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Review

G-quadruplex in animal development: Contribution to gene expression and genomic heterogeneity

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ABSTRACT

During animal development, gene expression is orchestrated by specific and highly evolutionarily conserved mechanisms that take place accurately, both at spatial and temporal levels. The last decades have provided compelling evidence showing that chromatin state plays essential roles in orchestrating most of the stages of development. The DNA molecule can adopt alternative structures different from the helical duplex architecture. G-rich DNA sequences can fold as intrastrand quadruple helix structures called G-quadruplexes or G4-DNA. G4 can also be formed in RNA molecules, such as mRNA, lncRNA and pre-miRNA. Emerging evidences suggest that G4s have crucial roles in a variety of biological processes, including transcription, recombination, replication, translation and chromosome stability. In this review, we have collected recent information gathered by various laboratories showing the important role of G4 DNA and RNA structures in several steps of animal development.

1. Introduction

Development is a process that involves continuous global changes of gene expression. It is widely accepted that the information stored in the genes of the DNA is the responsible for the success of development. However, transcription of specific genes does not occur all the time or in all cell types simultaneously. Such genes, stimulated or deactivated, can produce or not proteins or RNAs that alter both the shape and the behavior of each cell. The turn on or shut-off of genes, depend on both particular DNA sequences that bind trans factors (mainly regulatory proteins) and DNA accessibility restricted by the local chromatin structural conformation.

The DNA molecule can adopt alternative structures different from the broadly known helical duplex architecture discovered by Francis Crick and James Watson in 1953. Alternative non-canonical DNA structures, although less familiar than duplex DNA, are not less biologically relevant since they have functions that go beyond storing and transmitting genetic information. In the past few decades, a large number of scientific papers, both computational and experimental (*in vitro* and *in vivo*) have demonstrated that G-rich DNA sequences can fold as intrastrand quadruple helix structures called G-quadruplexes or G4-DNA. G4-DNA is formed by G-quartets or G-tetrads, planar arrays of four guanines paired by Hoogsteen hydrogen bonding and stabilized by monovalent alkali cations, mainly K^+ or Na^+, located in the central cavities of the structure (Fig. 1A). G4-DNA folding frequently occurs at the telomeric regions, ribosomal DNA, immunoglobulin heavy chain class switch recombination region, and in transcriptional regulatory regions of multiple genes and oncogenes (Maizels and Gray, 2013). Genome-wide surveys based on a quadruplex folding rule have been performed, which have identified 376,000 ***p***utative G-***q***uadruplex ***s***equences (PQS) in the human genome, although probably not all of these form *in vivo* (Guilbaud et al., 2017; Hänsel-Hertsch et al., 2016, 2017; Sahakyan et al., 2017). The emerging evidences suggest that G4-DNA have crucial roles in a variety of biological processes, including transcription, recombination, replication, and chromosome stability (Bochman et al., 2012; Cahoon and Seifert, 2009; Cheung et al., 2002; Rodriguez et al., 2012; Siddiqui-Jain et al., 2002). Except for telomeric overhangs, a prerequisite for the formation of G4 is the destabilization of the highly stable B-DNA double-strand helix. The current hypothesis states that transcription bubble generates regions of positive and negative supercoiling, which can be spread along the DNA-helix until reaching sites susceptible of structural transitions. G4-DNA may affect gene transcriptional activity by either upregulation or downregulation (Fig. 1B). Therefore, a shift in DNA structural conformation could be another layer of non-genetic or epigenetic regulation of gene expression and thereby an important determinant of cell fate.

G4 are also found in RNA molecules. Indeed, G-rich RNA sequences in protein coding RNAs (mRNAs), long non-coding RNAs (lncRNAs), and short RNAs such as human telomerase RNA component (hTERC or hTR) and precursor microRNA (pre-miRNA) may fold into G4-RNA. G4s

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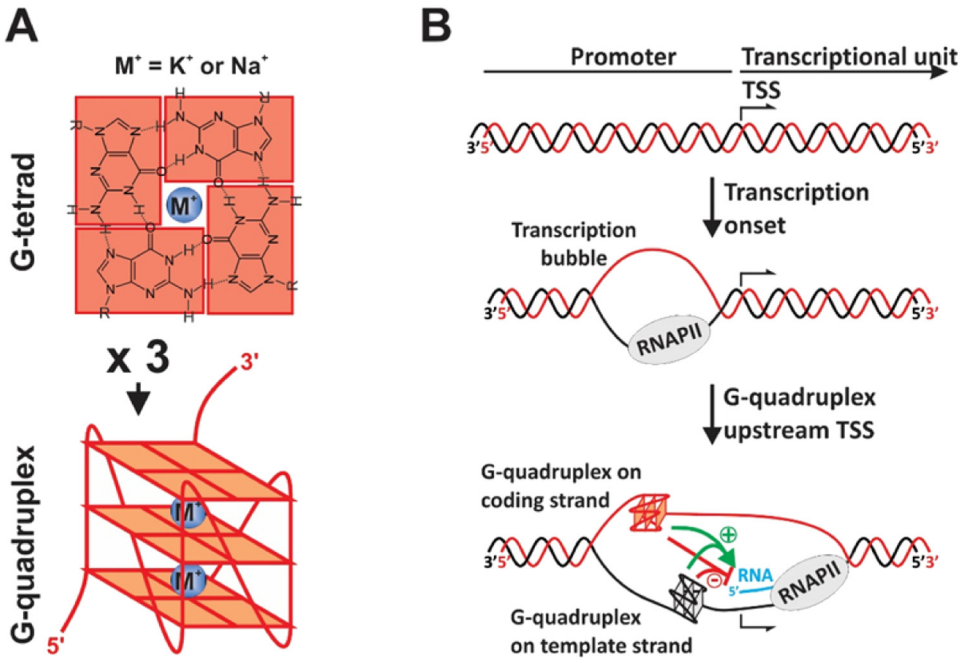

Fig. 1. G4-DNA structure and its roles in transcriptional control. (A) G4-DNA is formed by the stacking of two or more planar arrays of four guanines (G-quartets or G-tetrads) paired by Hoogsteen hydrogen bonding and stabilized by monovalent alkali cations, mainly K^+ or Na^+, located in the central cavities of the structure. (B) Transcription bubble generates regions of positive and negative supercoiling in the promoter region of DNA, which can be spread along the DNA-helix until reaching sites susceptible of G4-DNA folding in both strands that may affect gene transcriptional activity by either upregulation or downregulation.

associated to mRNAs are now widely accepted as critical regulators of pre-mRNA processing (splicing and polyadenylation), mRNA turnover, mRNA location and translation (Millevoi et al., 2012). G4 motifs mapped to ncRNAs, such as lncRNAs (Jayaraj et al., 2012), and to pre-miRNAs suggest a potential of G4-RNA to regulate post-transcriptional gene expression in *trans* and to control miRNA biogenesis (Kwok et al., 2016; Mirihana Arachchilage et al., 2015; Pandey et al., 2015).

Ontology studies revealed that genes containing sequences with high potential to fold as G4-DNA in their promoter regions are related to particular processes, such as development, neurogenesis and cell proliferation/cell-cycle regulation (David et al., 2016; Huppert and Balasubramanian, 2007). However, scant information has been gathered so far about the role of G-quadruplex and its associated proteins in these processes. This issue has become one of the major challenges for scientists interested in fully understanding the role of G4 in developmental biology.

In this review, we have collected recent information gathered by various laboratories showing the important role of G4 structures in several steps of animal development.

2. Role of G4 resolvases in mice spermatogenesis and embryonic development

2.1. Ablation of G4 resolvases causes mice azoospermia

The precisely spatial and temporal assembly and disassembly of G4 may require the action of G4 chaperones and resolvases. Several G4-resolvases have been reported to catalyze the resolution of G4 structures back to the single-stranded DNA or RNA. An increasing number of different diseases have been associated with the inappropriate regulation of G4 resolution showing the potential importance of these structures on development and human health (Cammas and Millevoi, 2016; Maizels, 2015).

The RNA helicase associated with AU-rich element (RHAU, also known as DHX36 or G4R1) is a major source of G4-resolvase activity in HeLa cell lysates (Gueddouda et al., 2017; Lattmann et al., 2010; Vaughn et al., 2005). RHAU, a member of the ATP-dependent DEX(H/D) family, is a highly conserved gene displaying high expression in testis germ cells (Fu et al., 2002). It was recently reported that the generation of germ-cell-specific RHAU knockout mice causes azoospermia. The abnormality begins with the blockage of spermatogonia differentiation, and subsequent meiosis initiation arrest. RHAU deletion affects the proliferation of differentiating but not of undifferentiated spermatogonia. The raise of G4-DNA because of the RHAU ablation in differentiating spermatogonia may trigger both cell apoptosis and low cell proliferative activity, thus eventually disrupting the progression of meiosis. Apparently, higher apoptosis is mediated by the downregulation of *c-kit* in mouse testes (Gao et al., 2015), in agreement with reports showing a transcriptional role of G4 upon the *c-kit* proto-oncogen expression (Patel et al., 2007).

2.2. Ablation of G4 resolvases causes mice embryonic lethality

Inbreeding of $RHAU^{-/+}$ knockout mice produced either $RHAU^{+/+}$ or $RHAU^{-/+}$ but no $RHAU^{-/-}$ offspring. This points out that the $RHAU^{-/-}$ phenotype is embryonic lethal. The finding of $RHAU^{-/-}$ embryos dying at around 7.0 days post-coitus suggests that RHAU activity is required for proper gastrulation. The functional consequences of knockdown of RHAU are impaired telomerase assembly and changes in telomere length (Booy et al., 2012). $RHAU^{-/+}$ mice did not show different phenotype from wild type mice. Identical numbers of born embryos were observed when crossing $RHAU^{-/+} \times RHAU^{-/+}$ and $RHAU^{-/+} \times RHAU^{+/+}$, suggesting that RHAU ablation does not impair embryonic implantation (Lai et al., 2012).

3. G4 in tissues and organs development

3.1. G4-DNA cause genetic heterogeneity during Caenorhabditis elegans organ development

Inaccuracies during DNA replication cause genomic instability, as replication forks are prone to stalling and collapse, resulting in DNA damage. DNA replication enables G4 formation because the DNA becomes single-stranded (Bochman et al., 2012; Lipps and Rhodes, 2009; Rhodes and Lipps, 2015). Although the lagging strand template may be susceptible to the formation of secondary structures because of the discontinuous manner in which it is replicated (Cheung et al., 2002), several studies show that formation of G4 predominates in the leading strand template (Lopes et al., 2011; Sarkies et al., 2012). G4 structures constitute replication-blocking DNA structures, and specialized DNA

resolvases are required for their resolution and for replication to proceed (Mendoza et al., 2016; Sauer and Paeschke, 2017). The persistence of G4-DNA motif may significantly impair DNA replication and repair, leading to DNA nicks, gaps, and breaks. Deletion of the *dog1* gene (the orthologues of human FANCJ, Fanconi anemia group J protein) in *Caenorhabditis elegans* results in the accumulation of small deletions of ~50–300 nt upstream from PQS (Kruisselbrink et al., 2008). In the same genetic background, a single unresolved G4 structure remains stable through multiple divisions with no change in conformation, thus contributing to the generation of genome variability during *C. elegans* development. Indeed, the presence of G4 origins deletions larger than 100 bp in the genome because unresolved replication barriers promote the formation of DNA double-strand breaks (DSBs) (Koole et al., 2014; van Kregten and Tijsterman, 2014). Endogenous DNA lesions cause severe genomic rearrangements during *C. elegans* development (Roerink et al., 2014) similar to the replication-associated mutagenesis found at genomic sequences able to adopt a G4-DNA conformation (Cheung et al., 2002; Koole et al., 2014; Kruisselbrink et al., 2008; Pontier and Tijsterman, 2009). When G4-resolvases fail to resolve a G4-DNA structure, thus favoring the permanence of this DNA secondary structure through multiple mitotic divisions, failed replication across a G4-DNA motif causes single-strand DNA gaps giving rise to DSBs in subsequent cell divisions. The persistence of the G4 on the sister chromatid prevents DSB repair by homologous recombination. An alternative pathway, the polymerase θ-mediated end joining (TMEJ) pathway, generates small insertions and/or deletions (in-dels) on this chromatid, whereas the other chromatid propagates the pre-mutagenic lesion containing a G4 and a single-stranded DNA gap (Fig. 2). In this context, a single G4 in a fertilized zygote could spawn multiple deletions at the same genomic *locus* in descending cells supporting the existence and maintenance of a local pre-mutagenic substrate. This hypothesis seems to be true since individual animals containing differently sized deletions in two typical endogenous G4 *loci* present in the *C. elegans* genome were found. Deletion frequencies within affected animals were at least fourfold higher than the stochastic deletion frequency in the animal population. Importantly, multiple unique genomic deletions mapped at fixed locations, which may reflect stalling events at *in vivo* G4 folds. Data led to the suggestion that persistent G4-DNA motif through multiple S-phases causes genetic heterogeneity during *C. elegans* organogenesis (Lemmens et al., 2015).

3.2. Putative roles of G4-DNA in DNA replication and epigenesis

The early embryonic cell cycles of most metazoans are usually shorter compared to those of somatic cells (Yasuda and Schubiger, 1992). In the majority of animals, embryonic cell divisions are very rapid and highly synchronous (Hörmanseder et al., 2013) including a replication phase (S-phase) and a division phase (M-phase), with short or absent intermediate G1- and G2- (gap) phases (Farrell and O'Farrell, 2014). In early embryos, replication origins are more abundant than in somatic cells (Hyrien et al., 1995; Mahbubani et al., 1992; Sasaki et al., 1999; Walter and Newport, 1997). The high density of active replication origins contributes to the accelerated rate of S-phase in correlation with cell cycle shortening. The rapid early divisions are followed by the mid-blastula transition (MBT), during which the cell cycle elongates and zygotic transcription begins (Tadros and Lipshitz, 2009). This elongation of the cell cycle is coincident with a reduction in the density and synchrony of DNA replication initiation events (Blumenthal et al., 1974; Hyrien et al., 1995; McKnight and Miller, 1977), likely due to the increasing nuclear to cytoplasmic (N/C) ratio and the consequent titration of replication initiation factors (Collart et al., 2013). With the exception of certain lower eukaryotes, replication origins do not have a well-defined DNA sequence (Bell and Stillman, 1992). The presence of G4 has recently been proposed to play an important role in the initiation of DNA replication in chicken cells (Besnard et al., 2012; Cayrou et al., 2012; Valton and Prioleau, 2016). Cooperating with a *cis*-regulatory element, G4s seem to contribute not only to predict replication origins position but also to account for replication origins distribution, usage efficiency and timing (Besnard et al., 2012; Cayrou et al., 2012; Prioleau, 2017; Valton et al., 2014). The RECQL4 helicase was found as one of four replication initiation factors limiting DNA synthesis rates in *Xenopus* egg extracts in an N/C ratio-dependent manner (Collart et al., 2013). RECQL4 does not possess G4-unwinding activity *in vitro* (Rossi et al., 2010), although the N-terminal region binds to G4 structures (Keller et al., 2014). No experimental evidence showing G4s controlling DNA synthesis rates during the early stages of embryonic development have yet been reported. Therefore, it would be interesting to test experimentally the role of both G4s and G4-resolvases in replication control mechanisms during animal embryonic development.

G4s have also been implicated in the induction of epigenetic instability (Cheung et al., 2002; Koole et al., 2014; Lopes et al., 2011;

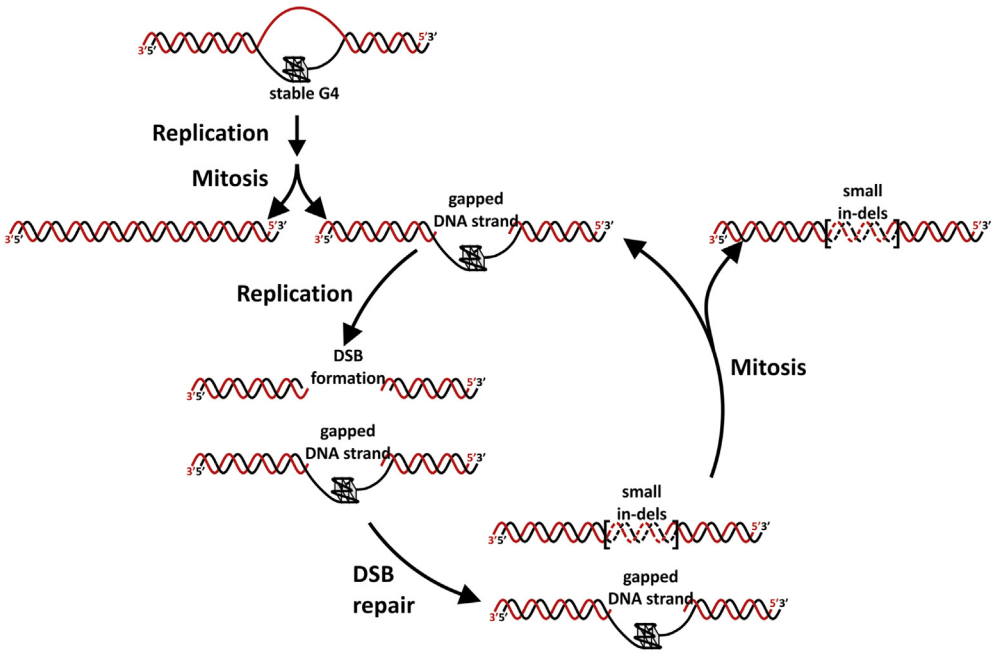

Fig. 2. Roles of G4-DNA in genetic heterogeneity generation. Stable replication-blocking G4 causes ssDNA gap across the G4-containing strand. Mitotic inheritance of stable G4 originates a new ssDNA gap and a double-strand break (DSB). G4 prevents DSB repairing through homologous recombination. DSB is thus repaired *via* TMEJ, generating small in-dels and genetic heterogeneity among proliferating cells. Adapted from Lemmens et al. (2015).

Piazza et al., 2012; Ribeyre et al., 2009; Sarkies et al., 2010, 2012). Cultured avian cells lacking G4 resolvases exhibit epigenetic instability in the vicinity of G4 (Sarkies et al., 2010, 2012) because G4 motifs replication uncouples the DNA helicase and DNA polymerase due to impairment of DNA polymerase advance, resulting in the formation of post-replicative gaps distant from the replication fork. These gaps are replicated either by the release of the blocked fork or by a fork arriving from the other direction, resulting in a tract of biased new histone incorporation. Therefore, newly synthesized histones that do not bear the parental post-translational modifications are incorporated interrupting the flow of epigenetic information from the parental to daughter DNA strands (Papadopoulou et al., 2015; Sarkies et al., 2010, 2012; Schiavone et al., 2014). Nonrandom segregation of sister chromatids with different epigenetic marks, proposed by the 'silent sister' hypothesis, may be a mechanism to direct differential gene expression and cell fate in stem and progenitor cells, and may be a relevant mechanism for G4 regulated cellular differentiation during development (Lansdorp, 2007). Whilst the consequences of G4s in the epigenetic information inheritance may be relevant for cellular commitment and differentiation during development, it is worth noticing that results reported in this topic were obtained in a cell line-based model system. A comprehensive and integrated understanding of the mechanism of altered epigenetic information inheritance induced by G4s remains elusive.

3.3. G4 might mediate cardiovascular lineage commitment

LncRNAs have emerged as important regulators of development and disease. Studies have highlighted diverse cellular roles for lncRNAs across eukaryotes such as X chromosome inactivation, genomic imprinting, cell-cycle regulation, embryonic stem cell pluripotency, and lineage commitment (Flynn and Chang, 2014; Lee et al., 2016). The mouse lncRNA Braveheart (*Bvht*) has been reported as acting in *trans* regulating cardiovascular lineage commitment (Klattenhoff et al., 2013). The *in vitro* study of the secondary structure of the full-length *Bvht* lncRNA revealed that the transcript is organized into a modular structure including a 5′ asymmetric G-rich internal loop (AGIL). Deletion of this loop in mouse embryonic stem cells impairs cardiomyocyte differentiation, likely due to the failure to activate key cardiac transcription factors during the transition from nascent mesoderm to the cardiac progenitor state. Different algorithms predict that the *Bvht* asymmetric G-rich internal loop can form a G4 structure. Interestingly, this G-rich loop is specifically bound by CNBP (Xue et al., 2016), a zing finger protein highly conserved in vertebrates (Calcaterra et al., 2010) that binds G-rich single-stranded DNA and RNA (Armas et al., 2008). CNBP acts as a nucleic acid chaperone and can control the formation of G4 structures in both DNA and RNA molecules (Armas et al., 2008; Benhalevy et al., 2017; Borgognone et al., 2010; Chen et al., 2013). Results suggest that *Bvht* influences the commitment of cardiovascular lineage cells by antagonizing CNBP, which may function as a negative regulator of this process (Fazal and Chang, 2016). Thereby, it is possible that *Bvht* and CNBP function together to regulate cardiac cell differentiation by controlling G4 structures (Fig. 3).

Cnbp homozygous mutant mouse embryos exhibit severe forebrain truncation and heterozygotes display various craniofacial defects (Chen et al., 2003). Further studies performed in zebrafish revealed that CNBP is required for proper neural crest expansion and the development of skeletogenic derivatives (Weiner et al., 2007, 2011; Sdrigotti et al., 2017) and that its overexpression prevents severe craniofacial aberrant phenotypes in a Treacher Collins-like zebrafish model (Porcel De Peralta et al., 2016). A substantial reduction in cell proliferation was observed in the cephalic regions of $Cnbp^{(-/-)}$ mouse embryos at gastrulation and neural-fold stages. In these regions, *c-Myc* expression was not detected, suggesting that CNBP targets *c-Myc* in rostral head formation (Chen et al., 2003). Human *c-MYC* expression is enhanced by CNBP (Michelotti et al., 1995) likely by binding to a G4 found at the proximal promoter region (Armas et al., 2008; Chen et al., 2013). *c-Myc* is a key gene in the gene regulatory network governing both the neural crest formation and the size premigratory neural crest stem cells (Barembaum and Bronner-Fraser, 2005; Bellmeyer et al., 2003; Kerosuo and Bronner, 2016). Therefore, it is tempting to speculate that the role of CNBP in embryonic development is performed, at least in part, through its capability to act upon G4 folding/stability.

4. G4 and the embryonic development of vertebrates

During embryonic development, gene expression is orchestrated by specific and highly evolutionarily conserved mechanisms that take place accurately, both at spatial and temporal levels (Wolpert, 1994). An intricate array of *cis*-regulatory sequences controlling individual genes leads to a fine-tuning of gene expression in different developmental processes, which in turn may set up specific phenotypes in both health and disease. The last decades have provided compelling evidence that not only protein-mediated transcriptional control but also chromatin state play essential roles in orchestrating all stages of embryonic development (Misteli, 2009; Rajapakse et al., 2009). In this context, G4-DNA structures have been recently suggested as additional members of the intricate array of *cis*-regulatory elements responsible for the transcriptional control during embryonic development (David et al., 2016). Several *in silico* studies using different algorithms had revealed the enrichment of PQS in the promoter regions of developmental regulated genes (Huppert and Balasubramanian, 2007). The presence of several of these PQS is conserved in vertebrates, not in primary sequences but in the positional level (David et al., 2016), suggesting a functional selection of G4-DNA motif likely participating in accurate transcriptional regulation throughout development. A major challenge was to demonstrate experimentally the existence of G4 *in vivo* and that G4-DNA formed in promoter regions of genes required for proper embryonic development are indeed involved in their gene expression regulation. Results from 2D NMR spectroscopy probed that G4-DNA exist in a folded state inside living *Xenopus laevis* oocytes for relatively long periods of time, suggesting that G4-DNA are resistant to numerous enzymatic activities and, at least for this case, can be consider as long-lived species (Salgado et al., 2015). Another attempt to test *in vivo* the existence of a G4 was performed by microinjecting zebrafish embryos with a small G4-stabilizing-ligand (furan-based cyclic homo-oligopeptide) and assessing the effect upon embryonic development. The presence of ligand inhibited the transcription of *cdh5* (N-cadherin), a gene required for proper vascular and cardiovascular development that contains a G4 in the upstream promoter region. Besides, treated embryos showed malformed and mispatterned intersegmental vessels along with reduced blood flow, thinning of vessels, and a reduced heart rate in the affected embryos, recapitulating phenotypic alterations observed when microinjecting N-cadherin antisense morpholino (Agarwal et al., 2014).

Additional evidences of the role of G4 *in vivo* on development were reported by David et al. (2016) assessing *in vivo* in zebrafish the behavior of a set of genes containing conserved PQS in their proximal promoter region (PPR; arbitrarily defined as the 1000 bp upstream from the TSS) and that had been identified as required for proper embryonic development. Among the potential candidate genes, *nog3*, *fzd5* and *col2a1* were selected for further studies because well-described developmental phenotypes had been reported as consequence of their depletion by different experimental approaches (Cavodeassi et al., 2005; Gansner and Gitlin, 2008; Mangos et al., 2010; Ning et al., 2013). The hindering of G4 folding in the PPR of these three genes was achieved by injecting in one-cell zebrafish embryos small antisense oligonucleotides (ASO) complementary to the PQS thus competing with G4 formation and favoring double-stranded DNA formation (Fig. 4A). Injected embryos were allowed to develop until appropriate developmental stages at which phenotypes, relative level of transcripts, and spatial and temporal expression pattern were assessed. Both a decrease in the levels of transcripts of the three genes and a reduction in their expression

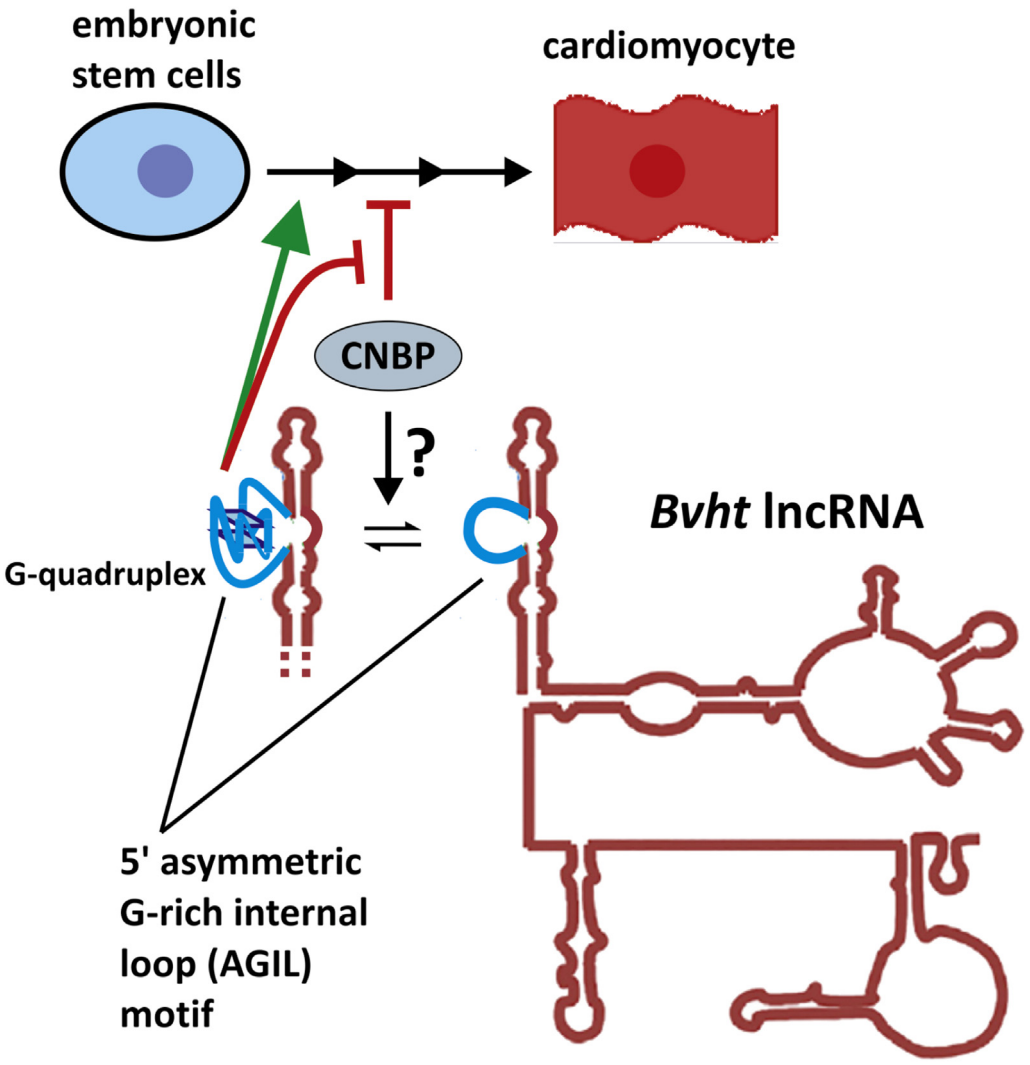

Fig. 3. Potential role of G4 in cardiomyocyte commitment. In *Braveheart* lnc-RNA, an 11-nt asymmetric G-rich internal G-loop (AGIL) with potential to fold as G4 is essential for cardiomyocyte differentiation and interacts with CNBP, a putative antagonizing of AGIL, to coordinate cardiac-lineage commitment.

territories were observed along with the appearance of phenotypes mimicking those ones reported for *nog3*, *fzd5* and *col2a1* morphants or mutant zebrafish specimens. Briefly, the intervention of the G4 present in the PPR of *col2a1* affected notochord development and 4 days post-fertilization staged larvae displayed significant shortening in the body length (Fig. 4B). Embryos displaying eyes reduced in diameter were observed in the presence of ASO complementary to the PQS located to the *fzd5* PPR (Fig. 4C). The blockage of G4 formation in the PPR of *nog3* affected the development of pharyngeal craniofacial cartilages; phenotypes were rescued by the injection of *in vitro*-synthetized *nog3*-mRNA (Fig. 4D).

The zebrafish genome possesses lower number of PQSs within PPRs than the mouse and the human ones (David et al., 2016). However, 50% of PQS present in PPRs identified in zebrafish are conserved in mouse and human orthologous genes, thus suggesting that similar mechanisms of gene expression control is taking place in vertebrate embryonic development. Mammalian PPRs contain higher number of PQSs due to a genome CG-enrichment along evolution; thereby, it is likely that the contribution of G4 to the transcriptional regulation of mammalian developmental related genes is even more relevant than in zebrafish (David et al., 2016).

5. Main conclusions and perspectives

In the last years, there have been notable advances in the understanding of the molecular mechanisms governing gene expression during embryonic development. DNA and histone modifications regulate transcriptional activity and thus represent valuable targets to reprogram the activity of genes. The basic principles of epigenetic reprogramming in embryos have been known and studied for many years; however, major aspects remain enigmatic. Results generated in an avian differentiated-cell system showed that G4-mediated DNA replication blockage triggers local epigenetic plasticity, thus favoring transcriptional activity reprogramming of certain genes. However, do G4s play similar roles during the deep epigenetic reprogramming happening during gametogenesis and immediately after fertilization? Whilst this hypothesis is very exciting, the scarcity of data generated until now in multicellular organisms development does not allow assuring such G4s roles.

Here we have pointed out that G4 collaborate in the replication origins positioning and one of the replication factors identified as participants in the replication rate lowering belongs to the Rec G4-resolvase family. Two questions arise from these observations: 1) Are G4s involved in establishing the increased number of replication origins during early embryogenesis? 2) Are G4 and G4-resolvases involved in the lowering of replication origins (and the consequent slowing of the

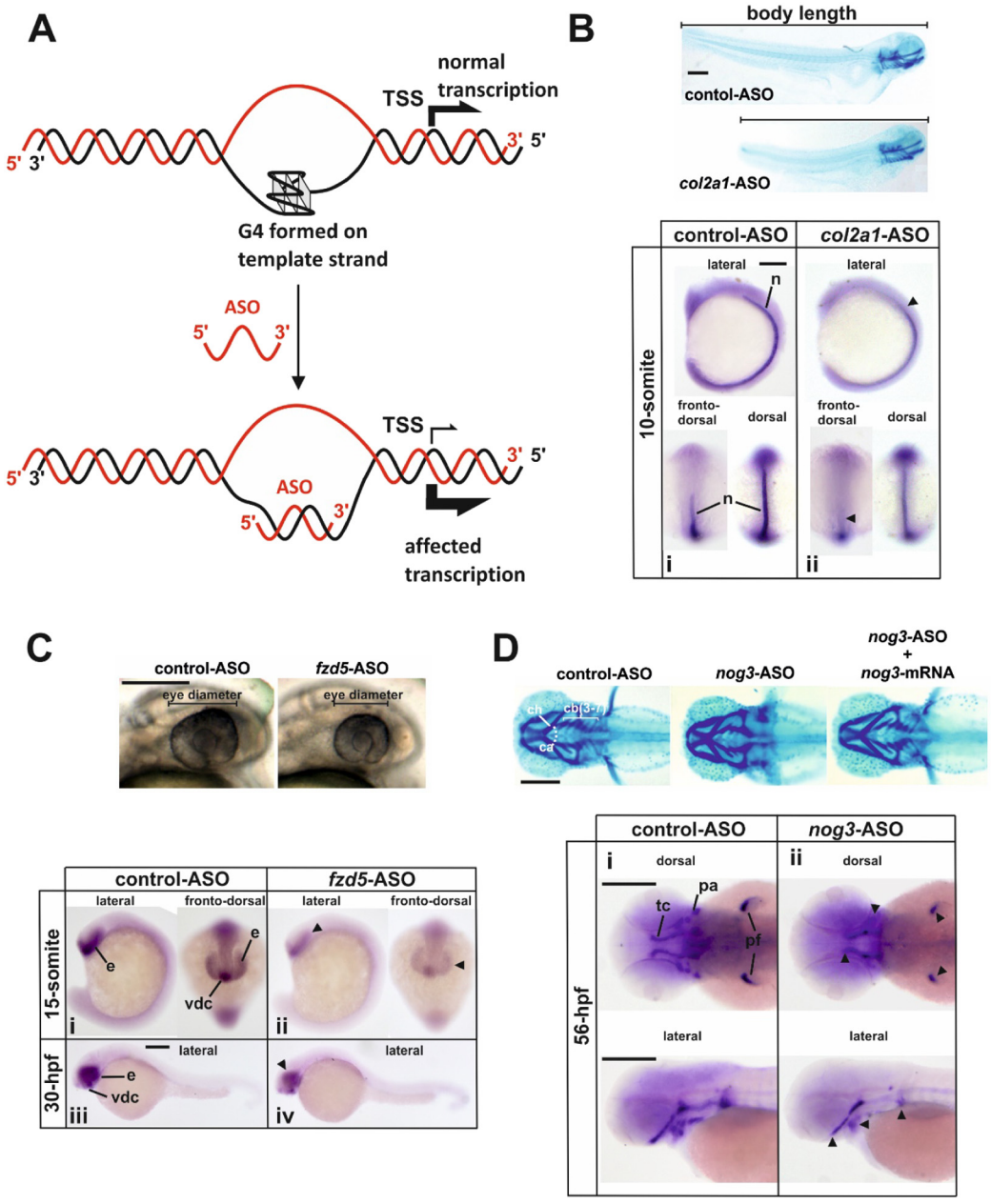

Fig. 4. G4 *in vivo* function during zebrafish development. (A) Approach for intervention of G4 controlling transcription using antisense oligonucleotides (ASO) complementary to the PQS to compete with G4 formation. (B) Effect of *col2a1* G4 disruption by *col2a1*-ASO injection in zebrafish embryos. Upper panel shows 4 days post-fertilization (dpf) staged larvae injected with control or *col2a1*-ASO and stained with Alcian Blue (AB) to determine the body length. Lateral views, anterior to the left. *col2a1*. Lower panel shows whole-mount *in situ* hybridization (WISH) detecting *col2a1* mRNA in 10-somite staged embryos injected with control (i) or *col2a1*-ASO (ii). Arrowheads point regions of lower expression. In lateral views anterior is to the left, and in dorsal and fronto-dorsal views anterior is up. n: notochord. (C) Effect of *fzd5* G4 disruption by *fzd5*-ASO injection in zebrafish embryos. Upper panel shows eye diameter of 30 h post-fertilization (hpf) staged embryos injected with control or *fzd5*-ASO. Lateral views, anterior to the left. Lower panel shows WISH detecting *fzd5* mRNA in 15-somite (i and ii) and 30-hpf (iii and iv) staged embryos injected with control (i and iii) or *fzd5*-ASO (ii and iv). Arrowheads point regions of lower expression. In lateral views anterior is to the left, and in fronto-dorsal views anterior is up. e: eye; vdc: ventral diencephalon. (D) Effect of *nog3* G4 disruption by *nog3*-ASO injection in zebrafish embryos. Upper panel shows AB staining of craniofacial cartilages in 4-dpf staged larvae injected with control, nog3-ASO or nog3-ASO + *nog3*-mRNA. Ventral views, anterior to the left. Lower panel shows WISH detecting *nog3* mRNA in 56-hpf staged larvae injected with control (i) or *nog3*-ASO (ii). Arrowheads point regions of lower expression. In lateral and dorsal views anterior is to the left. ca: ceratohyal cartilages angle; cb (3–7): ceratobranchial cartilages 3 to 7; ch: ceratohyal cartilage; pa: pharyngeal arches; pf: pectoral fin; tc: trabeculae cranii. Scale bars represent 200 μm.

replication rate) observed after MBT? Further experimental data generated in multicellular living organisms are needed to answer these questions.

On the other hand, several works have highlighted the contribution of imprinting, pre-mRNAs post-transcriptional modifications, microRNAs and lncRNAs in the understanding of the molecular mechanisms governing gene expression during embryonic development. Nonetheless, the role of the DNA secondary structures upon transcriptional control during development has been scarcely explored. To our knowledge, all the relevant existing literature relative to the role of G4 in development has been discussed in this review. Most of this consists of indirect evidences gathered from mutations or ablations of G4-resolvases. Several proteins were identified capable of resolving, stabilizing or binding G4 structures. Therefore, it is interesting to consider

the implications of G4–protein interactions as a pair. In this context, variations in protein G4-binding domains may contribute to the fine-tuning of transcriptional control during development. The work reported showing the role of G4-DNA by ASO intervention during zebrafish development is the unique so far showing straightforward participation of the G4 in the transcriptional control of specific genes during embryonic development. The extensive knowledge recently generated on the importance of G4 s in cellular biology, in addition to the enrichment of PQS in the promoter regions of key developmental genes, suggest a relevant role of the G4 in the transcriptional control of genes required to guarantee the success of development. Additionally, G4 may also be relevant switches in post-transcriptional regulation mechanisms of developmental control involving pre-mRNA alternative processing, miRNA biogenesis and targeting, lncRNAs function or mRNA turnover, localization and translation. However, the participation of G4 in such RNA regulative processes is barely understood at the cellular level and even less at the whole-developing organism level. Further studies are needed to fully understand the multiple gene expression regulation levels at which G4 structures may be playing important roles during animal cell differentiation and development.

Abbreviations

AGIL	5′ asymmetric G-rich internal loop
ASO	small antisense oligonucleotides
CNBP	cellular nucleic-acid binding protein
DSBs	DNA double-strand breaks
FANCJ	Fanconi anemia group J protein
G4	G-quadruplex
MBT	mid-blastula transition
lncRNA	long non-coding RNA
ncRNA	non-coding RNA
PPR	proximal promoter region
PQS	putative G-quadruplex sequence
pre-miRNA	precursor microRNA
RHAU	RNA helicase associated with AU-rich element

Conflict of interest

The author has declared no conflict of interest.

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