Dark DNA and stress (Review)

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Abstract. Over the past few decades, research at the molecular level has focused on the part of the genome that does not encode protein sequences. Since the discovery of transcriptional evidence from the hitherto considered 'junk' DNA, this region of the genome, which is currently termed dark DNA, is constantly gaining interest. The term borrows an analogy from the corresponding eminent fields of dark matter and dark energy in physics and cosmology. In fact, an increasing number of attempts are being made to enhance the current understanding of the non-coding RNA (ncRNA) transcripts produced by such regions. Although the base-pair length and gene number appear to be very diverse between species, it appears that the amount of the non-coding regions of the genome of an organism is a sign of evolutional superiority. ncRNA molecules are able to orchestrate the expression of genetic information in the most complex, rapid and reversible manner, participating in almost every major biological process. A prime example of such a process is the maintenance of homeostasis, the internal physiological balance, despite internal and external stressful stimuli. These molecules have been shown to be excellent regulators of gene expression, with marked spatiotemporal specificity, rendering them ideal tools for regulating stress responses. Herein, an attempt is made to extract and fuse information from a repertoire of studies, which have demonstrated that the expression of a number of these molecules was modified following exposure to acute and chronic stress, as well as in patients with anxiety disorders and their respective animal models. All in all, ncRNAs have the potential to be used either as biomarkers or as therapeutic targets for disorders resulting from the loss of equilibrium, the disruption of homeostasis and the destabilization of the hypothalamic-pituitary-adrenal axis.

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Contents

1. Introduction

Dark DNA, the non-coding portion of the genome, constitutes ~98% of human DNA, while genomes of other large multicellular eukaryotes also appear to be mostly comprised of DNA that does not encode for proteins as well (1,2). New sequencing technologies made it possible to investigate this dark side of the genome, previously referred to as 'junk' (1). It appears that the extended DNA sequences of higher eukaryotic organisms that do not encode proteins are eventually transcribed in RNA to a very large percentage. Up to 80-90% of the genome of eukaryotic organisms is estimated to being transcribed, suggesting a potential role for such molecules (3). Furthermore, as the complexity of an organism increases, so does the percentage of dark DNA, while non-coding RNAs (ncRNAs) appear to dominate the regulation of its genome, in contrast to protein-coding genes (4).

Although the functions of all these transcripts were initially unknown, RNA has been shown to have a very wide range of biological functions. Specifically, RNA transcripts are being used as a means of gene regulation in higher eukaryotes, both by *cis* and *trans* mechanisms (5). Research has indicated

that up to 20% of these dark DNA regions play a vital role in controlling gene expression by regulating when and where a gene is activated or deactivated (6). ncRNAs appear to use a number of different mechanisms in order to regulate gene expression. RNA transcripts have the ability to function as transcriptional or post-transcriptional regulators or as regulators of epigenetic modifications (7,8). RNA transcripts also appear to have tissue-, time- and cell-specific expression. One such example is microRNA (miRNA/miR)-18a, a ncRNA involved in the site-specific regulation of glucocorticoid receptor (GR) expression and the stress response. This expression of this ncRNA appears to be regulated throughout the lifetime of an organism, with miR-18a levels declining from the embryonic stage to adulthood (9). Finally, given that the non-coding part of the genome is extremely larger than the protein-coding one, the genetic cause of numerous diseases may be related to mutations within ncRNAs, including neurological and psychiatric disorders, among several others (7,10).

Moreover, the expression of these transcripts is dependent on the specific developmental stage of the organism in order to optimally regulate its internal dynamic equilibrium. ncRNAs are also essential for the function of organisms, since they allow them to respond to environmental signals, with a prominent position among them being to maintain their homeostasis in response to stress, a state where homeostasis is threatened (11). Differentiation, development, the maintenance of homeostasis, stress responses and plasticity are, therefore, mediated via epigenetic mechanisms, such as ncRNA expression (7).

ncRNAs are thus ideal molecules to mediate stress responses, and research has suggested that ncRNAs are key players of epigenetic responses in the brain (12). Other studies have demonstrated that exposure to stress induces brain-specific alterations in both long ncRNA and small ncRNA expression levels (13,14). The present review aimed to collect the information available thus far concerning ncRNA alterations under stress conditions in order to highlight their mediating role in the stress response.

2. Classification of non-coding RNAs

ncRNAs can be divided into two main categories, structural and regulatory (11). The former group includes well-known classes of ncRNAs, such as ribosomal, transporter and small nuclear RNAs. Regulatory ncRNAs, although a slightly more difficult to classify, can be divided into small ncRNAs (sncRNAs) and long ncRNAs (lncRNAs) depending on their length. sncRNAs include miRNAs, small interfering RNAs (siRNAs), P-element induced wimpy testis (PIWI)-interacting RNAs (piRNAs) and small nucleolar RNAs (snoRNAs). There are also ncRNAs derived from enhancer RNAs (eRNAs), super-enhancers RNAs (seRNAs) and ncRNAs derived from introns and telomeric sequences (TERRA) (15-20).

miRNAs are single-stranded RNA molecules that suppress translation in a number of eukaryotic organisms through incomplete mating, of six to eight nucleotides in length, with their target mRNAs. In the case of perfect complementarity with their mRNA targets, they cause their degradation via the RNA-induced silencing complex, a phenomenon known as RNA interference (16,21). miRNAs are derived from introns and exons of both protein-coding and non-coding transcripts synthesized by RNA polymerase II (21); thus, a particular set of miRNAs can be synthesized under specific conditions and in certain cell types only (22). By controlling various biological processes and strictly regulating their expression, miRNAs are central players in a wide range of developmental processes, such as cell proliferation or stress responses (23).

siRNAs, produced from longer double-stranded RNAs or long hairpins (often of exogenous origin), are considered to be more specific than miRNAs and usually target homologous sequences for gene silencing (17). However, both miRNAs and siRNAs are produced by similar pathways and have similar mechanisms of action; therefore the distinction between them becomes blurred (17). While siRNAs were originally considered to be primarily an antiviral mechanism, other findings suggest that they ultimately play a much broader role in genome regulation (16,17).

piRNAs are integrated into the PIWI subfamily of Argonaute proteins. piRNAs in mammals appear to function primarily in the reproductive cell line, where they target and suppress the expression of transposable elements in order to maintain genomic stability (15). The reason transposons, although comprising up to 70% of the genome, have not led organisms to extinction is that they are controlled by a number of mechanisms in the cell, including piRNAs (24). It is worth mentioning that transposons themselves can regulate gene expression through their ncRNAs. The expression of transposons responds to environmental signals and a number of them are activated under various forms of cellular stress, sometimes resulting in inherited mutations of certain genes (25).

lncRNAs are a heterogeneous class of ncRNA regulators, >200 nucleotides in size. lncRNAs have minimal conservation, unlike other classes of ncRNAs, although their promoters are highly conserved (18,26,27). It has been suggested that their conserved secondary structure is the key to their interaction with protein molecules (11). A number of nuclear lncRNAs appear to be involved in gene-regulating processes, where they can regulate both their neighboring environment and act in distant genomic loci. In other words, they may be involved in the specific repression of a promoter or transcription activation (8,28). They also apppear to play a critical role in genomic imprinting, the process through which a gene expresses only one of its two alleles (29). Based on their modes of action, they can be classified as 'signals' for the incorporation of temporal, spatial and developmental information, as baits with the ability to isolate RNA and protein molecules in order to suspend their functions (decoys), as 'guides' that lead molecules to specific genomic sites, or finally, as scaffolds for the creation of macromolecular complexes with various functions (Fig. 1) (26). By modulating transcription, lncRNAs can be considered as sensors of environmental signals, such as stress, playing a key role in regulating transcriptional responses to external, as well as developmental stimuli through their interaction with transcription factors (15).

Sense and antisense transcripts are also derived from certain enhancers and are therefore termed eRNAs (19). The majority of these eRNAs are transcribed at lower levels than other ncRNAs and are rapidly degraded by protein complexes, such as the human PM/Scl complex, while they can also

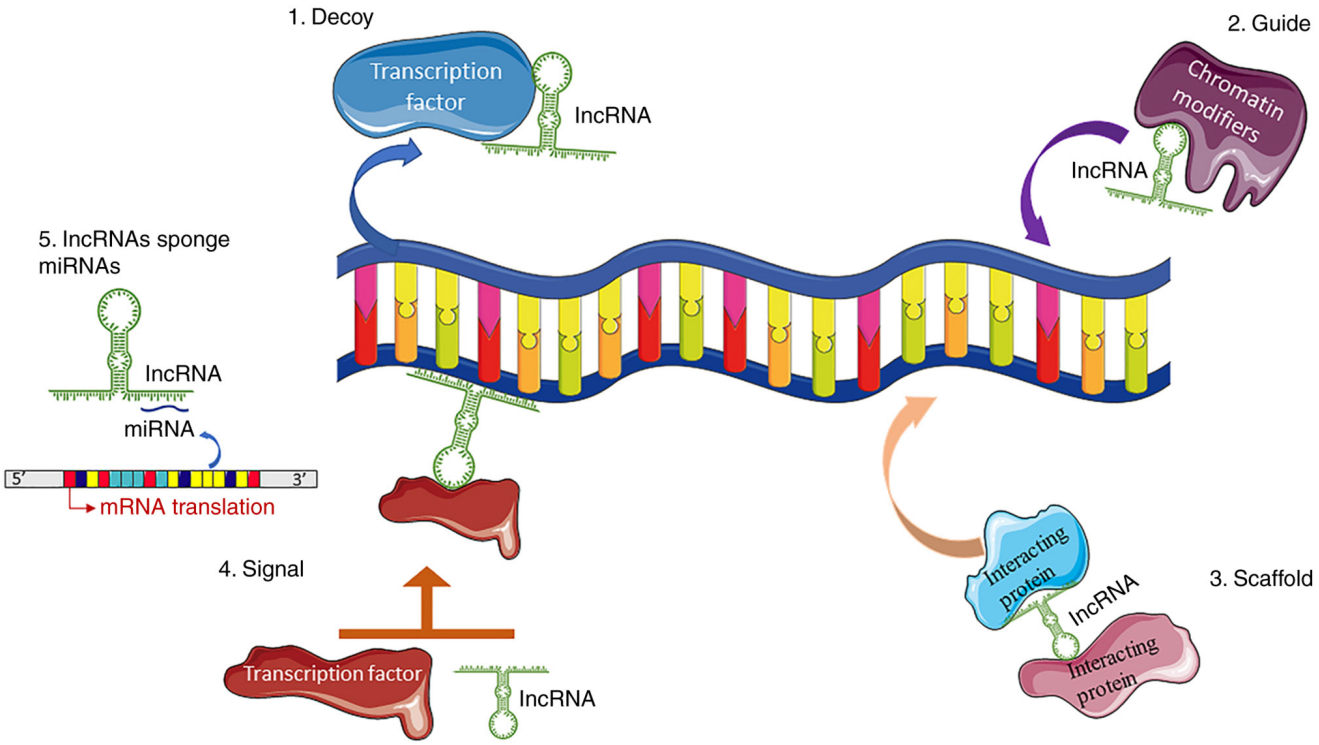

Figure 1. Schematic diagram illustrating the mode of action of lncRNAs. lncRNA, long non-coding RNA.

undergo methylation (30). They are divided into two categories: Small, bidirectional, non-polyadenylated eRNAs and long, unidirectional, polyadenylated eRNAs (31). Enhancer transcripts may play a structural role in creating or stabilizing enhancer-promoter loops. The transcription of eRNAs has been found to be associated with mRNA synthesis in neighboring genes, suggesting their involvement in transcriptional regulation, while subsequent studies have demonstrated their ability to orchestrate time and tissue-specific gene expression (19,32). Super-enhancers (SEs) are areas where multiple enhancers are assembled together. They also have the ability to transcribe, producing seRNAs, which exert their action through *cis* and *trans* mechanisms (31).

A large number of functional ncRNAs are produced in introns, including snoRNAs, piRNAs and lncRNAs (20,33). Although introns are believed to degrade immediately following cleavage by primary transcripts, there is strong evidence to indicate that intron RNAs can be processed into smaller RNAs with significant half-lives and specific subcellular locations, while the splicing of introns appears to provide plasticity to the type of RNA produced from a genetic locus (34). Additionally, ncRNAs also appear to be produced by pseudogenes, offering another mechanism to control gene function (35).

lncRNA molecules may also be transcribed from the sub-telomere sequences. These lncRNAs carry telomere repeats in their sequences, and are involved in the maintenance and regulation of telomere homeostasis. The heterogeneous lncRNA produced by these regions is termed TERRA (36), and it has been shown that it may be subject to developmental regulation and, in turn, may play a key role in orchestrating certain aspects of the complex chromosomal transactions that occur during cell differentiation (37).

3. Stress and ncRNAs

ncRNAs have been linked to a variety of disorders of the stress system, such as anxiety, and major depressive and bipolar disorders (38) (Table I). These RNA molecules appear to be a conserved mechanism of how genes are being regulated in response to a stressor among numerous animals (39).

A number of studies have demonstrated that that the expression of specific miRNAs changes in response to stress. For example, a clear association between the manifestation of anxiety behavior and the differential expression of specific miRNAs has been observed in mice (40), while exposure to environmental stressors regulates the expression not only of miRNAs, but also of factors involved in their biogenesis (41). lncRNAs have also been shown to regulate gene expression in various mental illnesses (42,43). In addition, research on the effects of early-life stress in rodents has revealed long-term effects that vary, depending on both the genetic background and exposure to stressors, which may lead to epigenetic alterations in genes involved in the stress circuit (14). Furthermore, researchers have demonstrated that acquired changes due to traumatic stress, as well as regulated fear responses, can be inherited for up to two generations in mice (44). Research on *C. elegans* and mice has demonstrated that sncRNAs mediate a non-Mendelian inheritance of traits or phenotypes acquired during life. sncRNAs are abundant in germ cells and are influenced by environmental factors, such as early traumatic stress, contributing to the possible onset of pathological features later in life (45).

As regards the roles of miRNAs, the latter appear to be responsible for the regulation of genes associated with the activity of the hypothalamic-pituitary-adrenal (HPA) axis (38). Their levels are altered by stress, glucocorticoids and

Table I. Stress and ncRNAs.

ncRNAs and their stress-related targets			
ncRNAs	Stress-related targets of ncRNAs	Effect/function	(Refs.)
miR-34c	CS-R1 mRNA	Downregulation	(40)
miR-132 and miR-128	BDNF gene	Downregulation	(48)
miR-34b and miR-27a	CRHR1 mRNA	Downregulation	(49)
miR-15a	FKBP51 gene	Downregulation	(9,50)
lncRNA GAS5	GR	GRE decoy in regulating glucocorticoid feedback	(51-53)
Lethe lncRNA	NF-κB	Inhibition of NF-κB/DNA binding and inhibition of activation of NF-κB target genes	(57,58)
IL1β-eRNA	IL1β and CXCL8	Positive correlation	(65)
ncRNAs regulated by stress induction			
ncRNAs		Effect after stress induction	(Refs.)
miR-186 and miR-381		Overexpression	(47)
miR-709		Downregulation	(47)
lncRNA Gomafu		Downregulation	(14)

CS-R1, corticosteroid type 1 receptor; BDNF, brain-derived neurotrophic factor; CRHR1, corticotropin releasing hormone receptor 1; FKBP51, FKBP prolyl isomerase 5; GR, glucocorticoid receptor; CXCL8, C-X-C motif chemokine ligand 8; GRE, glucocorticoid response element.

mood stabilizers, suggesting that miRNAs may be vital to the aetiology of anxiety disorders (40), in which stress is a critical factor both in influencing their onset and maintenance (46). miRNAs target and regulate stress-related proteins and play a role in the specific regulation of genes associated with susceptibility to anxiety disorders. An example of a protein targeting miRNA, is miR-34c, which targets the corticosteroid type 1 receptor and facilitates the recovery process following stressful situations (40). Moreover, in a previous study, the exposure of rats to mild restraint stress for 2 weeks altered the expression of certain miRNAs in the cerebellum; miR-186 and miR-381 were overexpressed, while miR-709 was underexpressed; these changes may be involved in the long-term adaptation of organisms to stress (47). An additional two miRNAs, miRNA-132 and miRNA-128, have also been found to affect stress by targeting the brain-derived neurotrophic factor gene (48), while the expression of numerous miRNAs has been found to be increased in primary hypothalamic neurons following stress. miR-34b and miR-27a, for example, have been found to be negatively associated with corticotropin releasing hormone receptor 1 (CRHR1) mRNA levels. In particular, the overexpression of miR-34b reduces both the CRHR1 mRNA and protein levels, thereby reducing the effects of the HPA axis and stressful behavior (49) (Fig. 2). In the amygdala, miR-15a has been found to play an essential role in regulating behavioral responses to chronic stress. As a target of miR-15a, the FKBP51 gene, is known to play a role in the transcriptional activation of glucocorticoid receptor following an increase in cortisol levels, and has been found to be involved in a number of stress-related psychiatric disorders (50). Mice expressing decreased levels of miR-15a in the amygdala following exposure to chronic stress also exhibit severe anxiety behavior. In humans, exposure to a traumatic event in childhood has also been found to be associated with elevated levels of miR-15a in peripheral blood (9).

It is now known that lncRNAs regulate genes in mental illnesses, such as post-traumatic stress disorder (PTSD), major depressive disorder, schizophrenia and autism spectrum disorders, and have been associated with >200 illnesses (42,43). lncRNAs are also involved in the regulation of the HPA axis negative feedback. Growth arrest-specific transcript 5 (Gas5), a lncRNA that interacts with glucocorticoid receptors, interferes with the binding of the glucocorticoid response element (GRE) to DNA and, thus inhibits its transcriptional activity (51). Thus, Gas5 can act as bait and GRE decoy in regulating glucocorticoid feedback. Gas5 can alter the fate of cells by making them more or less sensitive to apoptotic or other growth-related stimuli by regulating the activity of glucocorticoids and, perhaps, other steroid hormones. This lncRNA is involved in regulating certain immune functions, as well as the pathogenesis of autoimmune, inflammatory and infectious diseases, such as multiple sclerosis, partly through modulating GR transcriptional activity (52,53) (Fig. 3). It is important to note that Gas5 can be used as a biomarker for personalized therapies and a novel therapeutic target (54). In another study, the expression profiles of lncRNAs in the medial prefrontal cortex revealed that the lncRNA Gomafu was significantly downregulated in adult mice following the induction of fear. On the other hand, stress reactivity and anxiety behaviors have also been reported following mutations in this lncRNA, while

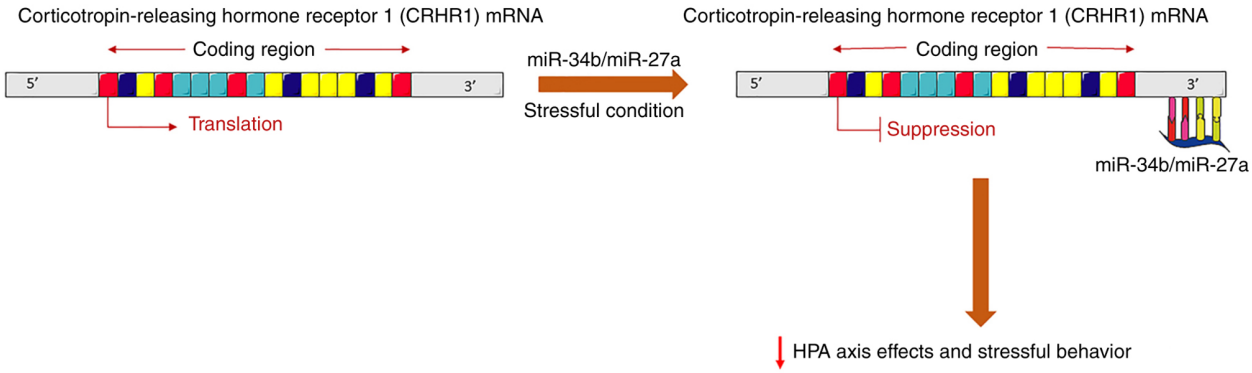

Figure 2. Schematic diagram illustrating the mode of action of miR-34b and miR-27a that downregulate the expression of corticotropin releasing hormone receptor 1 mRNA and subsequently, its protein levels, leading to the reduction of the effects of the HPA axis and to stressful behavior. HPA axis, hypothalamic-pituitary-adrenal axis.

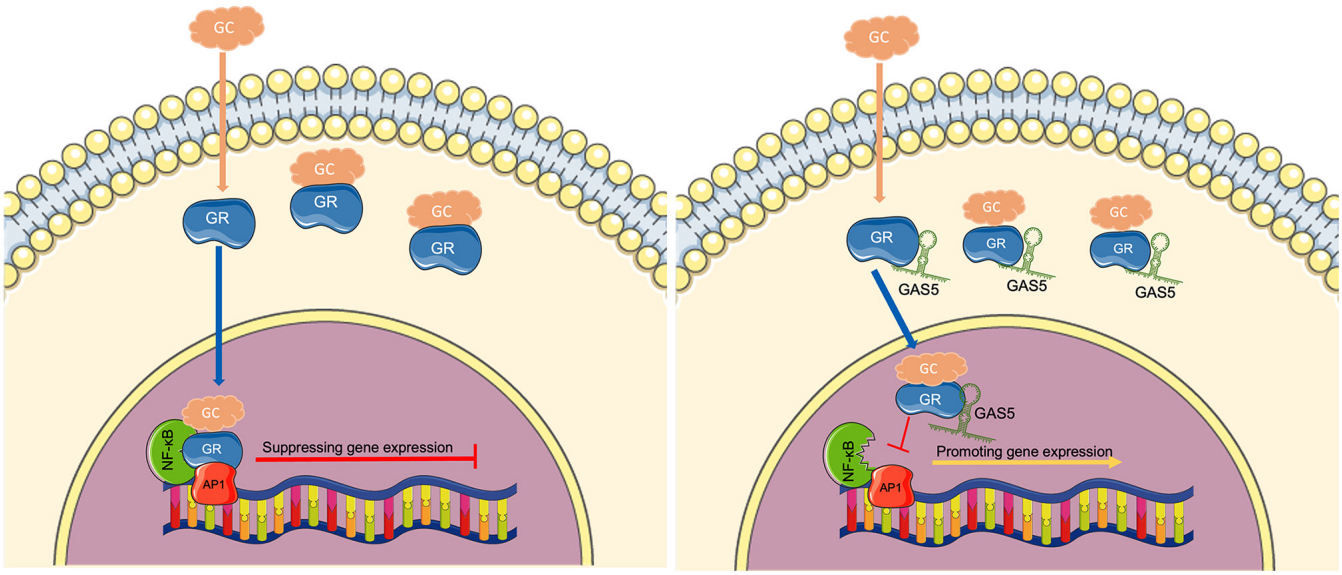

Figure 3. Schematic diagram illustrating the action of lncRNA GAS5. GAS5 acts as a decoy by interacting with glucocorticoid receptor, modulating GR transcriptional activity and involving in the regulation of numerous biological functions. GAS5, growth arrest-specific transcript 5; GR, glucocorticoid receptor; GC, glucocorticoid.

the reduction of Gomafu expression in the brain cortex has also been associated with schizophrenia (14). The lncRNA, Neat1, has also been shown to be involved in stress signaling in the brain, and enhances adaptive behavior in response to stress, while its loss in mice leads to hyperactivity, deficits in social interaction, and a panic escape response (55).

The pro-inflammatory cytokine, TNFα, which has been found to regulate hundreds of lncRNAs, functions through the transcription factor, NF-κB, thus playing a role in a variety of processes, such as innate and adaptive immunity, inflammation, apoptosis and aging (56). Lethe is a lncRNA derived from a pseudogene that is selectively induced by proinflammatory cytokines and interacts with an NF-κB subunit to inhibit its binding to DNA and the subsequent activation of NF-κB target genes. The loss of Lethe ncRNA expression is age-related and may be one of the causes of increased NF-κB activity in aging. Notably, Lethe is also selectively induced by dexamethasone, suggesting that a potential anti-inflammatory therapeutic effect may be due in part to the activation of Lethe's negative feedback system (57). Finally, Lethe also exerts a protective effect on sepsis-induced brain injury in mice, by regulating autophagy, which is generally known to be controlled by glucocorticoids in mouse cortical neurons (58).

A ground-breaking discovery in nematodes has provided information on the mechanisms through which the nervous system communicates through sncRNAs within the gamete line in order to influence animal behavior across generations (59). That research demonstrated that small RNA molecules in the nervous system can regulate genes in the reproductive line, allowing specific behaviors to be modified for a number of generations. These findings suggest that small RNAs, particularly piRNAs, play a critical role in the epigenetic inheritance of learned behaviors, enhancing an organism's chances of survival. One of the proposed mechanisms for transmitting learned behaviors to offspring is the natural transfer of small RNAs from neurons to gametes resulting in inheritable changes (44). It has also been documented that piRNAs mediate the suppression of retrotransposon mobilization, a mechanism that contributes to different transfer rates in brain regions involved in learning and memory (44). It is also important

to state that the mobility of retrotransposons increases with age, which can contribute to the observed neuronal decline associated with age. Thus, activating the expression of certain piRNAs, which show age-onset rhythmicity, may be a novel strategy for maintaining the genomic integrity, which is threatened by stress as an organism grows (60).

According to several studies, changes in eRNA levels are also observed under conditions of stress, as their up- or downregulation appears to lead to pathology. In models of mice with myocardial infarction and transaortic constriction, the expression of various eRNAs was induced, suggesting that these molecules are differentially expressed in response to stress and may promote abnormal transcription (61). Another study revealed that hypoxia-inducible factor 1α-activated eRNA (HERNA1) was a defining factor in heart disease, and it was shown that the inactivation of HERNA1 by antisense oligonucleotides *in vivo* prevented cardiac pathogenesis and improved the overall survival of ill mice (62,63). Other studies have shown that in the human BEAS-2B cell line, there are certain eRNAs responsible for controlling anti-inflammatory genes, whose expression is regulated by glucocorticoid receptor in association with NF-κB (64). eRNAs also have been found to regulate the expression of their target genes, such as IL1β-eRNA, a molecule that regulates the expression of genes involved in inflammation (65).

4. Possible applications

As miRNAs can cross the blood-brain barrier, they could potentially be quantified by routine examinations as markers for various neurodegenerative and neurodevelopmental disorders (66). The experimental up- or downregulation of certain miRNA expression which is altered following stress have been shown to influence stress-associated behavior in animal models of anxiety disorders (67). So far, several studies have linked circulating miRNAs to perceived stress and anxiety. For example, before their final examinations, the stress levels of medical students have been shown to be significantly associated with blood miR-16 levels (68). Furthermore, miR-125a expression has been shown to be reduced in individuals with PTSD, while blood levels of miR-22, -138-2, -148a, -339, -488 and -491 have been found to be associated with panic and phobic disorders (69). It should be noted that extracellular miRNAs are quite stable in the circulation. This makes them attractive candidates for monitoring the progression of a disease and its treatment. Another advantage of using ncRNAs as biomarkers is that changes in their expression in body fluids are also predicted to occur earlier than changes observed in conventional biomarkers (70). Similar to miRNAs, lncRNAs can be released into the extracellular space and detected in body fluids or as circulating lncRNAs. Despite a large number of publications on the use of circulating ncRNAs as potential clinical biomarkers, to date, only one ncRNA has been converted to an FDA-approved diagnostic marker (71).

Recent developments in basic and clinical research have demonstrated that RNA molecules are a valuable tool in the therapy of neurodegenerative diseases (72). ncRNAs could, therefore, become novel targets for therapeutic interventions. For example, miR-223 may be a neuroprotective agent after a stroke, as it has been observed to minimize the death of neuronal cells following an ischemic attack (73). Moreover, several different artificial oligonucleotides have been used to inhibit the activity of miRNAs in cell lines or *in vivo*. For example, 2'-O-methyl-oligonucleotides inhibit let-7b and miR-124, thereby inducing nerve stem cell proliferation (73). The main issue with oligonucleotide therapy appears to be the way in which the active oligonucleotide would be 'delivered' to its site of action in the cytosol or in the cell nucleus within tissues (74). Nevertheless, efforts have been made to overcome these obstacles, as novel lipid nanoparticles are being developed to shield miRNAs for tumor-targeted delivery to combat metastatic cancers (75).

There are also evolved regions in the dark genome that are related to stress and that have been associated with schizophrenia and bipolar disorders or even with psychosis and suicide. Those dark genome parts are specific to humans as they have not been found in the genomes of other vertebrates. It is possible that these regions evolved rapidly in humans as our intelligence and cognitive abilities developed. However, it appears they are easily disrupted, leading to the manifestation of schizophrenia and bipolar disorders. This is a breakthrough, as it applies to numerous individuals in modern societies and as many of the available drugs are designed to target gene-encoded proteins and not the dark part of the genome. Thus, there is hope and potential in the future treatment of schizophrenia and bipolar disorder as novel pharmacological targets may be identified.

5. Conclusions and future perspectives

At the beginning of the 21st century, it was demonstrated that, although only ~2% of the human genome encodes proteins, ~80% of it is transcribed to RNA. Thus, the idea of the existence of 'junk DNA' was debunked, and many of the transcribed non-coding RNAs were shown to be involved in almost every level of gene expression regulation, with their expression being quickly adjusted to environmental changes. The latter property renders them ideal for regulating the stress response. In fact, research has indicated that the various classes of non-coding RNAs, such as miRNAs, lncRNAs, piRNAs, etc., and the factors involved in their biogenesis, show variations in their levels in response to stressful stimuli. A prime example is that of the glucocorticoid receptors, which are targeted by a variety of miRNA molecules, as evidenced by decreased expression of these receptors by miR-18α or by miR-124. It is known that the regulation of gene expression is mainly aimed at helping organisms adapt to their environment and to ensure the optimal changes for their survival. The use of ncRNA molecules as mediators of these responses has ultimately proven to be a truly intelligent mechanism of adaptation. Thus, ncRNAs may be used both as biomarkers and as therapeutic molecules; their potential clinical uses are a very promising field of research.

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Authors' contributions

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Ethics approval and consent to participate

Not applicable.

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Competing interests

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