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REVIEW

New Insights into the Functions of Nucleic Acids Controlled by Cellular Microenvironments

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Abstract

The right-handed double-helical B-form structure (B-form duplex) has been widely recognized as the canonical structure of nucleic acids since it was first proposed by James Watson and Francis Crick in 1953. This B-form duplex model has a monochronic and static structure and codes genetic information within a sequence. Interestingly, DNA and RNA can form various non-canonical structures, such as hairpin loops, left-handed helices, triplexes, tetraplexes of G-quadruplex and i-motif, and branched junctions, in addition to the canonical structure. The formation of non-canonical structures depends not only on sequence but also on the surrounding environment. Importantly, these non-canonical structures may exhibit a wide variety of biological roles by changing their structures and stabilities in response to the surrounding environments, which undergo vast changes at specific locations and at specific times in cells. Here, we review recent progress regarding the interesting behaviors and functions of nucleic acids controlled by molecularly crowded cellular conditions. New insights gained from recent studies suggest that nucleic acids not only code genetic information in sequences but also have unknown functions regarding their structures and stabilities through drastic structural changes in cellular environments.

Keywords Nucleic acids · Quadruplex · Molecular crowding · Phase separation · Membrane-less organelles · Senescence

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1 Introduction

Intracellular environments are densely occupied by nucleic acids, proteins, polysaccharides, small molecules, various ions, and so on—a phenomenon known as molecular crowding. Molecular crowding is crucial for efficient biological processes to take place and may be controlled precisely at specific locations and at specific times [1–12]. Liquid–liquid phase separation (LLPS) is an essential example of a phenomenon induced by molecular crowding environments [13–18]. Various types of biological condensates induced by LLPS are increasingly being observed in various activities, including cell division [17], development [19], neurodegenerative disease [20], oncogenesis [21], and signaling pathways [22–24]. Therefore, studies on the behavior of macromolecules in molecular crowding environments would allow the understanding and regulation of the mechanisms of these biological processes.

Physicochemical properties, such as viscosity, osmotic pressure, and dielectric constant, in a molecularly crowded solution are significantly different from those in dilute solution. Changes in these properties affect both the structure and stability of the nucleic acids [25, 26], in which the affecting factors are hydrogen bonding, base stacking, conformational entropy, hydration, and cation binding [27–30]. Especially in the molecular crowding environment, hydration and cation binding are the main factors affecting the structure and stability of nucleic acids [31–37]. To understand biological processes in cells, it is crucial to investigate the behavior of nucleic acids in these crowded environments, although it remains difficult to evaluate the thermodynamic behavior of intracellular nucleic acids quantitatively and directly. To overcome this difficulty, various cell-mimicking systems using molecular crowding agents have been established to quantitatively assess the thermodynamics of nucleic acids in cells [25, 26]. Here, we review progress in research into the interesting characteristics and functions of nucleic acids in molecular crowding environments, including in vitro and in cells. We also describe recent studies showing the relationship between non-canonical structures of nucleic acids and disease, including cancer, neurodegenerative disease, and senescence that induces age-related disease, from the viewpoint of molecular crowding.

2 New Characteristics of Nucleic Acids in Molecular Crowding

2.1 The Environment Mimicking Intracellular Molecular Crowding Using Cosolutes

Over the last few decades, many studies have evaluated the interesting behavior of nucleic acids in cellular environments using various cosolutes [37–41]. Polyethylene glycol (PEG) is a typical molecular crowding agent that is widely adopted as it has useful properties for experiments owing to its high solubility

in water, relatively low vapor pressure, and ability to be synthesized in a variety of molecular weights. Moreover, there is a correlation between PEGs and cellular environments. For example, it was recently revealed that the chemical environments in the nucleolus are similar to a solution of PEG with an average molecular weight of 200 (PEG200) [12]. Therefore, PEGs have contributed greatly to the accumulation of basic knowledge regarding the thermodynamic stability and structure of nucleic acids in cells. The G-quadruplex is a typical non-canonical structure, whose behavior in cells can be investigated using PEGs. G-quadruplexes are formed from guanine-rich sequences in nucleic acids and are composed of several stacks of G-quartets, in which four guanine bases interact circularly and planarly with Hoogsteen hydrogen bonds. Various topological structures of G-quadruplexes, such as parallel, anti-parallel, and hybrid-type structures, exist based on the direction of the strand, glycosylic torsion angles, and the composition of loops. Previous studies have shown that both DNA and RNA G-quadruplexes are stabilized by PEGs [42–46]. One of the factors that stabilizes G-quadruplexes is the decrease in water activity caused by PEGs because G-quadruplexes are dehydrated during their formation. Other molecular crowding reagents such as 2-methoxyethanol, ethylene glycol, 1,2-dimethoxyethane, 1,3-propandiol, and glycerol can stabilize DNA G-quadruplexes by decreasing water activity in the surrounding solution [42]. Interestingly, PEG200 can induce a topological change from anti-parallel to parallel for telomeric repeat DNA of *Oxytricha nova*, $d(G_4T_4G_4)$ and human telomeric repeat DNA, $d(G_3(T_2AG_3)_3)$ [47, 48]. The topological change in DNA G-quadruplex that is accompanied by dehydration is caused by molecular crowding. 1,2-Dimethoxyethane and 1-propanol efficiently induced topological change of G-quadruplex of the*Tetrahymena* telomeric repeat from hybrid to parallel topology [37]. On the other hand, RNA G-quadruplexes have a monomorphic structure, existing mostly as parallel structures, even in the presence of PEGs.

Recently, the effect of molecular crowding with PEGs on the thermodynamic stability of RNA G-quadruplexes was investigated systematically and quantitatively [49]. The thermodynamic stability at 25 °C ($-\Delta G^\circ_{25}$) of RNA G-quadruplexes with two to four G-quartets and two to four bases in loops was evaluated in the presence of 0–40 wt% PEG200 as a molecular crowding agent [49]. As a result, G-quadruplexes with three or more G-quartets were stabilized by PEG200, while G-quadruplexes with two G-quartets were not. The effect of PEG200 was evaluated quantitatively by $\Delta\Delta G^\circ_{25}$ as the subtraction of $-\Delta G^\circ_{25}$ in the absence of PEG200 from $-\Delta G^\circ_{25}$ in the presence of 40 wt% PEG200. $\Delta\Delta G^\circ_{25}$ for G-quadruplexes with two, three, and four G-quartets is −0.1 to 0.1 kcal mol^{-1}, 2.0–3.2 kcal mol^{-1}, and 3.8–4.0 kcal mol^{-1}, respectively, which indicates that the sensitivity to molecular crowding stimuli is modulated by the number of G-quartets. Based on the results determining the thermodynamic stabilities of RNA G-quadruplexes, novel motifs for exploring G-quadruplexes in transcriptomes are provided. The exploration of G-quadruplexes in human non-coding RNA using the novel motifs has shown that the distribution of G-quadruplex differs depending on the number of G-quartets. G-quadruplexes with two G-quartets are concentrated in signal recognition particle (SRP) RNAs, while G-quadruplexes with three G-quartets are concentrated in

precursor and miscellaneous RNAs [49]. This result suggests that RNA G-quadruplexes with different numbers of G-quartets have specific functions depending on the number of G-quartets, which allows the development of rational strategies for drug discovery that selectively target RNA G-quadruplexes.

The structural transitions between canonical and non-canonical structures should be considered to examine the functions of nucleic acids in cellular environments. Therefore, in addition to understanding the structural transition of non-canonical structures in various cellular mimic conditions [50–53], a method to predict the stability of DNA duplexes is required. The most convenient prediction method based on the nearest-neighbor (NN) model predicts stability from the base sequence of the DNA duplex, RNA duplex, or RNA/DNA hybrid [54–56]. However, it is difficult to apply classical NN parameters to predictions in intracellular environments that are molecularly crowded and have low salt concentrations, since these parameters are determined under conditions of 1 M NaCl. Recently, the NN model for DNA duplexes was shown to be valid even under molecular crowding conditions with 40 wt% PEG200 in 100 mM NaCl [57]. NN parameters for RNA duplexes under molecular crowding with 20 wt% PEG200 were also validated [58]. Moreover, a general method was developed to predict the stability of DNA duplexes in the different cosolutes by considering the relationship between duplex stability and the water activity of the cosolute solution [59]. These improved NN parameters are applicable to predicting the stability of DNA duplexes in solution, which accurately mimics the environments in nuclear and nucleolar environments [12]. It is expected that this method will accelerate studies on biological processes that occur under specific intracellular crowded conditions.

2.2 Compartment Environments

Biomolecules are confined mostly to the lipid membranes of organelles in cells, which are of nanometer-scale volumes. Confinement on this scale could change the properties of biomolecules and produce a heterogeneous environment in cells that differs from that in a dilute solution [10, 60]. To investigate the behavior of DNA inside a confined environment that mimics the interior of an organelle, an experimental system has been established using reverse micelles (RMs) (Fig. 1a) [61]. RMs are simple artificial systems that mimic intracellular environments [62], and their radial dimensions are similar to those of confined aqueous compartments in cells [63].

The structures of human telomeric DNAs, d[AGGG(TTAGGG)$_3$] (22AG) and d[(CCCTAA)$_3$CCCT] (22CT) were examined in bis(2-ethylhexyl)sulfosuccinate (AOT) RMs [61]. Circular dichroism (CD) spectroscopy showed that 22AG and 22CT formed mixed structures of duplex and tetraplex structures of G-quadruplex or i-motif in RMs at 37 °C, although both DNAs formed duplexes only in a diluted solution. Moreover, the conversion between duplexes and tetraplexes depends on micellar size, which is characterized by the parameter $\omega = [H_2O]/[\text{surfactant}]$. The stabilization of G-quadruplexes in RMs is caused by the excluded volume effect [48, 62], in which encapsulation of DNA within nanosized water pools of the RMs

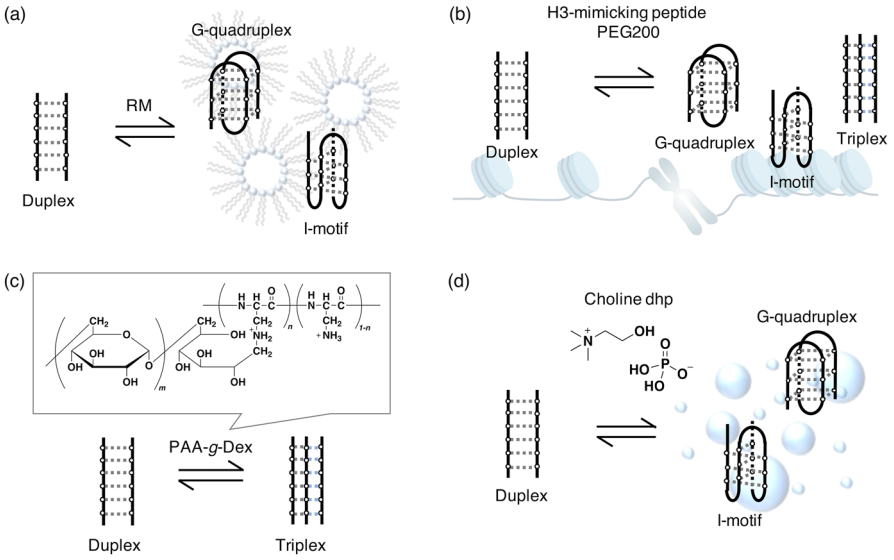

Fig. 1 Behaviors of nucleic acids in cellular mimic environments, such as environments **a** confined by reverse micelle (RM), **b** in the presence of histone H3-mimicking peptide, **c** in the presence of cationic polyelectrolyte, and **d** in the presence of choline dihydrogen phosphate (dhp)

reduces the available volume. Moreover, both PEG200, as a molecular crowding reagent, and a histone H3-mimicking peptide stabilized DNA triplexes and G-quadruplexes compared with a diluted solution (Fig. 1b) [64]. Not only cationic peptides, such as H3-mimicking peptides but also polycationic molecules, such as poly(lysine)-graft-dextran (PLL-*g*-Dex) and poly-(allylamine)-graft-dextran (PAA-*g*-Dex), can stabilize triplex DNA with Hoogsteen hydrogen bonds (Fig. 1c) [65, 66]. These results suggest that non-canonical structures of DNA are preferred not only in confined environments mimicking intracellular organelles but also in the presence of a cationic polyelectrolyte mimicking the environment in the nucleus.

2.3 Ionic Liquids

Choline is a small molecule that is essential for the synthesis of phosphatidylcholine (PS) and sphingomyelin (SPH), which are major components of the cell membrane. Choline is also essential as a precursor for the neurotransmitter acetylcholine and the methyl group donor *S*-adenosylmethionine. The effect of choline dihydrogen phosphate (choline dhp) as a representative ionic liquid (IL) on DNA duplexes was previously evaluated (Fig. 1d) [67]. The melting temperatures (T_ms) of 10-mer DNA duplexes, whose content of A–T base pairs was different, were assessed in a solution of 4 M choline dhp (80 wt% choline dhp) and in a solution of 4 M NaCl. The T_m values of the DNA duplexes in the choline dhp solution increased as the A–T content increased, although the T_m values of DNA duplexes in a dilute solution decreased as the A–T content increased. This result suggests that an A–T base pair is more

stable than a G–C base pair in the choline dhp solution. The ΔT_m values, calculated by subtracting the T_m value obtained from a diluted solution from that in the choline dhp solution, showed that the correlation between ΔT_m values and the A–T contents of the duplexes is linear. The choline dhp solution stabilized the DNA duplex with more than 61% A–T content. In other words, the choline dhp solution destabilized the DNA duplex with more than 39% G–C content. Moreover, Hoogsteen base pairs in triplexes and G-quadruplexes are stabilized in this solution, and, interestingly, the i-motif is more stable than the G-quadruplex in the choline dhp solution [68, 69]. The detailed mechanisms of how choline ions modulate the stability of DNA duplexes with A–T base pairs and triplexes are studied using 20 ns molecular dynamic (MD) simulations [70, 71]. The results from MD simulations showed that choline ions stabilized the A–T base pairs in the DNA duplex by preferentially binding to them in the minor groove [70, 71]. The multiple hydrogen bonds between choline ions and DNA, which are provided by the narrow groove of the A–T base pairs, modulated the stability of DNA duplexes with A–T base pairs. As phosphocholine and choline levels are found to be elevated in various cancer cells [72, 73], the results of the experiments on choline dhp solution suggest that the structural change in DNA as a response to the surrounding environments during oncogenesis could contribute to the regulation of oncogene expression.

3 Revealed Functions of Nucleic Acids in Molecular Crowding

3.1 Transcription Regulated by Non-canonical DNA Structures

The non-canonical structures of triplexes, G-quadruplexes, and cruciforms, which release water molecules during formation, are largely stabilized under molecular crowding conditions, as compared with the canonical duplex structure [33, 42, 74–77]. The fact that non-canonical structures are drastically modulated by their surrounding environments suggests that these structures could play important roles in biological processes such as gene expression and maintenance of genome stability.

Transcription is a biological process that involves nucleic acids, and is the first step in the regulation of gene expression. Transcription is carried out by RNA polymerase along the template DNA strand. Transcription is inhibited by the presence of stable non-canonical structures such as hairpins [78, 79], Z-form [80], triplexes [81], and G-quadruplexes [82–86], on a template DNA. Recently, it has been shown that the various non-canonical structures could cause arrest, slippage, or pause of transcription depending on their stabilities. For example, the effect of the formation of hairpins and G-quadruplexes on transcription elongation has been evaluated quantitatively (Fig. 2a) [87]. Ten different template DNAs, containing hairpin and G-quadruplex, with different thermal stabilities were designed and synthesized. In addition to these 10 sequences, others that contained only the non-structured region were used as controls. The results of gel electrophoretic analysis of the RNA transcribed from multiple turnover transcription at 37 °C showed that the amount of full-length RNA decreased dramatically when templates formed non-canonical structures, whereas transcription

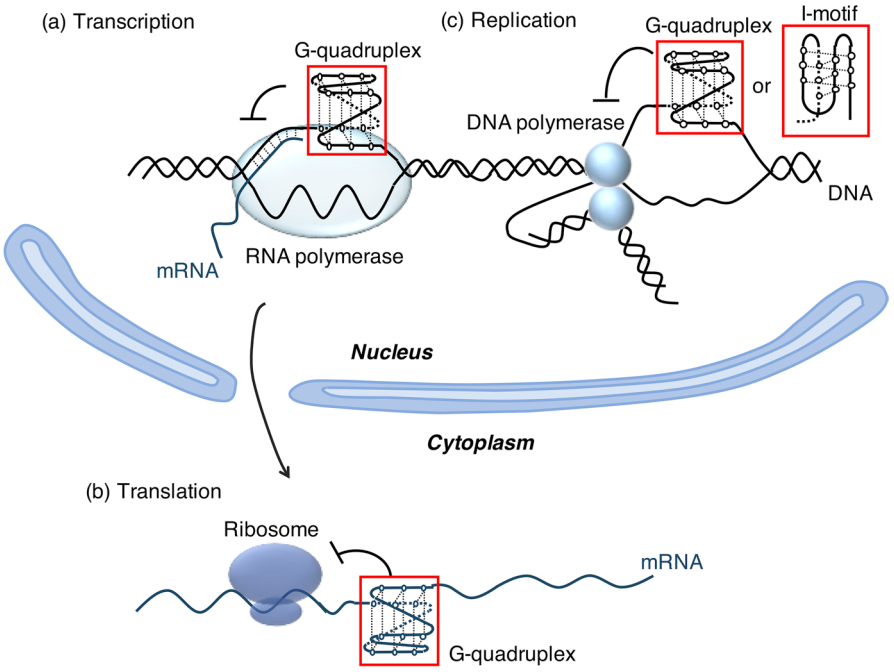

Fig. 2 Effects of DNA and RNA G-quadruplex formation on **a** transcription, **b** translation, and **c** replication

proceeded to the end of the control DNA templates without any structures, resulting in the production of full-length RNA. Transcription of a DNA template with a hairpin formation produced RNA that was approximately 10 nt longer or shorter than full-length due to slippage in transcription. On the other hand, transcription of a DNA template with a G-quadruplex formation produced RNAs shorter than full-length due to the arrest of transcription. Moreover, the arrest in transcription was induced by the G-quadruplex with a stability greater than 14.3 kcal mol^{-1} of $-\Delta G^{\circ}_{37}$. The transcription efficiency decreased with an increase in the stability of hairpins and G-quadruplexes, and the extent of transcription inhibition due to G-quadruplexes was greater than that due to hairpin formation.

As polymerase could cause crowding environments around the template DNA during transcription, resulting in modulation of the stability of the structure on the template [87], the values of stability ($-\Delta G^{\circ}_{37}$) for the non-canonical structures on the templates in the presence of 20 wt% PEG200 as molecular crowding conditions were examined. As a result, the correlation between the thermal stability of non-canonical structures with 20 wt% PEG200 and the transcription efficiency of full-length ($TE_{run\text{-}off}$) was linear. This indicates that $TE_{run\text{-}off}$ decreased with an increase in the stability of non-canonical structures in the presence of 20 wt% PEG200.

3.2 Translation Regulated by Non-canonical RNA Structures

In prokaryotes, shortly after transcription, mRNA is translated by ribosomes. In eukaryotes, in contrast, processing of the precursor RNA, such as capping, splicing, and 3′ polyadenylation, is conducted soon after transcription. RNA folding during transcription, referred to as co-transcriptional folding, could affect translation by acting as a roadblock for ribosomes or processing to interact with a specific protein. To evaluate the environmental effects on RNA folding dynamics during transcription, co-transcriptional G-quadruplex formation was investigated under molecular crowding conditions [88, 89]. Co-transcriptional folding dynamics of G-quadruplex were analyzed by monitoring the fluorescence of G-quadruplex binding ligands in real time. Furthermore, the formation of RNA G-quadruplex, which can transform between hairpins and G-quadruplexes depending on the surrounding environment, was monitored in real time during transcription. As a result of this experiment, RNA was found to preferentially fold into a hairpin structure shortly after transcription and subsequently form G-quadruplex. This result indicated that a balance between the rates of transition from the metastable hairpin-like structure to the stable G-quadruplex structure and translation rate should play a key role in gene expression when metastable structures are formed on mRNA before the formation of the G-quadruplex [90]. It was also demonstrated that the time lag between transcription and translation is also a crucial factor affecting the formation of the G-quadruplex, which suppresses translation both in vitro and in *Escherichia coli* cells [91]. These results show that considering the conformational dynamics of RNA folding is important for understanding the contribution of mRNA structures to controlling gene expression.

The effect of thermodynamically stable non-canonical structures on the regulation of translation has been demonstrated. Many G-quadruplex-forming sequences have been found in the 5′ untranslated region (5′ UTR) of mRNAs [92], and the G-quadruplexes have been shown to regulate translation [93–96]. For example, RNA G-quadruplex-forming sequences found in the 5′ UTR of *NRAS* repressed translation in a cell-free translation system. Not only the effect of G-quadruplex in the 5′ UTR but also that in open reading frames (ORFs) has been demonstrated previously [90, 97–100]. Features of mRNAs such as rare codons and secondary structures in the ORF can cause arrhythmic rather than uniform translation. Rare codons and certain stable secondary structures in ORFs slow or stall translation elongation [101]. Stalling of translation elongation at a specific position on mRNA affects protein expression through modulation of translation initiation of a second ORF or inducing ribosomal frameshifting [101–105]. In addition, there is a correlation between the positions of rare codons and stable secondary structures on mRNA and the flexible linker region in protein structures [106–108]. Since proteins partially fold during translation elongation, the slowing down caused by the rare codons and secondary structures on mRNA likely affects the tertiary structure of full-length proteins. This observation suggests that mRNA sequences and structures could contribute not only to the expression levels but also to the processing of proteins. To further investigate the suppression of translation elongation mediated by the G-quadruplex and how long and where translation is suppressed, the effect of G-quadruplexes in the ORF

on translation was evaluated using a synchronized translation system [97–99]. This method enables a time-course analysis of translation elongation in vitro. G-quadruplex-forming sequences from ORFs of natural genes that form parallel G-quadruplexes were incorporated into an mRNA. Consequently, translation elongation stalled before the G-quadruplex-forming region (Fig. 2b) [99]. Moreover, a mass spectrometric analysis of the translated products showed that the ribosome stalled six or seven nucleotides before the G-quadruplex, suggesting that the G-quadruplex structure blocks further entry of the mRNA into the ribosome [99]. G-quadruplex-forming sequences derived from an ORF of the human estrogen receptor alpha (*hERα*) formed parallel G-quadruplexes and inhibited translation elongation in vitro [98]. When the full-length *hERα* and variants containing synonymous mutations in the G-quadruplex-forming sequence were expressed in cells, translation products cleaved at the specific site were detected, with the amount depending on the thermodynamic stability of the G-quadruplex. These results indicate that G-quadruplexes in ORFs regulate the folding and proteolysis of proteins by slowing or temporarily halting translation elongation.

3.3 Replication Regulated by Non-canonical DNA Structures

Non-canonical nucleic acid structures are involved not only in gene expression but also in genome stability, as mentioned above. DNA replication is a highly regulated process that accurately conveys genomic information throughout the cell cycle. If stable non-canonical structures on DNA are not unwound by helicases before replication, DNA polymerase would stall during replication reaction, leading to the destruction of genomic DNA. Werner's syndrome (a progeroid syndrome) and Bloom's syndrome (a multi-cancerous disorder) lack specific helicases, suggesting a relationship between the effect of non-canonical structures of DNA on replication and disease such as cancer and aging-related diseases [109, 110]. The presence of G-quadruplex structures may induce replication stress and DNA damage [111–113]. In fact, stable G-quadruplex structures induced by peptide nucleic acids inhibit polymerase extension [114]. Recently, the effect of non-canonical DNA structures, such as G-quadruplex and i-motif, on replication were investigated in the presence PEG200 as a molecular crowding reagent (Fig. 2c) [115]. It was found that not only G-quadruplex, but also i-motif formation decreased the rate of replication by Klenow fragment DNA polymerase (KF) and stalled DNA polymerase immediately before the i-motif-forming region. The rate constant of replication (ln$k$) decreased linearly with an increase in the thermodynamic stability ($-\Delta G^{\circ}_{37}$) of non-canonical DNA structures. This result indicated that the extent of inhibition of replication depends on the thermodynamic stability of non-canonical structures. Although the thermodynamic stability of the i-motif is comparable to that of the G-quadruplex and hairpin structures, the i-motif inhibited DNA polymerase progression more efficiently. The slope of the ln$k$ versus $-\Delta G^{\circ}_{37}$ showed that the activation free energy ($\Delta G^{\circ}_{37}{}^{\ddagger}$) required to unwind the i-motif structures was approximately 17- or 3-fold higher than that required to unwind hairpins or G-quadruplexes with mixed topology. The structural change and (de)stabilization effect by the surrounding environment differ

depending on the non-canonical structures. Therefore, non-canonical structures may modulate replication through changes in their structures and thermodynamic stabilities in response to the surrounding environments. The formation of non-canonical structures could inhibit DNA replication, leading to genomic instability, which is involved in development and senescence.

4 Do Non-canonical Structures of Nucleic Acids Induced by the Surrounding Environment Determine Cell Fate?

4.1 Cancer

During tumorigenesis, the chemical environments of cells are significantly changing. The metastatic ability of cancer cells depends on their intracellular chemical environments. One of the typical features of aggressive cancer cells is the overexpression of potassium channels, which induce significantly lower intracellular K^+ concentrations in transformed cells than those in normal cells [116–118]. Moreover, the dielectric constant (ε_r) in cancerous cells is lower than that in normal cells [119]. As the stability of G-quadruplexes depends strongly on the K^+ concentration, gene expression may be modulated in response to changes in the cellular chemical conditions during tumor progression through changes in the stability of G-quadruplexes in oncogenes (Fig. 3a). This is also supported by the presence of many G-quadruplex-forming sequences in the promoter region of oncogenes [120–123].

The effect on transcription of DNA templates with non-canonical structures of chemical environmental changes during tumor progression has been evaluated [124]. To investigate the production of full-length transcripts from G-quadruplex-forming templates in cells, DNA template sequences were inserted upstream of the *firefly luciferase* gene in a plasmid, while the phRL plasmid encoding *renilla luciferase* was used as a control. The plasmids were co-transfected, RNAs were isolated, and full-length transcripts were quantified by quantitative real-time PCR. Analysis of transcriptional efficiency in normal and cancerous cells showed that 1.1- to 1.7-fold higher transcript levels were produced from templates with G-quadruplex-forming sequence in Ras-transformed and highly metastatic breast cancer cells (MDA-MB-231) than in non-transformed and control MCF-7 cells. Importantly, unfolding of G-quadruplex was observed during tumor progression by immunofluorescence with G-quadruplex-binding antibody in cells. These results indicate that potassium ions can suppress the transcription of certain oncogenes through the stabilization of G-quadruplex in normal cells.

The effect of lower intracellular K^+ concentrations should be more critical in the cytoplasm than in the nucleus, as the cytoplasmic environment is influenced more directly by extracellular stimuli compared with the nucleus, which is separated by a nuclear membrane. Since RNA G-quadruplexes are also stabilized by K^+, are more stable than DNA G-quadruplexes, and do not have the complementary strand, it is necessary to consider their functions in the pathology of cancer [96, 125]. G-quadruplex in mRNA regulates the translation of specific genes related to tumorigenesis. RNA G-quadruplexes are found in the 5′ UTR, ORF, and 3′ UTR regions of

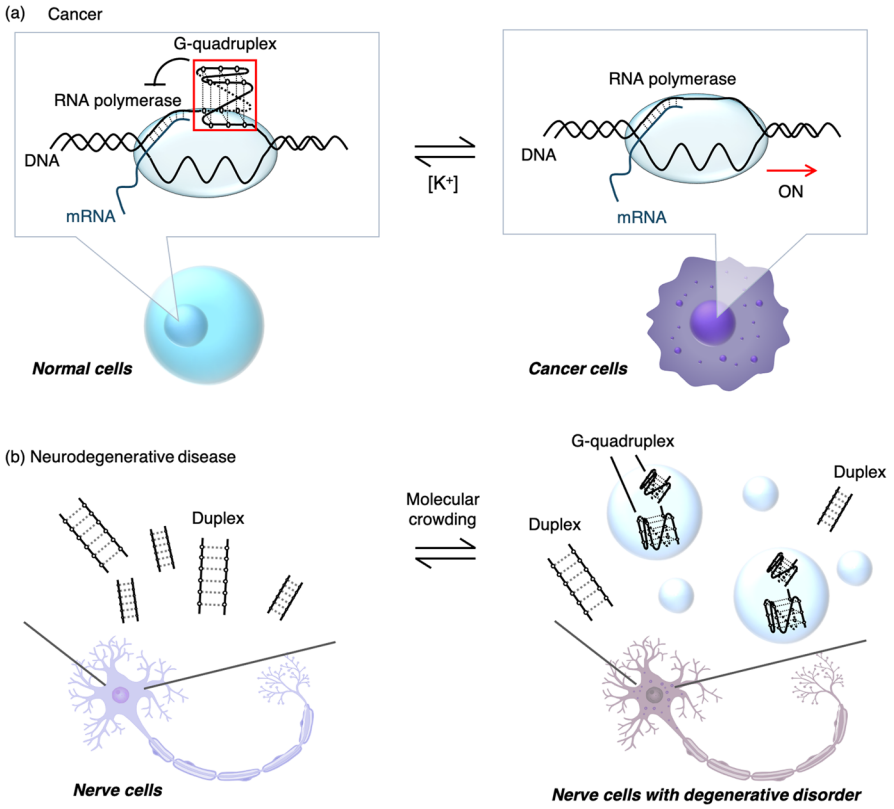

Fig. 3 Possible contributions of non-canonical structures of DNA and RNA in disease onset, such as **a** cancer and **b** neurodegenerative disease. **a** Enhanced transcription by destabilized G-quadruplexes responding to the decrease of potassium ions in cancer cells. **b** Repeat expanded RNA, which can fold into G-quadruplex, could promote RNA gelation by molecular crowding

oncogenes, such as *NRAS*, *MYC*, *MYB*, and *CDK6* [126]. In addition to the inhibition of translation, RNA G-quadruplex can be a signal for translation initiation by the initial ribosome entry site (IRES). G-quadruplex in the 5′ UTR of *FGF2* mRNA could act as a signal for initiation [127]. In contrast, stabilization of G-quadruplex within the IRES of *VEGF* showed inhibition of IRES-derived initiation, which indicated that modulation of stability of the RNA G-quadruplex is a possible strategy for anti-angiogenic therapies [128]. Future studies disclosing the mechanisms of regulation of translation modulated by G-quadruplex in changing chemical conditions are required.

Not only regulation of translation but also interaction between RNA G-quadruplex and oncoprotein could contribute to malignant transformation. A highly proliferative state is one of the features of cancer cells. To maintain the continuous proliferation, various growth factor in signaling pathways are highly activated in cancer cells. Recent studies have highlighted that LLPS plays important roles in signaling

pathways such as the T cell receptor signaling pathway [22], innate immune signaling activated by cyclic GMP-AMP synthetase [23], and signaling by activated cAMP-dependent protein kinase [24]. LLPS provides specific interaction between substrates and enzymes by producing a specialized microenvironment to achieve an efficient reaction. Signaling pathways to maintain proliferation may be accelerated by LLPS, and the non-canonical structures of DNA and RNA may work as scaffolds interacting with proteins. In fact, RNA G-quadruplexes tend to be included in phase-separated condensates [129, 130]. Therefore, small molecules that bind to G-quadruplexes [131, 132] and our knowledge of the effect of molecular crowding on the interaction between small molecules and G-quadruplexes would led to an increased focus on G-quadruplex-targeted anticancer therapeutic strategies [133, 134]. In addition, as a new anticancer strategy, it may in the future be possible to alter the structure of nucleic acids by changing the microenvironments in the cell rather than the structures themselves.

4.2 Neurodegenerative Disease

Non-canonical structures may contribute not only to cancer but also to repeat-expansion disorders, which cause neurodegenerative diseases. RNA repeats transcribed from repeat expansions frequently accumulate and form RNA gels, which are induced by LLPS, resulting in disease onset [135, 136]. The transcribed RNA repeats typically fold into non-canonical structures, such as G-quadruplexes and hairpins [137, 138]. As the structures of nucleic acids are altered by the surrounding molecular crowding conditions in a cell through the stabilization of Hoogsteen base pairing [25], the accumulation of RNA repeats may be affected greatly by the structural changes of RNA responding to changes in the chemical environment during disease onset. It was quantitatively determined that chemical environments, both in vitro and in cells, affect the accumulation of the RNA repeats $(CAG)_n$, $(CTG)_n$, $(GGGGCC)_n$, and $(GGGTTA)_n$, which form hairpin and G-quadruplex structures [130]. Therefore, the accumulation of G-quadruplex-forming RNA repeats is greatly accelerated, while no acceleration is induced by hairpin-forming RNA repeats (Fig. 3b). The change in melting temperature of RNA G-quadruplex (ΔT_m) indicates a correlation with the time required for gelation (t_{max}), indicating that RNA gelation is facilitated by an increase in thermal stability of G-quadruplex. Moreover, RNA gelation increases with a decrease in the dielectric constant (ε_r). t_{max} values are lower under conditions with a lower ε_r, such as those with PEG200 and PEG8000, while gelation progresses at a lower rate in ethylene glycol and glycerol, leading to a relatively higher ε_r, similar to that in the absence of cosolutes. This result suggests that RNA gelation is regulated by electrostatic interaction and indicates that abnormalities in the cellular environment of neurons, especially a decrease in the dielectric constant, could induce RNA accumulation. Approaches that normalize the dielectric constant of neurons may be promising for the treatment of neurodegenerative diseases.

In addition to the accumulation induced by RNA repeats, there may be a relationship between neurodegenerative diseases and aggregates through LLPS. Some

proteins containing stress granules, such as RNA-binding protein FUS [139], TDP-43 [140] and hnRNPA1 [141, 142], have been implicated in the pathology of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Stress granules are membrane-less organelles found in eukaryotic cells; their formation is induced by diverse stresses and regulated by LLPS [143]. By binding with RNA-binding proteins, RNAs are recognized as important elements in the formation of membrane-less organelles. A decrease in the amount of mRNA in the cytoplasm by inhibition of transcription and nuclear export prevents the formation of membrane-less organelles, while an increase in mRNA induces the formation of stress granules [144]. Not only mRNA but also long non-coding RNAs (lncRNA) contribute to LLPS. NEAT1 is an essential component of paraspeckle in the nucleus [145]. As the expression level of NEAT1 changes in the central nervous system in some neurodegenerative diseases, NEAT1 may accompany neuronal damage. Interestingly, G-quadruplex-forming sequences in NEAT1 have been confirmed [146], suggesting that G-quadruplexes in NEAT1 promote LLPS and play roles as molecular hubs for cellular processes during pathology. Although the contribution of RNA to LLPS is drawing attention, the type of RNA and how it induces the formation of a membrane-less organelle remains unknown.

4.3 Senescence

A factor determining the chemical conditions in cells is size. Significant changes in cell size occur during senescence and tumor progression. The size of both senescent and cancer cells is heterogeneous and fluctuates greatly. The size of cancer cells is often smaller [147], while that of senescent cells is larger [148]. Although proper cell size is crucial to maintain cell functions, how cells detect and regulate their size is currently unknown. Moreover, whether the intracellular water concentration increases during the expansion of cell volume in vivo remains unknown. Recently, it was found that expanded young cells showed characteristic phenotypes in senescent cells, such as a delay in cell division, increases in DNA damage, low sensitivity to pheromones, and changes in whole transcription activity in the cells [149]. Importantly, the extent of molecular crowding in the cytoplasm of cells decreases during cellular senescence [149]. This result suggests that changes in molecular crowding through changes in cell size promote cellular senescence. How does a cell express the senescence phenotype by dilution of the cytoplasm? One possibility is the assembly and disassembly of membrane-less organelles, which are likely controlled by changes in the physicochemical environments of cells. It was proposed in 1995 that compartmentalization by LLPS was caused by macromolecular crowding in cytoplasm [13]. Recently, it was suggested that entropy-driven LLPS is enhanced by molecular crowding [150]. Therefore, a decrease in the extent of molecular crowding could prevent the formation of membrane-less organelles or cause the melting of a membrane-less organelle, which may contribute to the expression of various phenotypes to promote senescence. Interestingly, various stresses to cells during the early stage of senescence could induce the formation of stress granules in the cytoplasm; however, the number of stress granules formed during senescence decreases

as senescence progresses [151]. This result suggests that the loss of ability to form stress granules caused by diluted cytoplasm could accelerate senescence. In addition to stress granules, the number of processing bodies (p-bodies) formed in the cytoplasm is increased by specific stresses [152, 153] such as glucose starvation [154] and osmotic stress [154]. P-bodies may also play a role in protecting cells from various stresses during the early stage of senescence.

To consider the effects of cell volume change during senescence, not only on stress granules and p-bodies but also on other membrane-less organelles, characteristics of membrane-less organelles are summarized in Table 1 and Fig. 4. Based on Table 1, membrane-less organelles are categorized into two types. One type contains the membrane-less organelles in the cytoplasm, such as stress granules and p-bodies, whose size and number increase with stress. The other contains membrane-less organelles in the nucleus, which assemble and disassemble throughout the cell cycle. Most membrane-less organelles found in the nucleus assemble at the G_1 phase, increase in size, and disassemble by the mitotic (M) phase in the cell cycle [155–160]. Importantly, the extent of molecular crowding in the nucleus increases from the G_1 phase to the M phase [11]. This observation suggests that the increase in the extent of molecular crowding through progression of the cell cycle may contribute to assemblies of membrane-less organelles in the nucleus. However, it still remains unknown how membrane-less organelles behave in the nucleus of senescent cells, whose cell cycle is arrested. Studies regarding changes in molecular conditions in the nucleus of senescent cells through an expansion of cell volume are required.

Regarding membrane-less organelles in the nucleus of senescent cells, it was shown that the nucleolus expands during senescence [182]. The nucleolus is a membrane-less organelle located in the nucleus that is assembled by LLPS [183]. The nucleolus is the location of ribosome biogenesis, including transcription from rDNA to rRNA and processing of rRNA. As oncogenic stress enhances rRNA transcription and replicative stress delays rRNA processing, increasing nucleolar RNA content and inducing of senescence [182], the increase in pre-rRNA may promote expansion of the nucleolus through LLPS. The increased pre-rRNA may enhance transcription, and newly transcribed pre-rRNA could further accelerate molecular crowding in the nucleus. In fact, an increase in the excluded volume by molecular crowding could promote transcription by increasing the affinity between promoter DNA and RNA polymerase [184, 185].

Interestingly, pre-rRNA, which can contribute to the acceleration of molecular crowding in the nucleus, has a GC-rich sequence prone to folding G-quadruplexes, and G-quadruplexes in rDNA and large subunit rRNA are present in the human genome [186]. Moreover, the exploration of G-quadruplex-forming sequences by recently verified motifs [49] identified 107 G-quadruplex-forming sequences in human 45S pre-rRNA, indicating eight G-quadruplexes per 1 kb. As the frequency of G-quadruplexes in whole human non-coding RNAs is 1.3 sequences per 1 kb, G-quadruplex-forming sequences are concentrated in 45S pre-rRNA. It is well known that RNA G-quadruplexes can be recognized by proteins [96]. For example, a ribonucleoprotein (RNP) in the nucleus, nucleophosmin (NPM1), can recognize RNA G-quadruplexes [187]. Moreover, in conditions of molecular crowding, water activity should decrease compared to diluted solutions, and phenomena that are

Table 1 Membrane-less organelles in the nucleus and cytoplasm through cell cycles

Appearance location	Organelle[a]	Appearance phase	Nucleic acids[b]	Proteins	Biological role
Nucleus	Nucleolus	G_1–G_2 [155] and G_0 [161]	rDNA and rRNA [155]	Fibrillarin [162], Nucleolin [163], and Nucleophosmin	Ribosome biogenesis
	Paraspeckles [164]	G_1–G_2 [156]	NEAT1 [145] and Ctn	PSPC1, NONO/P54NRB, and SFPQ/PSF	Regulation of gene expression
	Nuclear speckles [157, 165, 166]	G_1–G_2 [157]	Poly A^+ mRNA and MALAT1	SRSF	Storage of splicing factors
	Cajal bodies [158]	G_1–G_2 [158]	snRNA, snoRNA [167], and scaRNA [168]	Coilin and SMN	Regulation of snRNP maturation
	Histone locus body [169]	S [170]	Histone pre-mRNA [171]	NPAT, FLASH, and U7 snRNP	Processing of histone pre-mRNAs
	PML bodies [172]	G_1–G_2 [159, 160]	None [173]	PML	Regulation of transcription and protein storage
Cytoplasm	Stress granules [174]	Stressed cells [151, 175]	Poly A^+ mRNA [176]	FUS and hnRNPA1 [177]	Storage of translationally stalled mRNA and proteins of the translational machinery
	P-bodies	G_1–G_2 [178] and stressed cells [152–154, 179]	mRNA [180]	Pdc1, Dcp2, and Edc3 [181]	mRNA processing and decay

[a] *PML bodies* promyelocytic leukemia bodies, *P-bodies* processing bodies

[b] *rDNA* ribosomal DNA, *rRNA* ribosomal RNA, *snRNA* small nuclear RNA, *snoRNA* small nucleolar RNA, *scaRNA* small Cajal body-specific RNA

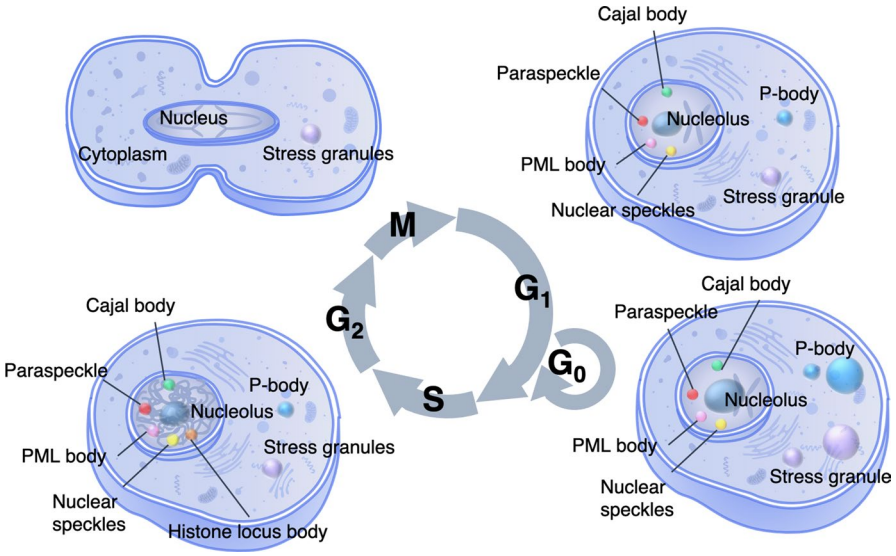

Fig. 4 Assembly and disassembly of membrane-less organelles in the nucleus and cytoplasm through the cell cycle. Most membrane-less organelles, such as nucleolus, Cajal bodies (green), paraspeckles (red), PML bodies (pink), nuclear speckles (yellow), and p-bodies (light blue), appear at the G_1 phase and disappear during the mitosis phase. Histone locus bodies (orange) appears at the G_2 phase and disappear during the mitosis phase. Stress granules appear regardless of phases. Nucleolus (dark blue), p-body (light blue), and stress granules (purple) expand by stressed cells (G_0 phase)

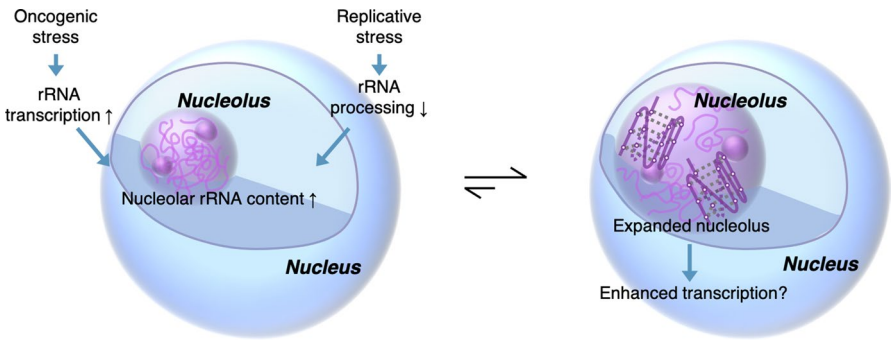

Fig. 5 Hypothesis to promote senescence whereby expansion of the nucleolus could modulate the molecular crowding environment in the nucleolus and further promote transcription of rDNA

accompanied by dehydration, such as the formation of G-quadruplexes and interactions between RNAs and proteins, are preferable. Therefore, interactions between G-quadruplexes in rRNA and nucleolar proteins could change the properties and the extent of molecular crowding conditions in the nucleolus, which may affect the activities of transcription (Fig. 5). Moreover, since sizes of molecular crowding reagents do not depend monotonically on transcription reactions [188], the effects of different sizes and shapes between 45S pre-rRNA and complexes between 45S

pre-rRNA and nucleolar proteins on LLPS and transcription should be considered. Not only the increase in the amount of pre-rRNA but also the formations of G-quadruplexes within pre-rRNA could contribute to expanding the nucleolus. This might be one example in which non-canonical structures of nucleic acids could promote the assembly of membrane-less organelles during an increase in molecular crowding. The hypotheses of new functions for nucleic acids that work as sensors of cell size through changes in molecular crowding are expected to be studied in the near future.

5 Concluding Remarks and Future Prospects

Nucleic acids may prefer non-canonical structures to canonical structures under cellular environments such as molecular crowding and confined environments. Such non-canonical structures of nucleic acids regulate biological processes, such as transcription, translation, and replication. Moreover, recent progress discloses that the non-canonical structures responding to surrounding environments during disease onset can contribute to disease progression. In addition to the regulation of gene expression and genome stability, it is possible that nucleic acids could induce drastic changes in cell morphology, such as the construction of functional complexes and formation of membrane-less organelles, by modulating the strength of interaction with proteins or small molecules. The canonical structures have roles for "maintenance" of information of a genome, and the non-canonical structures have roles for "regulation" of gene expression and genome stability. The significance of the non-canonical structure of nucleic acids in the regulation of cellular function, despite the presence of proteins that control cellular function, could be a quick response to changes in surrounding environments by structural changes in nucleic acids. Drastic changes in the nucleic acids likely contribute to the formation and destruction of membrane-less organelles. Moreover, RNA sequences contribute to modulate the production of peptides that induce LLPS. Repeat expansion in mRNA, which can form hairpin or G-quadruplex structures, affects cell fate through the production of dipeptides that induce LLPS by repeat-associated non-AUG translation [189, 190]. Recently, it has been indicated that Z-form DNA and ADAR2 protein, Z-DNA binding protein, regulate extinction memory in the prefrontal cortex of mice [191]. This result suggests a relationship between the non-canonical structures of nucleic acids and higher brain functions. Immediate structural changes in nucleic acids are convenient for cerebral functions that require quick responses. The unveiling of the underlying mechanisms of cellular functions that can only be achieved by nucleic acids is expected, taking into account the environmental changes that happen in cells. Another possibility is a relic of the RNA world, which is a hypothesis that RNA first emerged and played crucial roles in life activities in the early evolution of life. Considering the energy costs of translation and transport between the nucleus and cytoplasm, it would be preferable for cells to utilize functional RNAs.

It is of growing significance to uncover the functions of non-canonical structures in specific locations and at specific times in various types of cells. These kinds of studies will allow the development of therapeutic strategies for cancer,

neurodegenerative diseases, and aging-related diseases by targeting non-canonical structures of nucleic acids. Nucleic acids flexibly change their structures as a strategy to adapt to changes in their surrounding environments; targeting these specific cells can be a promising option for the treatment of disease in the near future.

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Declarations

Conflict of interest The authors declare no competing financial interest.

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