpins, stem-loops, duplex-, triplex- and quadruplex-motifs, which allow interactions with both proteins and other nucleic acids (NAs) (15). Among RNA structures, RNA G-quadruplexes (rG4s), which may occur in guanine (G)-rich sequences, are organized in stacks of tetrads and are stabilized by metal cations in physiological conditions (16). rG4s have been described in human mRNAs, telomeric RNA and in viral RNA genomes, where they have been validated as regulators of key biological processes, such as transcription termination, pre-mRNA processing, mRNA targeting, mRNA translation and maintenance of telomere homeostasis (17,18). G4s have been also linked to several human diseases (19), including can-

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cer (20,21), neurological disorders (22,23) and infectious diseases (24–26). Despite rG4s forming in ncRNAs may hold great potential as mediators of structure-based functions, this field has started to be explored only recently. An increasing number of reports on the presence of rG4s in some types of ncRNAs, such as microRNAs (miRNAs) (27) and long noncoding RNAs (lncRNAs) (28), is currently being released, suggesting a possible role of rG4s in ncRNA-mediated regulation of gene expression, in controlling miRNA biogenesis and in mediating miRNA/mRNA interactions.

Here, we provide a comprehensive survey of the emerging functional rG4s in miRNAs and lncRNAs and critically analyze their mechanisms of action linked to human diseases. We intend to (i) offer insights into the general features and biological roles of rG4s; (ii) highlight the involvement of rG4s in the regulation of ncRNA function; (iii) explore the possibility to interfere with disease-associated miRNAs/lncRNAs by exploiting rG4 binders able to modulate rG4 conformation and rG4/protein interactions.

NONCODING RNA BIOLOGY AND FUNCTION

Conventionally, ncRNAs are classified into small or long transcripts, depending on whether they are shorter or longer than 200 nucleotides (2,29). The most so far studied small ncRNA class, estimated to modulate up to 60% of the human protein-coding genes at the post-transcriptional level, is that of miRNAs, single stranded RNAs of about 23 nucleotides in length (30), the expression and maturation of which are regulated through a multiple step process (Figure 1A). The genes that encode for miRNAs are transcribed by RNA polymerase II or III, and the derived transcripts adopt a long hairpin-like structure called primary miRNA (pri-miRNA) (31,32). Then, the pri-miRNAs are cleaved by the microprocessor complex composed of Drosha, DiGeorge critical region 8 (DGCR8) and other additional variable proteins, thus generating the precursor miRNA (pre-miRNA), characterized by a stem-loop structure (33). The exportin protein Exp5 next exports the pre-miRNA to the cytoplasm, where the stem-loop is cleaved by the Dicer exonuclease, generating a short RNA duplex. Finally, one of the two strands of the duplex is degraded while the other one is loaded onto the AGO2 protein within the RNA induced silencing complex (RISC), becoming the mature miRNA able to bind and repress target mRNAs (34). The subsequent post-transcriptional repression varies in its mechanism depending on the extent of miRNA complementarity to its target mRNA. If pairing is extensive, the target mRNA is sliced, a process typically found in plants. In contrast, in humans and bilaterian animals, where miRNAs usually bind with imperfect complementarity to sites present in the 3′ untranslated regions (3′-UTR) of the target mRNAs, the dominant mechanism relies on deadenylation of the poly(A) tail, with subsequent mRNA destabilization and decay, and/or inhibition of translation (35). The fact that partial pairing is sufficient to direct target repression makes it possible for a single miRNA to regulate dozen or even hundreds of different targets (36). A growing number of reports have shown that subsets of miRNAs have clinical relevance as biomarkers (37) and some miRNA-based therapeutics have reached clinical trials to be tested for the treatment of different human diseases (38). Because alterations in miRNA expression and/or processing can lead to various pathological phenotypes including cancer (39,40), neurodegenerative diseases (41) and cardiovascular diseases (42), miRNAs are considered important therapeutic targets.

Other classes of small ncRNAs include PIWI-associated RNAs (piRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNA) and tRNA-derived fragments (tRFs), all characterized by different mechanisms of action and/or expression in specific cell types and conditions (reviewed in (43,44)). Since investigation regarding the presence and role of rG4s in such transcripts is still at a very early stage, with few reports available (45–47), only the miRNA class of short ncRNAs will be considered in this review.

According to the current size-based definition, lncRNAs are a highly heterogeneous collection of transcripts characterized by low or null protein-coding potential. Though representing the major fraction of the human transcriptome, their mechanisms of action are not completely elucidated (48,49). Most lncRNAs are transcribed by RNA polymerase II, thus sharing similarities with mRNAs, including a 5′ 7-methylguanosine cap and a 3′ poly(A) tail (50). According to the position and direction of transcription relative to other genes, lncRNAs may be classified into different subtypes, including antisense, intergenic, overlapping, intronic, bidirectional and processed transcripts (51,52). From the functional point of view, lncRNAs are not characterized by a common mode of action and may sustain both gene inhibition and gene activation in a number of different ways, also depending on their nuclear or cytoplasmic localization. Moreover, gene regulation may occur *in cis* (in close proximity to the transcribed lncRNA) or *in trans* (at a distance from the transcription site) (53). The so far reported mechanisms of action of lncRNAs have been nicely reviewed by Statello *et al*. (54). Briefly, nuclear lncRNAs can regulate gene expression by altering the chromatin state, through guiding or sequestering (also known as decoy mechanism) chromatin modifiers to or away from the promoters of target genes (Figure 2A). In addition, nuclear lncRNAs can directly activate/inhibit transcription of target genes in an RNA-dependent and/or independent manner, with the latter mode of action intimately linked to lncRNA transcription itself. In either cases, transcriptional regulation may rely on the interaction with proteins, including transcription factors, RNA polymerase and chromatin looping factors, or with the DNA, through creation of RNA-DNA-DNA triple helices (the so-called triplexes) (55) (Figure 2B). Some lncRNAs serve as primary transcripts to form miRNAs (56,57) (Figure 2C). Both in the nucleus and in the cytoplasm, lncRNAs may also act post-transcriptionally to modulate mRNA splicing (Figure 2D), turnover or translation (Figure 2E) by titrating RNA binding proteins (e.g. splicing factors, proteins involved in mRNA degradation) or by forming RNA-RNA hybrids with target pre-mRNAs (58,59). Some cytoplasmic lncRNAs act as ‘sponges’ of miRNAs: in light of the presence of miRNA binding sites in their sequence, such lncRNAs can physically sequester miRNAs, thus reducing their availability (Figure 2F). lncRNAs may also act as scaf-

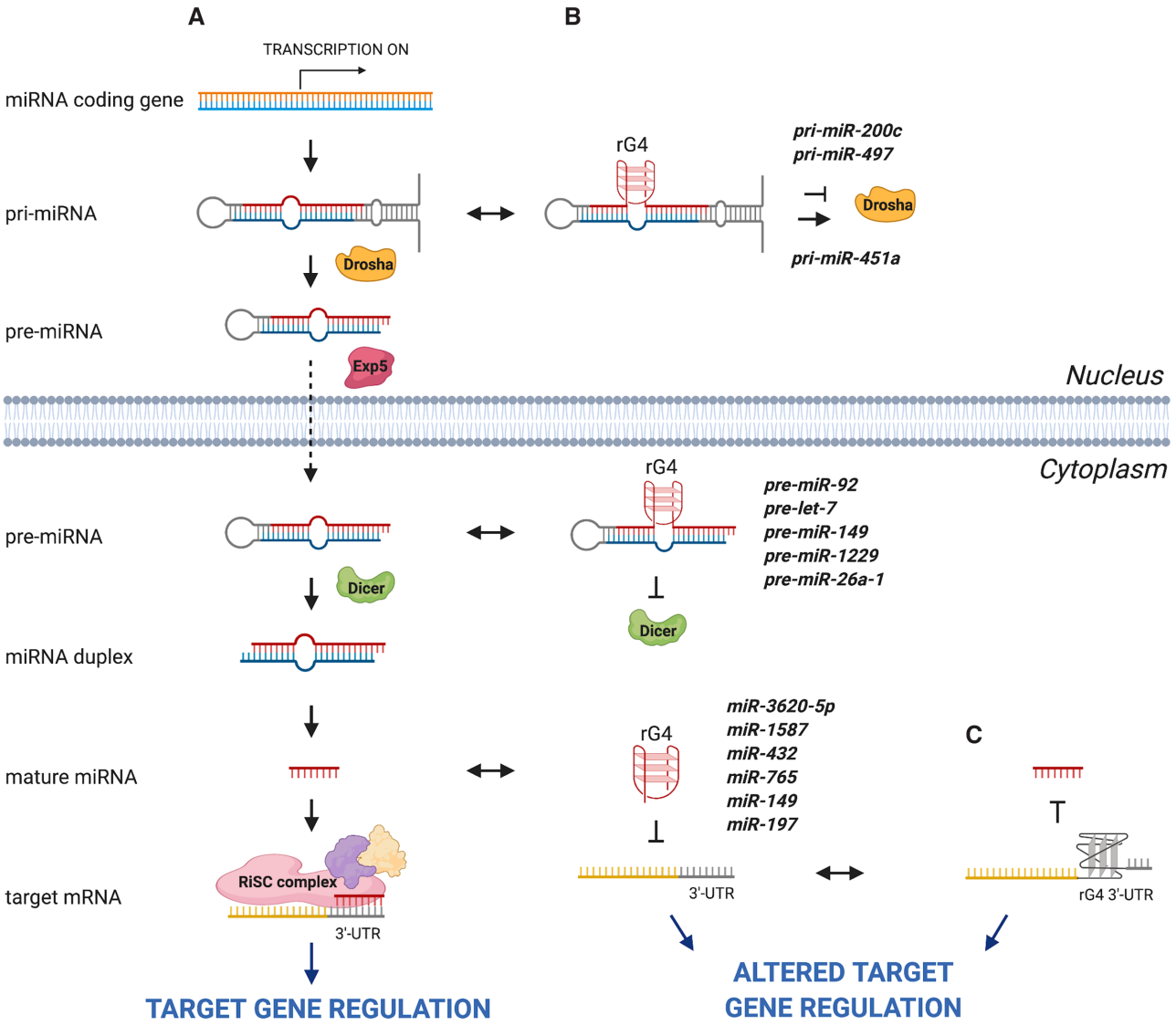

Figure 1. (**A**) The canonical miRNA processing pathway. In the nucleus, miRNA genes are initially transcribed into pri-miRNAs, which are then cleaved into pre-miRNAs by Drosha. Pre-miRNAs are exported to the cytoplasm by Exp5 and further processed into a miRNA:miRNA duplex by Dicer. The miRNA:miRNA duplex is unwound and the mature miRNA is then assembled into the RISC complex, where it binds to the target mRNA, thus finally regulating gene expression. (**B**) rG4s interfere with the biogenesis and function of miRNAs. rG4s found in pri-miRNAs and pre-miRNAs affect miRNA processing by altering Drosha and Dicer binding, respectively, while rG4s in mature miRNAs impede miRNA binding to its target mRNA. (**C**) rG4s in the 3′-UTR of mRNAs hamper the accessibility to miRNAs and prevent RISC-mediated degradation. Created with BioRender.com.

folds for the assembly of different nuclear condensates, such as paraspeckles, ribonucleoprotein complexes or organelles (Figure 2G).

Consistent with their pervasive transcription and increasingly recognized role in gene regulation, several lncRNAs have been shown to participate to the pathogenesis of a range of cardiovascular, viral and oncologic diseases (6,10,12,14,60).

THE RNA STRUCTUROME

Prerequisite to the understanding of ncRNA functions is the identification of features that allow RNA to interact with other molecules (15,56). In this regard, it is straightforward that RNA may use its primary sequence to bind to complementary RNA through classical Watson-Crick base pairing. Example of this 'sequence-based' mode of action is the interaction between miRNAs and their target mRNAs. RNA-RNA base pairing is also used in the molecular sponge mechanism by which lncRNAs can sequester matching miRNAs. Another process based on direct RNA/RNA interaction is target-induced miRNA degradation, occurring when binding of a given miRNA to high affinity sites present in mRNAs or lncRNAs triggers its degradation (61).

Less trivial is understanding how RNA may interact with double stranded DNA or proteins, as it occurs, for example, when RNA binds to RNA-binding proteins, including the interaction of pre- and pri-miRNAs with the processing enzymes Drosha or Dicer, and in all the above mentioned

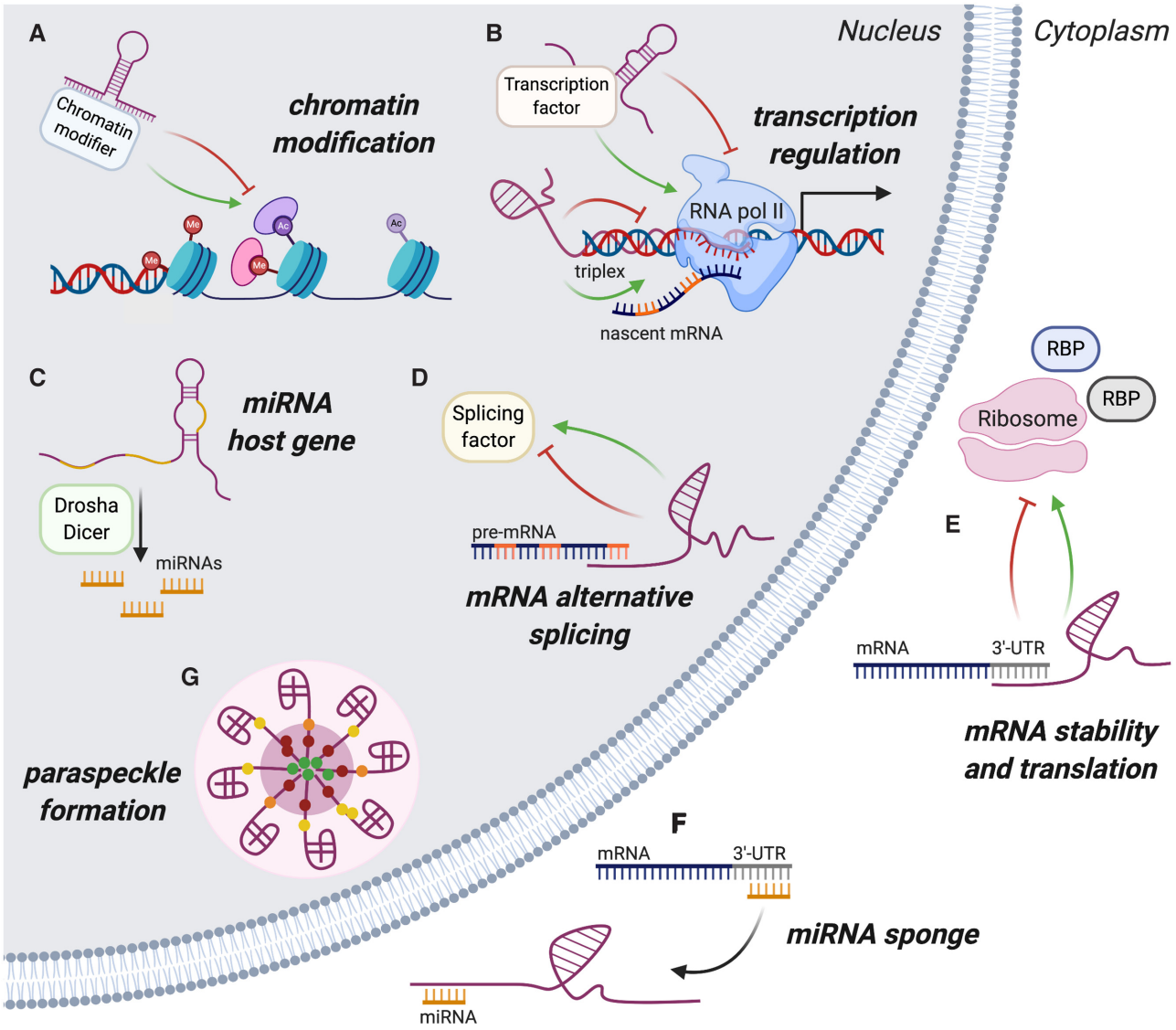

Figure 2. The repertoire of lncRNA modes of action. In the cell nucleus, lncRNAs can (**A**) modify the chromatin state, (**B**) activate/inhibit transcription of target genes, (**C**) serve as host genes for the transcription of miRNAs, (**D**) regulate mRNA alternative splicing. In the cell cytoplasm, lncRNAs can (**E**) modulate mRNA stability or translation and (**F**) act as sponges to sequester miRNAs. Some lncRNAs serve as scaffolds for the assembly of different organelles or nuclear condensates, such as (**G**) paraspeckles. All reported lncRNAs are depicted in purple. RPB: RNA binding protein. Created with BioRender.com.

reported modes of action of lncRNAs (62). In fact, if decoding of mRNA primary sequence into aminoacids can be used to identify known functional protein domains and ultimately predict the function of a still unknown protein-coding gene, prediction of lncRNA function is still challenging. The primary sequence is indeed poorly informative of lncRNA mode of action, as it is scarcely conserved across evolution, even for lncRNAs with closely related functions (63). However, multiple alignment analysis of lncRNAs has revealed that among large unconstrained sequences, short patches of conserved bases in correlated positions may be found, as well as a certain degree of conservation of secondary and higher order structures (64–66). This evidence would suggest that selective pressure may exist over structure rather than over the primary sequence and that, similarly to proteins, lncRNAs may work through discrete functional RNA domains/modules (63). Among these, for example, transposable elements have been shown to mediate lncRNA function and localization (57,67). Overall, these findings suggest that a number of RNA roles can be ascribed to 'structure-based' rather than to sequence-based mechanisms. In this context, figuring out RNA structures, motifs and interacting partners is crucial to understand RNA-mediated biological functions.

Primarily, single-stranded RNA can fold locally to create secondary structures such as hairpin stem or loops, bulges, three-way junctions, internal loops, pseudoknots, i-motifs and rG4s (15,68) (Figure 3). In these structures, base pairing can be canonical (A-U, G-C) or non-canonical (e.g. G-U; G-A), with each base-pair having an associated contextual free energy. Higher order intramolecular tertiary structures can then form among distantly located sequences in

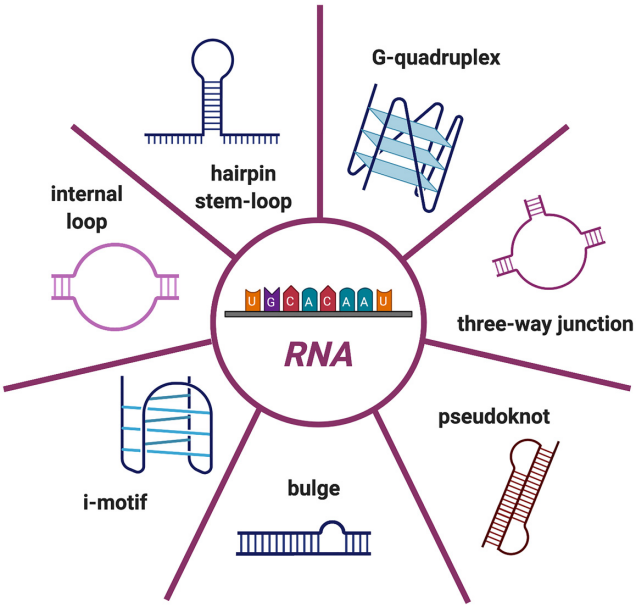

Figure 3. RNA can fold into several secondary structural motifs to fulfill its precise biological role in a specific cellular environment. These include hairpin stem or loops, bulges, three-way junctions, internal loops, pseudoknots, rG4s and i-motifs. It is to note that RNA i-motifs have only been observed *in vitro* (195) to date and their existence *in vivo* is controversial. However, Zeraati *et al*. speculate that the antibody fragment (iMab) (196), besides recognizing DNA i-motifs, also binds to RNA i-motifs in cells with high selectivity and affinity. Created with BioRender.com.

a hierarchical manner. In addition, intermolecular interactions with other molecules, including RNA, DNA and proteins, may result in complex quaternary assemblies (68). Examples of these are triplexes forming between the single-stranded RNA of lncRNAs and the double-stranded DNA of target genes, as is the case of *MEG3* (55). All of the above mentioned mechanisms in which lncRNAs act as scaffold, guide or decoy for proteins are also examples of quaternary assemblies.

A classical way to guess RNA folding at the computational level is based on free energy minimization, that is predicting the most thermodynamically stable structure as that having the minimum free energy (15). However, recent evidence suggests that the real RNA structure is more represented by a Boltzmann ensemble of suboptimal folding states rather than by a unique rigid conformation (69). RNA can indeed undergo dynamic changes between conformational states, with some conformations having higher probability than the others, thus accounting for a large proportion of the RNA population. In addition, some structures may form rapidly and others slowly. In living cells, the ensemble of RNA multiple conformations is dependent on a myriad of environmental and physical factors, such as the presence of cations, heavy metals, crowding agents, pH, temperature, as well as the interaction with ligands/small molecules or proteins (such as chaperones, helicases and target proteins). All these factors (cellular modifiers) can change the relative abundance of two or more pre-existing conformations, thus causing a shift in the ensemble distribution rather than changing or creating new structures (69). This suggests how flexibility in structure not only allows disparate mechanisms of action but also gene expression regulation in response to changing environmental conditions, thus making RNA a molecular sensor. In light of this, traditional techniques including nuclear magnetic resonance, X-ray crystallography and cryo-electron microscopy, that necessitate purified and almost static RNA molecules, are not fully suited to determine the structure of most RNAs, which instead exist in multiple dynamic conformations (70). New experimental procedures involving either enzymes or chemical reagents have emerged in the last years to more properly probe the structure of individual RNA molecules in living cells, as reviewed in (71,72). Importantly, coupling of these methods to next-generation sequencing is starting to provide hints on RNA structure at the transcriptome-wide scale, the so called 'RNA structurome' (73,74). In addition, methods to assess RNA–RNA, RNA–DNA and RNA–protein interactions are increasingly developed, as reviewed in (75,76).

RNA G-QUADRUPLEXES: GENERAL FEATURES AND BIOLOGICAL FUNCTIONS

Among RNA secondary structures found in G-rich sequences, rG4 structures stem from Gs self-assembling *via* Hoogsteen-type hydrogen bonds into a square planar arrangement called G-tetrad. Two or more tetrads stack on top of each other thus constituting the backbone of the rG4 structure, which is then stabilized by the coordination with monovalent cations (Figure 4), usually K^+, due to its highest abundance inside human cells. K^+ cations are located between the plane of two G-tetrads (Figure 4) and coordinate eight carbonyl oxygens, while smaller cations, such as Na^+, are deep-seated in the middle of a tetrad and coordinate only four carbonyl oxygens (16,77).

The use of biophysical methods has allowed to infer the features that determine rG4 conformations; these include the number of G-tetrads, the length/composition of the sequence connecting the tetrads (i.e. the loops), the occurrence of bulges, the sequence in flanking regions and the availability/nature of the central ion (78).

Given the chemical properties of RNA, rG4s are generally more stable than DNA G4s of the same sequence. Moreover, the presence of 2′-hydroxyl group in the ribose sugar of RNA significantly organizes the hydration shell and the hydrogen bond network, thus inducing steric constraints that promote a prevalently parallel rG4 topology, in which all four strands are oriented in the same direction. Recently, however, the Spinach artificial RNA aptamer demonstrated an unpredicted antiparallel conformation (79), suggesting that the range of rG4 topologies may be potentially wider.

Previous findings suggested that most rG4s exist in unfolded state *in cellulo* (80), thus making their existence in living cells a matter of debate. However, several methods have been more recently setup to visualize rG4s *in vivo*: the G4-specific antibody BG4 (81) allowed detection of G4s in fixed cells, while different G4-specific fluorescence and turn-on probes (82–85) allowed to visualize G4s and investigate their folding/unfolding dynamics in living cells. In addition, a transcriptome-wide method to map rG4s in hu-

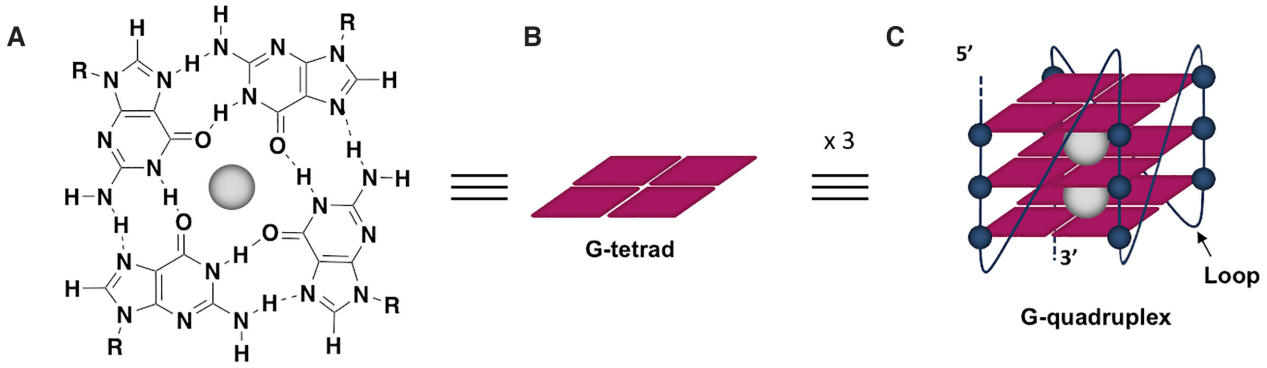

Figure 4. The rG4 structure. (**A**) Chemical structure and (**B**) schematic illustration of a G-tetrad (in purple), composed of four Gs linked together through Hoogsteen H-bonds; (**C**) example of intramolecular parallel rG4. Cations coordinated at the center of the tetrad are represented in gray, while Gs in blue.

man cells has been described, the so-called rG4-seq (86). These findings, along with the discovery of cellular proteins that specifically process rG4s, dampened the skepticism and consolidated a model of a dynamic rG4 folding equilibrium controlled by ions and by a protein machinery whose characterization constitutes an emerging challenge in the field (87). rG4 binding proteins (rG4BPs) are the main rG4 partners: they assist them in all their cellular functions and can alternatively stabilize, unwind or prevent their formation. Due to their chemical nature, RNA-recognition motif (RRM) and Arginine-Glycine-Glycine (RGG) motif are the protein domains most frequently reported to interact with rG4s (88). RRM domain is the most common RNA binding domain in higher vertebrates, shows a typical βαββαβα topology and is present in the HNRNP family of proteins, among which HNRNPF was shown to have some specificity towards rG4 recognition (89) and HNRNPA1 was reported to bind to the loop of the telomeric rG4 (90). HNRNPs also contain RGG or RGG-like domain(s), and together with Fragile X mental retardation syndrome-regulated (FXR) proteins and DEAD box helicases, including DDX3X, DDX17 and DHX36, are the most widely recognized RGG-containing rG4BPs (88). Among G4BPs, helicases have attracted great attention due to their ability to unwind both RNA and DNA G4s in different biological processes, thus affecting genome stability at multiple levels (for a detailed review on helicases see (91–94)). Aberrant rG4BP expression, rG4BP sequestration, altered rG4BP localization or impaired rG4-protein interactions can all dysregulate the finely controlled rG4 equilibrium, thus resulting in pathological conditions; in this regard, manipulation of rG4BPs may be viewed as an opportunity for the therapeutic targeting of rG4s (19). In addition to proteins, stabilizing cations (including K^+, Na^+, Mg^{2+}) and G4 stabilizing ligands (such as PhenDC3, BRACO-19, cPDS, Figure 5) contribute to shift the equilibrium towards the folded state, whereas destabilizing ions (such as Li^+) favor the unfolded conformations, thus increasing the complexity of rG4 folding equilibrium regulation (87).

In light of the intrinsic functional diversity of RNAs, rG4s were found not only in disparate classes of RNAs but also in different functionally distinct regions within a single RNA. In the human transcriptome, rG4s have been mainly described in mRNAs and pre-mRNAs, where they have been shown to regulate gene expression at any step, from transcription (both nuclear and mitochondrial) to protein synthesis, including mRNA processing, transport, localization, stability, turnover, targeting and translation. Indeed, rG4s are particularly represented in the functional regulatory regions of mRNAs, mainly 5′- and 3′-UTRs (95,96), where they modulate gene expression in different ways, sometimes with seemingly opposite roles. For example, selected 5′-UTR rG4s were shown to help translation, as in the case of *VEGF* (97), *FGF2* (98) and *TGFB2* (99), but, in most cases, rG4s in the 5′-UTRs inhibit translation (100).

Although being more abundant in UTRs, rG4s are also present in the intronic (especially in the very 5′ end of the first intron) and exonic regions of pre-mRNAs and, less frequently, in the coding region of mature mRNAs (101). rG4s in pre-mRNA introns or exons can enhance, inhibit or alternate their splicing by recruiting specific rG4BPs or inducing steric interference with regulatory elements in the proximity of the rG4; rG4s in coding regions mainly act as translational repressors (102).

In addition to mRNAs, rG4s have been found in both the telomerase RNA component *TERC* and the telomere repeat containing RNA *TERRA*. The rG4 structure at the 5′-end of *TERC* protects it from degradation (103) and it is recognized and unwound by the RNA helicase associated with AU-rich element (RHAU) (104), thus leading to P1 helix formation that is required for template boundary definition in mammalian telomerase. The telomere repeat-containing RNA *TERRA* is transcribed by DNA-dependent RNA polymerase II from subtelomeric loci in the C-rich strand of telomeric DNA. *TERRA* repeated sequences form multiple rG4s that interact with *TERC* and telomerase, thus inhibiting telomerase extension (105). rG4s are also found in intron lariats, where they control immunoglobulin class switch recombination (106). In recent years, evidence regarding the presence of rG4s in some types of ncRNAs, such as miRNAs (27) and lncRNAs (28), is flourishing, suggesting an even wider role of rG4s in the regulation of gene expression.

RNA G-QUADRUPLEXES IN MICRORNAS

In the context of small ncRNAs, rG4s have been linked to miRNA processing and function, possibly regulating each

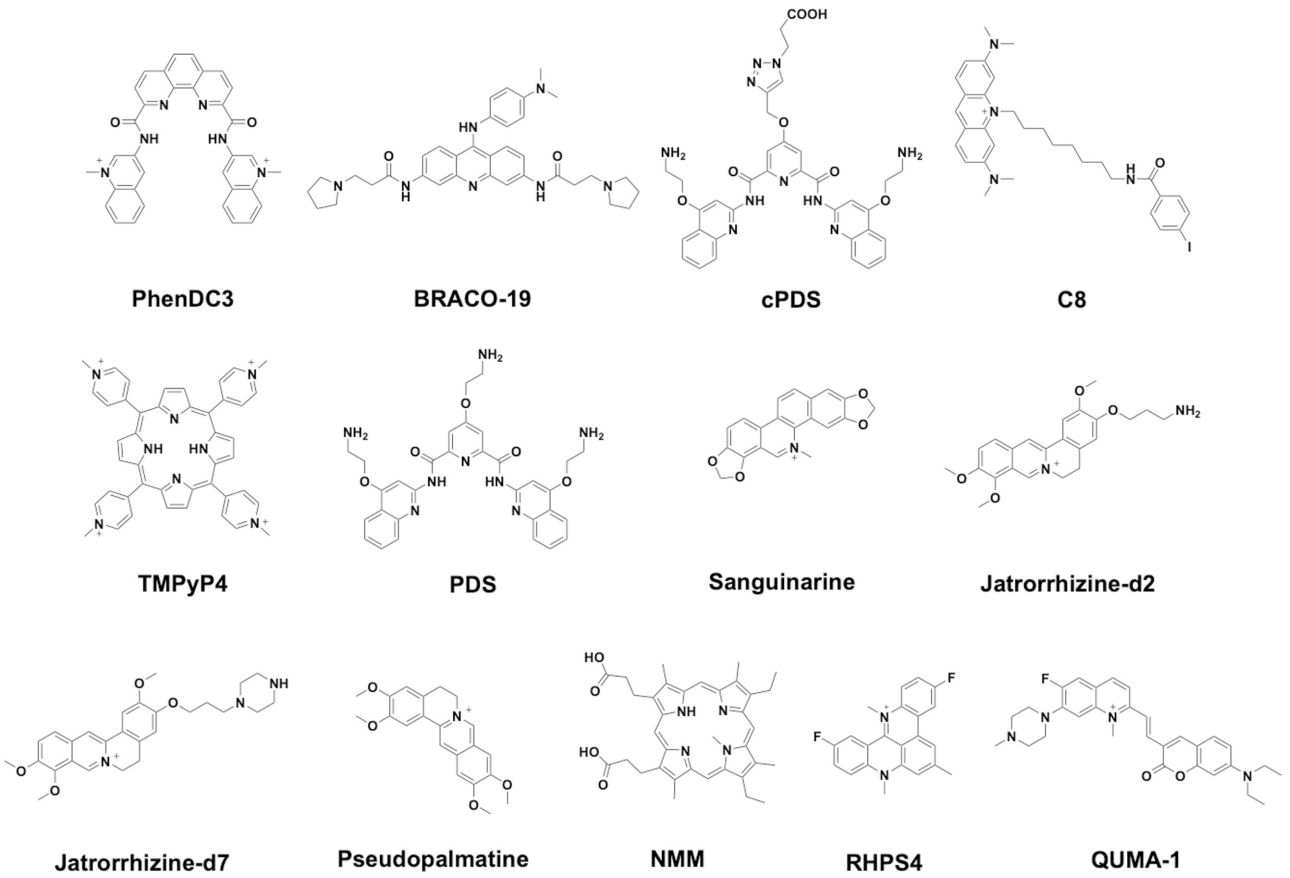

Figure 5. Chemical structures of the reported G4 ligands: PhenDC3, BRACO-19, cPDS, C8,TMPyP4, PDS, Sanguinarine, Jatrorrhizine derivative 2, Jatrorrhizine derivative 7, Pseudopalmatine, NMM, RHPS4, QUMA-1.

step of the canonical miRNA processing pathway (Figure 1B).

RNA G-quadruplexes in primary microRNAs

In 2018, Perreault *et al.* first explored the potential impact of rG4s in regulating the miRNA processing pathway at the pri-miRNA level (107). All the 2007 annotated human pri-miRNAs were screened for their potential to fold into rG4s in the region close to the Drosha cleavage site by G4RNA screener (108), a bioinformatic tool that combines three different G4 predictors: cG/cC (109), G4 Hunter (110) and G4NN (108). Depending on the stringency of the screen, between 9% (strict threshold, positive for all three predictors) and 50% (sensitive threshold, positive for at least one predictor) of all pri-miRNAs were predicted to have putative rG4-forming sequences, which may possibly modulate pri-miRNA processing. *Pri-miR-200c*, *pri-miR-451a* and *pri-miR-497*, the precursors of three known tumor suppressor miRNAs downregulated in many cancer types (111–113), were investigated in more detail for their potential to fold into rG4s. By reverse transcription stalling assay and in-line probing, all of them were confirmed to form rG4s, which in turn positively (*pri-miR-451a* rG4) or negatively (*pri-miR-200c* and *pri-miR-497* rG4) modulated the mature miRNA level, depending on a variety of factors, which included rG4 stability, loop length, rG4 position with respect to the Drosha cleavage site, the rG4 flanking sequence and the microprocessor complex protein composition. In fact, in many cases, the surrounding sequences/environment of the rG4 rather than the rG4 *per se* are critical for its biological effect (114). In addition, RBPs, such as helicases and hnRNPs, which have been reported to be components of the microprocessor complex (115), are particularly relevant: they can interact with, bind to, or even unfold the rG4, in turn impacting on how the rG4 influences pri-miRNA processing.

rG4s found in pri-miRNAs revealed to be quite unstable, thus being detected by *in vitro* methods only in the presence of ligands (107). Considering that RNA is a dynamic molecule existing in a myriad of different conformations that drive its biological function (see the 'RNA structurome' section) and that less stable rG4s are better suited for dynamic folding regulation, the authors proposed a possible dynamic shift between rG4 and hairpin folding, which still needs to be confirmed. This structural dynamic equilibrium has been first studied by Balasubramanian and collaborators, who demonstrated that the conformational preference strongly depends on the relative amounts of metal ions and can be effectively modulated by small molecules, thus highlighting a new and important level of complexity in RNA folding (116). In addition, Perreault *et al.* showed that mature miRNA levels could be exogenously modulated by small antisense oligonucleotides (ASOs) targeting either

the Gs involved in rG4 folding or the competing hairpin structures (107). These data indicate that rG4 motifs could contribute to the regulation of pri-miRNA processing. Such novel role for rG4s may be exploited to counteract the altered pri-miRNA processing found in pathological conditions, in particular in many cancer types where the processing, rather than the expression of the pri-miRNAs, is deregulated (117,118). To the best of our knowledge, the described report (107) is the only one on rG4s in pri-miRNAs. However, these findings may pave the way to future investigations that could both improve our understanding on the biological implications of rG4s and lead to the development of novel therapeutic approaches in pathologically relevant miRNA-linked contexts.

RNA G-quadruplexes in precursor microRNAs

rG4s in pre-miRNAs have been more extensively investigated as compared to those found in pri-miRNAs. In fact, using the bioinformatic tools QGRS mapper (119) and Quadfinder (120), two different studies concordantly proposed that 13-16% of pre-miRNAs harbor at least one rG4 motif in their sequence (121,122). Several studies have also proposed that rG4s in pre-miRNAs exist in equilibrium with the canonical stem-loop structure: their folding unwinds the stem–loop, thus hindering Dicer-mediated cleavage of the pre-miRNA and consequently affecting the pre-miRNA maturation process.

The first evidence of this type of rG4s was collected in 2015, when Arachchilage *et al*. reported a very stable rG4 involved in the maturation of the clinically relevant human *pre-miR-92b* (121). The extensively studied human *miR-92b* is upregulated and involved in drug resistance in non-small-cell lung cancer (123), it promotes hepatocellular carcinoma progression (124), it controls the G1/S checkpoint gene p57 in human embryonic stem cells (125), and it is overexpressed in glioblastomas operating as a potential oncogene *via* Smad3 targeting (126). Moreover it can be employed to differentiate primary brain tumors from metastatic ones (127) and it is considered a clinically significant biomarker for tumor monitoring of synovial sarcoma (128). In detail, a stable rG4 constituted by three tiers and very short loops was identified as alternative secondary structure to the canonical stem-loop of *pre-miR-92b.* By combining a multitude of techniques, including RNase T1 structure mapping, gel mobility shift assay and circular dichroism (CD), rG4 formation was proven to occur only in the presence of physiologically relevant K^+ concentrations, while it was prevented in the presence of Li^+ (121), which has been reported to disfavor rG4 formation without adversely affecting the stability of the stem-loop structure. Since Dicer enzyme is stem-loop structure specific (129), disruption of the stem-loop as a consequence of the ion-dependent rG4 formation was found to inhibit Dicer-mediated maturation of *pre-miR-92b in vivo*, leading to reduction of mature *miR-92b* and derepression of its targets (121). As *miR-92b* silences many important genes, including tumor suppressors, its downregulation is expected to impact on crucial physiological cell functions. In this regard, the equilibrium between rG4 and stem-loop was shifted toward the G4 conformation by a locked nucleic acid (LNA) rationally designed to specifically bind to a region near the 3′-end of *pre-miR-92b* (130). This reduced the amount of mature miRNA, thus resulting in a therapeutic effect, since PTEN tumor suppressor gene expression was rescued in human non-small-cell lung cancer cells (130). Additionally, Cruz and co-workers have recently proven that the fluorescently labelled rG4 forming sequence of *pre-miR-92b* can be also stabilized by acridine orange derivatives (including C8, Figure 5) and used, in a microfluidic platform, as a molecular recognition probe to selectively detect the biologically relevant biomarker nucleolin (NCL) in plasma samples from prostate cancer patients (131). Notably, the rG4-forming sequence within *pre-miR-92b* was found to be conserved in several species including primates, thus highlighting the key biological role of such structure (121). Altogether, these findings unraveled, for the first time, a completely new mechanism of regulation of pre-miRNA maturation at the RNA structural level and paved the way for the development of new strategies that target pre-miRNAs and inhibit their maturation.

Similarly to the above mentioned results, Pandey *et al*. found that rG4s in pre-miRNAs govern the biogenesis of mature miRNAs through a ‘structural interference’ mechanism (122). *Pre-let-7e*, chosen as representative pre-miRNA due to its dysregulation in different cancer types and biological processes (132), was found to form a two stacked rG4 that interfered with Dicer-mediated processing, thus leading to reduction of mature *let-7e* levels (122). To understand and establish the extent of rG4-mediated interference in pre-miRNA processing, cells were treated with the G4 ligand TMPyP4 (Figure 5) and production of mature miRNAs from 100 putative rG4-harboring pre-miRNAs was measured by qRT-PCR. A notable increase in the production of mature miRNAs was observed upon TMPyP4 treatment, possibly arising from the rG4 destabilizing effect of TMPyP4 that shifted rG4s/stem-loop equilibrium toward the stem-loops, which were in turn more efficiently cleaved by Dicer (122). In this regard, it is worth mentioning that TMPyP4 has been initially found to stabilize DNA G4 *in vitro* and *in vivo* (133–137). However, it has been also proven to unfold DNA G4s found in the fragile X mental retardation 1 (*FMR1*) gene (138) and in the anti-thrombin aptamer (139), along with several rG4s (140–142). Curiously, also short hairpin RNAs (i.e. the precursors of siRNAs in the RNA interference pathway), when rich in Gs, were shown to fold into rG4s that can be selectively stabilized by certain classes of small molecules, such as porphyrazines and bisquinolinium compounds, with the ultimate effect of inhibiting Dicer-mediated processing (143).

Balasubramanian and co-workers set up an new method called SHALiPE to map rG4s at the single nucleotide resolution (144). SHALiPE was able to overcome the limits of the canonical SHAPE (145), based on structure-dependent kinetics of acylation of 2′-OH of RNA, which is measured by reverse transcriptase stop at the 2′-O adduct, by introducing lithium ion (Li^+)-based primer extension (LiPE) with 2-methylnicotinic acid imidazolide. Among pre-miRNAs that contain putative rG4-forming sequences, they selected the conserved *pre-miR-149*, the processing of which results in two mature miRNAs, *miR-149* and *miR-149**, which function as oncogenic players in cancer (146–149). They proved that formation of an rG4 structure in *pre-*

miR-149, observed in the presence of K^+ ± the G4 ligand PDS (Figure 5), inhibited Dicer processing *in vitro* (144), thus providing both additional evidence of rG4-mediated pre-miRNA maturation and a revised approach to probe rG4s in the human transcriptome. In addition, Cruz *et al.* have recently demonstrated that the rG4-forming sequence found in *pre-mir-149* can be stabilized by the C8 acridine orange derivative (Figure 5) and used as supramolecular carrier for the cancer-selective delivery of the ligand, in light of the ability of such rG4 to bind to NCL protein overexpressed on the surface of prostate cancer cells (150).

rG4s in pre-miRNAs have also been linked to Alzheimer's disease, the most common age-related neurodegenerative disorder, which is characterized by the accumulation of extracellular beta amyloids (Aβ) that interfere with proper neuron-to-neuron signals at synapses (151). Very recently, *pre-miR-1229* has been shown to have six G-tracts that form a three layer rG4, which coexists in equilibrium with the canonical extended hairpin structure and regulates the production of the mature *miR-1229-3p* (152). *miR-1229-3p* regulates the translation of sortilin-related receptor (SORL1) (153), a protein whose downregulation has been reported to lead to amyloid precursor proteins being sorted into Aβ-generating pathways (154). Interestingly, the single nucleotide polymorphism (SNP) rs2291418 present in a *pre-miR-1229* variant (152) was shown to shift the rG4/hairpin equilibrium towards the hairpin structure, possibly due to the removal of loop–loop interactions that stabilize the rG4. Since rs2291418 is significantly correlated to Alzheimer's disease and mature *miR-1229-3p* production is increased in the rs2291418 *pre-miR-1229* variant (153), the rG4 could be therapeutically targeted for intervention purposes in the future. Ligands that stabilize it could hence be potentially used to decrease the levels of mature *miR-1229-3p* (152). This finding may also have wider implications, since the upregulation of *miR-1229* has been observed in different pathological contexts, including colorectal (155) and breast cancers (156).

A metastable two plane rG4 was also identified *in vitro* and *in vivo* in *pre-miR-26a-1* (157). G4 folding in *pre-miR-26a-1* inhibited mature *miR-26a* expression at the post-transcriptional level, therefore upmodulating *miR-26a* downstream targets and influencing *miR-26a* mediated-biological processes, including insulin sensitivity and hepatic metabolism, which are crucial in obesity (157). Interestingly, this rG4 was found to be highly conserved across almost all mammals, indicating an evolutionarily preserved role in *miR-26a* regulation. In addition, DHX36 was found to bind and unwind the rG4 structure in *pre-miR-26a-1*, thus promoting miRNA maturation. Its downmodulation led to the accumulation of rG4-folded molecules within *pre-miR-26a-1*, thereby impairing *pre-miR-26a-1* processing and *miR-26a* production. Taken together, these data reveal a dynamic DHX36/rG4/*miR-26a* regulatory axis in obesity and provide a new layer of regulation in *miR-26a* maturation *via* rG4.

Overall, all these reports strongly indicate that rG4s in pre-miRNAs act as regulatory switches that may form in response to appropriate stimuli and/or by interaction with protein partners and provide a finely tuned control mechanism for the production of mature miRNAs, in turn affecting the expression of target mRNAs.

RNA G-quadruplexes in mature microRNAs

The investigation of rG4s in mature miRNAs revealed a general and shared role of these structures in impairing miRNA binding to target mRNAs. miRbase (158) was screened for rG4-forming sequences using the G4P calculator developed by Maizels's lab (159), revealing that 152 G-rich mature miRNAs contained at least four runs of two Gs, with the potential to form intramolecular rG4s (160). Among these, the human *miR-3620-5p* was the first mature miRNA proven to fold into a very stable parallel rG4, composed of three layers, in near physiological conditions. A natural alkaloid screened from traditional Chinese medicine, termed sanguinarine (Figure 5), was able to bind with high affinity to *miR-3620-5p* rG4 by π–π stacking on the external tetrads. This resulted in an rG4 with increased stability, which impeded base-paring of *miR-3620-5p* with its target sequences. These results delineate a new strategy to regulate miRNA function by exploiting rG4 ligands and affecting miRNA-mRNA interaction (160).

By electrospray ionization mass spectrometry combined with CD, Yuan and co-authors demonstrated the *in vitro* formation of a stable intramolecular rG4 in the mature *miR-1587*, in the presence of K^+, Na^+ and low concentration of $NH4^+$. Interestingly, under high concentration of $NH4^+$ or molecular crowding environments, *miR-1587* folded into a dimeric rG4 possibly by 3′-to-3′ stacking of two rG4s, each one stabilized by two ammonium ions, with an additional $NH4^+$ positioned between the stacking interfaces, which contributed to the high thermal stability of the dimer. The dimerization was also induced by two synthesized jatrorrhizine derivatives (Figure 5) with terminal amine groups. This study expands our knowledge on high-order rG4 structures and highlights the potential of exploiting rG4 ligands to control *miR-1587*-regulated target gene expression (161). The same research group explored the impact of rG4 formation and stabilization on *miR-1587* biological functions: *miR-1587* binding to its target mRNA *TAGLN*, an early marker of smooth muscle differentiation and tumor suppressor, was impeded after rG4 formation due to inaccessibility of miRNA seed region, and further disrupted in the presence of pseudopalmatine (Figure 5), which improved rG4 thermal stability. As a consequence, miRNA-mediated inhibition of the target gene was dramatically reduced. On the other hand, rG4 disruption by TMPyP4 (Figure 5) improved *miR-1587* function, thus decreasing target gene expression. These data indicate for the first time that rG4s in mature miRNAs can be targeted in cells to enhance or impair miRNA function (162).

rG4 formation in mature miRNAs was also corroborated by Kwok *et al.*, who identified putative rG4-forming sequences in 6% of human miRNAs by bioinformatics analyses and characterized the rG4 structure and interaction with target mRNA at single nucleotide resolution by taking advantage of a refined RNA in-line probing assay (27). They selected a number of biologically relevant miRNAs (*miR-149*, *miR-197*, *miR-432* and *miR-765*) and confirmed, once again, the involvement of rG4s in miRNA-mediated post-

transcriptional regulation and the possibility to use a chemical ligand (NMM, Figure 5) to manipulate rG4 stability and folding.

RNA G-QUADRUPLEXES IN LONG NONCODING RNAS

The first evidence of rG4s in lncRNAs was reported in 2012 by Maiti and co-authors (28). Through an in-house prediction tool, Quadfinder (120), potential rG4-forming sequences having specific patterns in terms of length, loop composition and loop size were found in human lncRNAs. In particular, predicted rG4s were most represented in lncRNAs whose length ranged from 200 to 300 nucleotides and characterized by short loops (28), a feature that had previously been proven to favor rG4 stability (163,164). Some representative sequences were also demonstrated to have high propensity to fold into stable rG4s *in vitro* by biophysical methods, thus corroborating the *in silico* predictions (28). This finding is suggestive of a potentially novel mode of action through which lncRNAs may regulate cellular processes, namely by either direct or indirect rG4-mediated protein titration or sequestration.

A few years later, Kwok *et al.* generated a map of rG4s in the human transcriptome by employing the rG4-seq transcriptome-wide rG4 profiling method, which combines rG4-mediated reverse transcriptase stalling with next-generation sequencing. As expected, the vast majority of rG4s were found in mRNAs, but others were also detected in lncRNAs, including the well-studied metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) and nuclear paraspeckle assembly transcript 1 (*NEAT1*) (86). *MALAT1* is one of the most abundant lncRNAs in normal tissues and it is exceptionally conserved among mammalian species. It is involved in several physiological cell functions, including alternative splicing and regulation of gene expression at the transcriptional and post-transcriptional level. It is also linked to several human diseases: in particular, it is commonly dysregulated in different types of cancer, thus being proposed as a tumor biomarker in several studies (165–167). *NEAT1* is abundant in ovary, prostate, colon and pancreas, where it is involved in the sequestration of paraspeckle proteins and in the nuclear retention of mRNAs (168). It is upregulated in most, if not all, types of solid tumor, such as lung cancer, esophageal cancer and hepatocellular carcinoma (169), and emerging evidence shows that it is also essential in neurodegenerative diseases and viral infections (168). In light of the relevance of these lncRNAs in different biological contexts, identification of rG4s within *MALAT1* and *NEAT1* should prompt the exploration and dissection of the functions and mechanisms of action of such rG4s.

Subsequently, Monchaud's group set up the G4RP-seq protocol, combining G4-RNA-specific precipitation (G4RP) with sequencing, to identify rG4s in human cells (170). Beside proving the widespread existence of rG4s forming at least transiently *in vivo*, they surprisingly observed that lncRNAs, including *MALAT1*, X-inactive specific transcript (*XIST*) and ribonuclease P RNA component H1 (*RPPH1*), avoided rG4-formation and were mainly found in an unfolded state. However, this observation was ascribed to the fact that rG4 formation is actively counteracted by the actions of G4-destabilizing factors such as duplex structures, helicases and other rG4BPs. Moreover, since protein-RNA complex formation is faster than rG4 formation (171), the folding/unfolding kinetics must be also taken into consideration. Even more interestingly, they demonstrated that well-characterized rG4-stabilizing ligands, such as BRACO-19 (Figure 5) and RHPS4 (Figure 5), were able to change the whole rG4 transcriptomic landscape, most notably in lncRNAs (170). This observation strongly indicates that naturally occurring rG4s within lncRNAs can be accessed and stabilized by G4 ligands, suggesting that these rG4s may be druggable, an aspect that could be exploited *in vivo* to modulate lncRNA biological functions.

The same research group also developed two biotinylated biomimetic ligands, termed BioTASQs (BioTASQ v.1 and v.2), as molecular tools to isolate rG4s *in vitro* and *in vivo* through specific affinity pull-down procedures (172). BioTASQs were efficient in corroborating both the prevalence of rG4s in the human transcriptome in coding and non-coding RNAs, including the previously reported *MALAT1*, *XIST* and *RPPH1*, and the changes in their abundance in response to treatments with BRACO-19 (172), suggesting that this new-generation tools can be useful for the future identification of new cellular rG4s, including those found in ncRNAs.

To date, rG4s have been partially characterized from a functional point of view only in three lncRNAs: the aforementioned *NEAT1* (173), the cytoplasmic lncRNA *FLJ39051*, also termed *GSEC* (G4-forming sequence containing lncRNA) (174), and the *REG* family member 1 gamma pseudogene (*REG1CP*) lncRNA (175) (Figure 6). Nascent *NEAT1* transcripts have been indeed demonstrated to bind to the non-POU domain-containing octamer-binding protein (NONO) *in vivo* through conserved rG4 structural motifs (173) (Figure 6A). NONO plays a critical role in initial paraspeckle formation stabilizing *NEAT1* and providing the foundation necessary for the recruitment of the additional protein components needed for the subsequent steps of assembly and maturation (176). To reveal the crucial role of rG4 structures in mediating NONO-*NEAT1* association, the G4-distorting small molecule TMPyP4 was used to disrupt lncRNA/protein interaction (173). Similarly to *NEAT1*, both *FLJ39051 and REG1CP* were reported to be upregulated in colon cancer cells. *FLJ39051* was found to be required for colorectal cell migration/motility by binding to the RNA helicase DEAH box polypeptide 36 (DHX36) (174), which in turn regulates gene expression at the transcriptional and post-transcriptional levels (177,178). The interaction between *FLJ39051* and DHX36 was shown to be mediated by rG4 folding occurring in the region from nucleotides 11 to 26 of *FLJ39051* transcript, thus inhibiting DHX36 ATP-dependent rG4 unwinding activity (174) (Figure 6B). In line with several studies that have proposed the existence of decoy lncRNAs that mimic the consensus target sequences of NA-binding proteins and compete with their targets (179–181), formation of a rG4 structure within *FLJ39051* lncRNA seems to be crucial to exert its possible function as decoy molecule. *REG1CP* is the noncoding member of

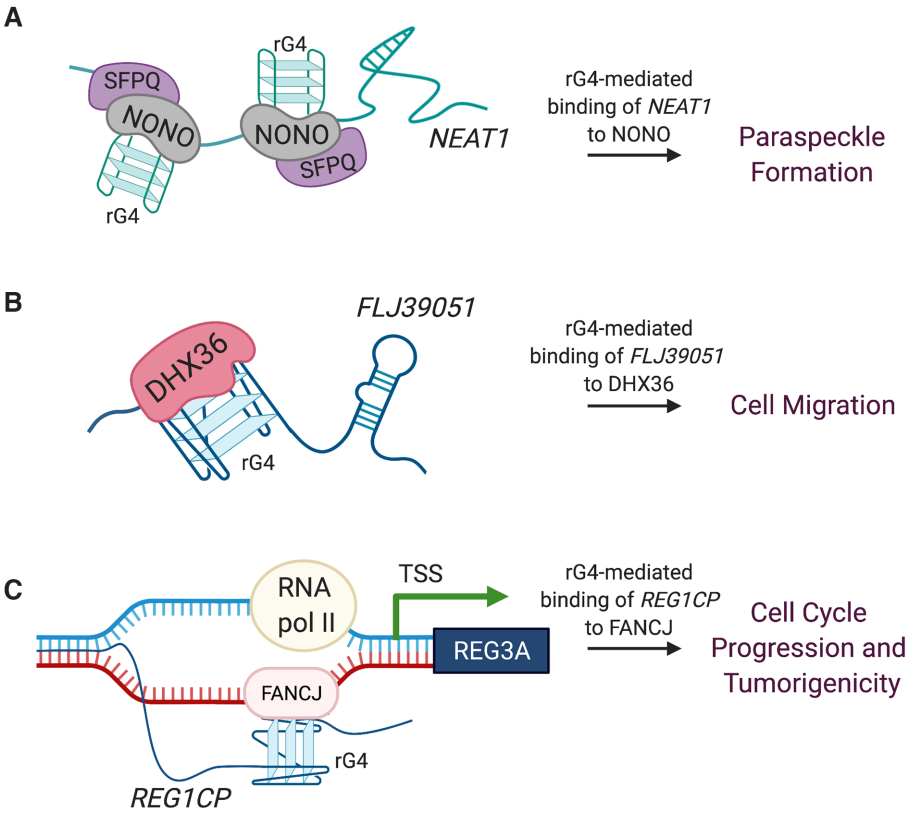

Figure 6. rG4s within lncRNAs influence the interaction between lncRNAs and their targets. (**A**) Nascent *NEAT1* transcripts bind to the NONO proteins through conserved rG4 motifs. The binding of NONO, along with the splicing factor proline- and glutamine-rich (SFPQ), favors the recruitment of additional protein components required for paraspeckle formation and maturation. (**B**) *FLJ39051* interacts with the RNA helicase DHX36 through its rG4 structure and inhibits DHX36 ATP-dependent rG4 unwinding activity, finally promoting motility of colorectal cancer cells. (**C**) *REG1CP* promotes cancer cell cycle progression and tumorigenicity by inducing *REG3A* gene transcription, which occurs as a consequence of the rG4-mediated binding of *REG1CP* to the helicase FANCJ, which is necessary to unwind the double-stranded DNA and derepress transcriptional inhibition. Created with BioRender.com.

the *REG* gene cluster, which encodes for proteins involved in protection against cell death and promotion of cell proliferation (182). High expression of *REG1CP* in colorectal cancer cells correlates with poor patient outcome. Indeed, the lncRNA promotes cancer cell cycle progression and tumorigenicity inducing *REG3A* gene transcription. This occurs through assembling of an enhancer complex relying on RNA-DNA triplex with the *REG3A* gene and recruitment of the DNA helicase FANCJ to *REG3A* core promoter. Binding of *REG1CP* to FANCJ, which is necessary to unwind double-stranded DNA and derepress transcriptional inhibition, occurs only in the presence of an rG4 structure formed within the lncRNA (175) (Figure 6C). It is to note that all the reported lncRNA rG4s apparently favor the interaction with proteins; however, it is reasonable to assume that such rG4s may also influence binding of lncRNAs to other targets, such and RNA and DNA. In this regard, whether rG4s may favor or impair lncRNA/target interaction still needs to be elucidated.

DISCUSSION AND FUTURE PERSPECTIVES

rG4s have long been studied *in vitro* and convincing evidence has been provided to prove both their existence *in vivo* and their regulatory roles in physiological and pathological processes, raising opportunities for therapeutic intervention. Several studies have unraveled association between aberrant ncRNA expression and various human diseases, with miRNAs being the most well-studied class of ncRNAs and lncRNAs being an emerging field of interest. Here, we have summarized the mounting evidence regarding the presence and role of rG4s in ncRNAs (Table 1). Overall, the reports mentioned throughout the review highlight (i) the widespread existence of rG4s in both miRNAs and lncRNAs; (ii) the functional link between rG4 formation within pri- and pre-miRNAs and the outcome of the miRNA biogenesis pathway; (iii) the involvement of rG4s in mediating the binding of miRNAs to their targets and of lncRNAs to interacting proteins.

Despite rG4s form more readily *in vitro* due to their increased thermodynamic stability and reduced steric hindrance, it is now well known that their folding *in vivo* is a transient phenomenon that tightly depends on the biological context and regulation by rG4BPs, ions and G4 ligands. This perfectly fits with the intrinsic nature of RNA, a flexible molecule that undergoes dynamic changes between conformational states in response to environmental and physical factors, and thus exerts its function through structure-based mechanisms. In light of this, it is not surprising that several rG4s in ncRNAs are quite unstable and can be detected only in the presence of ligands or ions. Again, rG4s in pri-miRNAs and pre-miRNAs exist in a finely tuned bal-

Table 1. Overview table summarizing known G4-forming ncRNAs. References to ncRNA G4-mediated mechanism of action, biological output in response to ncRNA G4 folding, molecules used to target ncRNA G4s and identified ncRNA G4 binding proteins are reported

	ncRNA	rG4-mediated mechanisms of action	Biological output	rG4 targeting molecule	rG4 binding protein	Reference
miRNA	*pri-miR-200c* *pri-miR-451a* *pri-miR-497*	prevent the Drosha-mediated cleavage[a]	modulation of the mature miRNA level	ASOs, cPDS, PhenDC3		(107)
	pre-miR-92b	prevent the Dicer-mediated cleavage	decrease of mature miRNA and derepression of its targets	LNA, C8	NCL	(121, 130, 131)
	pre-let-7			TMPyP4		(122)
	pre-miR-149			PDS, C8	NCL	(144, 150)
	pre-miR-1229					(152)
	pre-miR-26a-1			PDS	DHX6	(157)
	miR-3620-5p	impair miRNA binding to its target mRNA	derepression of miRNA targets	sanguinarine		(160)
	miR-1587			jatrorrhizine derivatives, pseudopalmatine, TMPyP4		(161, 162)
	miR-432 *miR-765* *miR-149* *miR-197*			NMM		(27)
lncRNA	*MALAT1*			BRACO-19		(86, 172)
	NEAT1	mediate the binding of *NEAT1* to NONO	initiation of paraspeckle assembly	TMPyP4	NONO	(86, 173)
	XIST			BRACO-19		(172)
	RPPH1			BRACO-19		(172)
	FLJ39051	mediate the binding of *FLJ39051* to the RNA helicase DHX3	inhibition of DHX36 ATP-dependent G4 unwinding activity		DHX3	(174)
	REG1CP	mediate the binding of *REG1CP* to the DNA helicase FANCJ	unwinding of dsDNA and derepression of transcriptional inhibition		FANCJ	(175)

[a]*hypothesized mechanism*.

ance with the hairpin structure, which responds to fluctuations in the intracellular conditions and, ultimately allows proper regulation of gene expression. In this regard, the structural dynamic shift between rG4 and hairpin folding has been proposed to depend on the relative amount of metal ions, especially potassium (116,121,122). Therefore, analysis of potassium ion dependence could be the best starting point when identifying and validating rG4 formation in pri-miRNAs and pre-miRNAs.

It is expected that an increasing number of biological functions will be found for rG4s as soon as all the heterogeneous modes of action of lncRNAs and the mechanistic features of their binding to all possible interactors (DNA, RNA, proteins) will be dissected. It is to note that in some cases (i.e. *MALAT1*) contrasting results have been obtained regarding whether rG4 folding is favored or repressed in lncRNAs. This outcome may be specifically linked to the distinct methodologies used to assess rG4 folding *in vitro* or *in vivo*, as well as to different physiological or pathological conditions that may modify rG4 formation.

The available literature shows that rG4 assembly within miRNAs and lncRNAs can be promoted or restrained by specific antisense oligonucleotides or G4-ligands. This aspect is particularly relevant for molecular studies aimed at investigating the relationships between ncRNA structure and function. Besides in humans, rG4s have also been characterized in several organisms, including protozoa (26), prokaryotes (183), viruses (184) and more recently, plants (185), highlighting common regulatory functions shared between different species and suggesting that similar ncRNA G4-mediated levels of gene expression regulation may be expanded to a wider range of organisms.

The possibility to interfere with ncRNA activity via rG4 stabilization or destabilization may also open new therapeutic opportunities for a variety of human disorders, including cancer, neurodegenerative conditions and infectious diseases. For example, it has been shown that the excessive levels of a given miRNA found in pathological conditions can be reduced through the rG4-based targeting of miRNA processing. Since a single miRNA can influence a multitude of

target genes belonging to a specific pathway, variation in mature miRNA level can in turn result in substantial biological and therapeutic consequences in the so called 'one hit multitarget approach'. In the era of precision medicine, it might be however necessary to modulate the expression level of a single and specific mRNA transcript. In this regard, it is noteworthy that a significant number of miRNA binding sites located in the 3′-UTR of mRNAs could themselves fold into rG4s, thus restricting the accessibility to miRNAs and preventing RISC-mediated degradation (186) (Figure 1C). Such mechanism could be exploited to target single miRNA/target mRNA pairs through rG4 stabilization. Similarly, rG4s can be found not only within lncRNAs but also in their target RNA or DNA. In this regard, the lncRNA *SMaRT* has been reported to bind to an rG4 structure forming within its target mRNA *Mlx-γ*, thus ultimately repressing its translation by counteracting the activity of the DHX36 RNA helicase (187). More in general, rG4s mediating interaction between disease-related ncRNAs and their targets/functional partners may be viewed as interesting platforms for the design of therapeutic interventions. In this context, the use of G4-ligands, i.e. small molecules that selectively recognize G4s over other NA structures, may prove effective. A G4-ligand may act by stabilizing or destabilizing the target G4-forming sequence, and/or preventing its interaction with protein partners: in all cases the result is disruption of the physiological G4 function. So far, many DNA G4-ligands have been developed mostly for anticancer and anti-infective purposes (20,184,188), with two of them reaching clinical trials as anticancer agents (189). In the recent years, research in this field has also advanced in the selective targeting of a single G4 structure (190,191), taking advantage of G4 diversity and polymorphism (78): microarray screening towards thousands of different G4s has demonstrated the possibility to identify small molecules that can preferentially recognize one single DNA G4 (192), with selective binding being rationalized by deep structural investigation of the single G4 structure (193). However, to date, only few ligands have displayed higher affinity towards rG4s, such as QUMA-1 (84) (Figure 5) and cPDS (81) (Figure 5), or have been shown to selectively bind parallel G4s, the typical topology of rG4s, such as the small molecules NMM (194) (Figure 5). In fact, small molecules that selectively recognize just one single rG4 have not been reported so far: rG4s display less diverse structural polymorphism, with loop sequence and length being the only major discriminants among rG4s. For these reasons, finding molecules specific for the single rG4 structure may prove more difficult, but the field is underexplored compared to the DNA G4 field, and thus applications such as microarrays or high-throughput screening methods towards different rG4 structures may provide hits or indication on the feasibility of such an approach. Moreover, rG4s display the advantage of occurring in ssRNA, where G4 formation and accessibility is favored with respect to dsNAs (18), a feature that could allow a further degree of targeting selectivity. As research on ncRNA rG4s expands, new functions of rG4 structures at the cellular level will be made available thus allowing the design and development of unprecedented rG4-mediated therapeutic applications.

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