Review

Regulatory Roles of Non-Protein-Coding RNAs in Cardiovascular and Hematopoietic Disorders

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Mammalian genomes encode numerous non-protein-coding RNAs (ncRNAs) including microRNAs and natural antisense transcripts. Functional validation studies indicate that ncRNAs are involved in regulation of various normal cellular pathways and a range of essential biological processes. Deregulations of ncRNAs are also documented in variety of human complex disorders including cardiovascular and hematopoietic disorders. This review provides an overview of different ncRNA classes and summarizes recent reports regarding involvement of ncRNAs in cardiovascular and hematopoietic disorders.

The human genome sequencing projects revealed that the human genome contains over 3 billion DNA base pairs, but only 20,000–25,000 protein-coding genes. In fact, only about 1.2% of the genome codes for proteins.[1] Surprisingly, the number of human genes seems to be almost equal to lower mammals like rodents, and less than a factor of two greater than that of many much simpler organisms, such as the roundworm and the fruit fly. On the other hand, recent studies have revealed that eukaryotic genomes are almost entirely transcribed,[2,3] generating an enormous number of non-protein-coding RNAs (ncRNAs).[4-6] Thus there may be a vast reservoir of biologically meaningful ncRNAs that greatly exceed the ~ 1.2% of the genome that corresponds to conventional protein coding genes.[7,8] Several classes of functional ncRNAs have been identified in recent years. Text box-1 provides a list of mammalian RNA species and various categories of ncRNAs. Natural antisense transcripts and microRNAs are two prominent and complex classes of ncRNAs.[1]

Natural antisense transcripts

RNA Natural antisense transcripts (NATs) are RNA molecules transcribed from the opposite strand of conventional (sense) genes and often overlapping in part with mature sense.[9,10](Fig. 1) Sense and antisense RNAs can both encode protein or be non-protein-coding transcripts; however, the most prominent form in the mammalian genome is a non-protein-coding antisense partner of a protein-coding gene.[11] NATs are shown to have tissue-specific expression pattern, supporting involvement of these ncRNAs in sophisticated regulatory functions of various organs.[12]

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Text Box 1. Some mammalian RNA species

1.Messenger RNA (mRNA), well known class of RNA with average size of 2 kb. It is transcribed from DNA and processed before leaving the nucleus. The processed mRNA, which is located in cytoplasm, contains polyA tail, cap structure, open reading frame and it is frequently spliced, in many cases alternatively.

2.MicroRNA (miRNA) is a small non-coding regulatory RNA. The miRNA precursor (pri-premiRNA) is transcribed into a single stranded RNA transcript of approximately 150-250 nucleotides in length. A 'hairpin' secondary structure is formed in pri¬premiRNA which is then processed by the enzyme Drosha and exported to the cytoplasm. Pre-miRNA is further processed by the enzyme Dicer to create a stable, ~22 nucleotide single-stranded mature miRNA from one arm of the hairpin. The mature miRNA sequence tends to be highly conserved.

3.Small nucleolar RNAs (snoRNAs) are a class of small RNA molecules that guide chemical modifications (methylation or pseudouridylation) of ribosomal RNAs (rRNAs) and other RNA genes (tRNAs and other small nuclear RNAs (snRNAs)). snoRNAs are commonly referred to as guide RNAs but should not be confused with the guide RNAs (gRNA) that direct RNA editing in trypanosomes. The snoRNAs are less than 70 nucleotides in length including 10-20 nucleotides of antisense elements for base pairing.

4.Small nuclear RNA (snRNA) is a class of small RNA molecules that are found within the nucleus of eukaryotic cells. They are involved in a variety of processes such as RNA splicing, regulation of transcription factors (7SK RNA) or RNA polymerase II (B2 RNA), and maintaining the telomeres.

5.Piwi-interacting RNA (piRNA) is a class of small RNA molecules that is expressed in mammalian testes and forms RNA-protein complexes with Piwi proteins. These piRNA complexes (piRCs) have been linked to transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, particularly those in spermatogenesis. Purification of these complexes has revealed that these oligonucleotides are approximately 29-30 nucleotides long.

6.Rapid associated RNA (RasiRNA): is presumably derived from long double stranded RNA (dsRNA) and match to repetitive sequence elements in antisense orientation. In the Drosophila germline, rasiRNAs ensure genomic stability by silencing endogenous selfish genetic elements such as retrotransposons and repetitive sequences

7.Natural antisense transcripts (NAT) are single-stranded RNAs that are complementary to mRNAs. NAT regulate mRNAs in a concordant or discordant manner. The average length of NAT is 2 kb, but in some cases it is extremely long (over 100 kb). NAT in some cases is spliced and contains polyA, cap structure or even open reading frame.

8.Other long non-coding RNA transcripts (sometimes referred to as macroRNA) are diverse and not necessarily well conserved; they are often processed, containing polyA tail and/or cap structure. There is no significant open reading frame for macroRNAs and their functions are largely unknown.

9.Ribosomal RNA (rRNA) and transfer RNA (tRNA) are well studied components of the protein synthesis machinery.

Recent research on NATs, including several large-scale expression-profiling studies,[11,12] has conclusively established the existence of NATs in eukaryotic genomes. In fact, the consensus opinion is that the mammalian genome encodes an enormous number of natural antisense transcripts, most of which represent ncRNAs.[11] However, there are many unanswered questions that still exist concerning NATs biological functions and their heterogeneous mode of actions in various cells. For instance, what fraction of NATs may have functional significance, and how many different regulatory mechanisms may exist for these RNA molecules? NATs appear to be utilizing various cellular pathways, but it is still not clear which intrinsic properties of natural antisense RNA molecules or extrinsic features, such as protein interactions, cellular and developmental context are decisive for any given pathway. How is the expression of these ncRNAs regulated in various cells, and what are the extrinsic factors that affect the regulatory output of antisense RNA transcripts? Based on what we know about the broad expression of NATs in different tissues and cell types, and

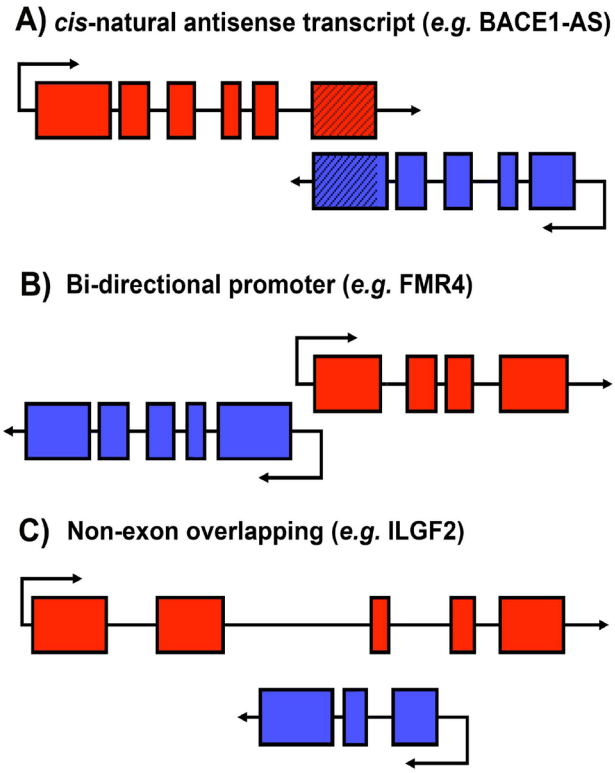

Figure 1. Illustration of prominent transcriptomics patterns relating to complex loci in human and mouse genomes. (A) cis-NATs, like BACE1-AS [9] or naPINK1 [38] in which two converging transcripts, from opposite strands of DNA, have overlapping exons shown as hashed parts. (B) Bi-directional promoters as for example for FMR4 [39] drive transcription of two RNAs in opposite directions. The transcripts may share the same transcription start site (TSS), or even exhibit overlapping 5'UTRs. (C) Full or intronic transcripts in which antisense RNA (blue) is inside the boundary of the sense transcript (red). Even if the fully processed RNAs do not contain overlapping sequences, RNA duplexes can still form between unprocessed transcripts. Alternatively, antisense RNA can bind to the DNA and exert its regulatory function.

their varied proposed functions, NATs appear to be a heterogeneous group of regulatory RNAs with a wide variety of biological roles.

Diverse regulatory mechanisms recruited by NATs

The NATs have been suggested to regulate gene expression by controlling various levels of gene expression including chromatin architecture/epigenetic memory, transcription, RNA splicing, editing, transcript localization, [9] translation and turnover. [13-16] NATs have been shown to be involved in methylation, [17] demethylation, [18] parental gene imprinting, [19] chromosome X inactivation, [20] splicing, [21-23] transport, [24] polyadenylation, [25-27] editing and stabilization. [28,29]

NATs in cardiac gene regulation

NATs are shown to be involved in cardiac gene regulation [30] as well as cardiac [31,32] and skeletal [33] myosin gene organization. Specifically, cardiac α and β myosin heavy chain gene switching is suggested to regulate through a mechanism involving naturally occurring antisense transcript. [31] Induction of hypothyroidism and diabetes states in rats were shown to alter the expression of NATs and subsequently the sense α and β myosin mRNAs. Additionally, in vitro stimulation of neonatal rat cardiac myocytes with either Triiodothyronine (T3) or phenylephrine is shown to alter α and β sense and antisense RNA level, in a concordant way. [32] Antisense transcripts are originated from bidirectional

transcription in the intergenic region of both α and β myosin heavy chain genes and could be accounted for cardiac α to β gene switching.

NATs in regulation of hemetopoiesis

Antisense transcript for PU.1 mRNA is a well documented case of translational inhibition mediated by NATs. Transcription factor PU.1 is an important regulator of hematopoiesis and suppressor of leukemia transformation. PU.1 mRNA translation is inhibited by a noncoding NAT.[34] Both sense and antisense transcripts of the PU.1 are co-regulated by an upstream regulatory element (URE). PU.1 antisense RNA is a polyadenylated transcript with a lower concentration but a longer half-life time than the sense PU.1 transcript and is equally distributed between cytoplasm and nucleus.[34] Processed antisense RNA in the cytoplasm may bind to the sense transcript and stall translation between initiation and elongation steps.[34]

NAT involvement in certain forms of anemia

In one inherited forms of anemia, α -thalassemia, a NAT has been reported to cause silencing of the α-globulin gene via methylation.[17] Tufarelli et al[17] found that a deletion in the globin gene locus of thalassemic patients relocates the constitutively active LUC7L gene 300 nucleotides downstream of alpha-2 globin (HBA2) gene. HBA2 encode hemoglobin alpha chain and antisense RNA causing promoter methylation and transcriptional silencing of HBA2 gene. This phenomenon results in anemia in patients because of reduction in hemoglobin alpha chain, which is major constituent of adult hemoglobin. A mouse model for genomic rearrangement (relocation of LUC7L) recapitulated the α-thalassemia disease phenotype and confirmed the role of cis-NAT in early developmental CpG island methylation.[17]

MicroRNA synthesis and function

MiRNAs are a class of small ncRNAs (19-25 nucleotides) that have recently generated much interest.[35-37] The enzymatic machinery and sequence of events, involved in the biogenesis of miRNAs are highly conserved across animals and plants (Fig. 2). Specifically, miRNA precursor (immature miRNA) is transcribed into a single stranded RNA transcript of approximately 50-120 nucleotides in length, which forms a 'hairpin' secondary structure.[38,39] This precursor miRNA hairpin is exported from the nucleus to the cytoplasm, where it is processed by Dicer, in combination with Argonaute proteins, and the RISC complex (RNA-induced silencing complex) to yield a stable, ~19 nucleotides single-stranded mature miRNA from one arm of the pre-miRNA hairpin.[39] This mature miRNA sequence is highly conserved across species.[35,36] In plants, miRNAs often demonstrate complete or precise complementary base-pairing with target mRNA transcripts,[40] resulting in the cleavage and degradation of target mRNA transcripts, via RNA interference (RNAi) machinery.[41,42] In contrast to plant, animal miRNAs are generally thought to recognize and bind to the target mRNA transcripts by incomplete complementary base pairing. Such imperfect base pairing with target transcripts results in translational inhibition and down-regulation of associated

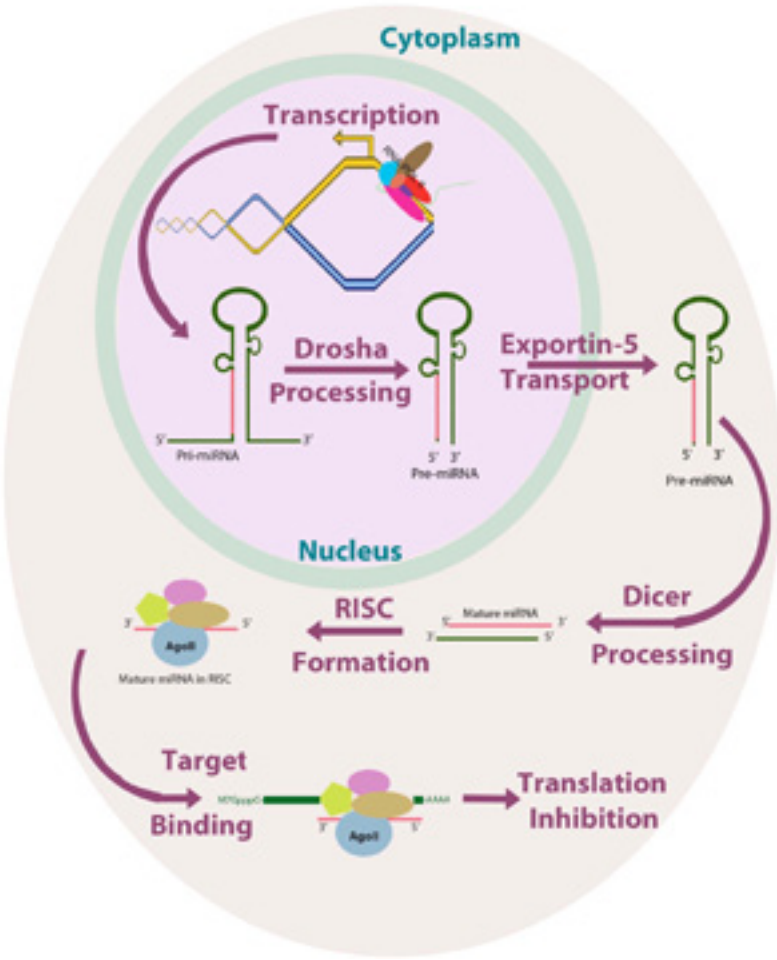

Figure 2. MicroRNA (miRNA) biogenesis: MiRNAs are transcribed by RNA polymerase II and usually form characteristic hairpin structure, which is termed primary miRNAs (pri-miRNAs).60 Pri-miRNAs are processed by ribonuclease enzyme, Drosha, to release a hairpin, which is called precursor miRNA (pre-miRNA). Exportin-5 is responsible for pre-miRNAs export from nucleus to the cytoplasm.61 Dicer is a member of the RNase III superfamily of ribonucleases that has been implicated in pre-miRNA cleavage, in the cytoplasm; to produce approximately 19 nucleotides long double stranded RNA. The resulting double-stranded RNA has two nucleotides 3' overhangs. Only one of the two strands is the mature miRNA, which is then incorporated into a multi protein complex called RNA-induced silencing complex (RISC). MiRNAs usually bind to target mRNA through nucleotide complementarities between miRNA "seed region" and 3' UTR region of the mRNA. Binding of miRNA to RNA transcript commonly caused translation repression of the targeted mRNA.

proteins. Thus, miRNAs may represent 'master regulators' of gene expression that orchestrate the expression levels of clusters of associated proteins.[43,44]

The miRBase Sequence Database provides a searchable online resource for entire published miRNA sequences.[45,46] The miRBase also contains predicted miRNA target genes. Latest release (Release-13.0) of the miRBase database contains 9539 hairpin precursor miRNAs, expressing 9169 mature miRNA products, in 103 species.[31] The data are freely available to all through the web interface at http://microrna.sanger.ac.uk.

Indeed, 713 human miRNAs have been experimentally identified and it has been estimated that more than 33% of human gene products may be regulated by miRNAs.[47]

MicroRNA and cardiovascular disorders

The essential role of miRNAs in divers biological processes, such as cell proliferation, differentiation, apoptosis and stress response has been described.[48,49] Importantly, there are several reports indicating pivotal role for miRNAs in cardiovascular physiologic function as well as various cardiac disorders. These reports include, but not limited to, involvement of miR-1 in cardiac arrhythmias,[50,51] miR-29

in cardiac fibrosis following myocardial infarction[52] and miR-133 in cardiac hypertrophy.[53]

Specifically, miR-133 has been shown to protect against cardiomyocytes hypertrophy both in human and mouse models of cardiac hypertrophy.[53] Both miR-133 and miR-1 are skeletal and heart muscle-specific miRNAs that control myogenesis, cardiac development and performance.[54] Over-expression of miR-133 or miR-1 caused inhibition of cardiac hypertrophy in vitro and blockage of miR-133 with antagomir caused sustained and marked cardiac hypertrophy in vivo. The effects of these miRNAs on cardiac hypertrophy is appeared to mediate through their binding and posttranscriptional regulation of RhoA, Cdc42 and Nelf-A/WHSC2 mRNAs.[53]

Another well documented miRNA involved in cardiac pathologies is the miR-21, which is shown to protect cardiomyocytes against reactive oxygen species.[55] Exposure of cardiac myocytes to hydrogen peroxide caused up-regulation of miR-21 and downregulation of its direct target gene, Programmed cell death 4 (PDCD4).[55] Moreover, miR-21 is shown to express in cardiac fibroblasts and regulate the ERK-MAP kinase signaling pathway. The ERK-MAP kinase signaling pathway is regulating fibroblast survival and growth hormone secretion and controlling the interstitial fibrosis and cardiac hypertrophy.[56] Blockage of miR-21, *in vivo*, in the mouse model of pressure-overload cardiomyopathy, attenuates cardiac dysfunction.[56] Therefore, deregulation of miR-21 might participate in several heart malfunctions such as cardiac hypertrophy, heart failure and myocardial infarction by increasing susceptibility to reactive oxygen species injuries.

Additionally, up-regulation of miR-1 and miR-206 is documented in rat model of myocardial infarction.[57] The target mRNA for miR-1 and miR-206 is appeared to be insulin-like growth factor 1 (IGF-1), which contains sequence complementary to both miRNAs within 3'-untranslated region (3'UTR) of IGF-1 mRNA.[57] Induction of myocardial infarction in rats caused down-regulation of IGF-1 protein, without obvious alteration of IGF-1 mRNA, which suggest a posttranscriptional regulation of this protein by miR-1 and miR-206.

Concluding remarks

In conclusion, enormous body of evidence indicates that there are widespread occurrences of non-protein-coding RNAs, including NATs and miRNAs in mammalian genomes and that many of these regulatory elements are indeed functionally relevant in controlling gene expression. Considering tissue-and cell type-specific expression patterns of ncRNAs and their heterogeneous proposed functions, it seems that we have, so far, only touched parts of an elephant in the dark. The big picture, in the light of future studies, probably will include these parts, but it could be dissimilar to our current understandings. I summarized here the recent reports regarding involvement of two ncRNA classes, NATs and miRNAs in various cardiovascular and hematopoietic disorders. Interestingly, most reported functional ncRNAs are helping to mediate precise gene expression in response to a variety of environmental stimuli and to keep tight regulation of protein expression by allowing proteins to perform their physiological functions while avoiding the serious consequences of over or

under expression. I present several examples of functional NATs and miRNAs to show involvement of these molecules in regulation of gene expression related to cardiovascular and hematopoietic disorders.

Although protein synthesis was the first assigned function for RNA molecules, it is becoming increasingly evident that the more pervasive function of RNA molecules is a regulatory one. This hypothesis is considerably supported in the light of present review in which we summarize regulatory functions of naturally occurring antisense transcripts and miRNAs in various cardiovascular and hematopoietic disorders. Considering other reported functional long ncRNA (macroRNA), and small regulatory RNA (such as miRNA, piRNA, rasiRNA) and their enormous physiological impact it is feasible to claim that the more frequent function of RNA molecules is a regulatory role, which is far greater than their assigned functions in protein synthesis as messenger, transfer and ribosomal RNA.

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