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Regulatory RNAs

Basics, Methods and Applications

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Preface

RNA molecules participate in and regulate a vast array of cellular processes besides being the physical link between DNA and proteins. They play several other key roles, which include RNA catalysis and gene regulation mediated mainly by noncoding RNAs. This regulation occurs at some of the most important levels of genome function, such as chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability, and translation. Further, harnessing the potential of RNA as a therapeutic or diagnostic tool, or as a central player in a fundamental biological process is becoming increasingly important to the modern day scientific community. Previously scientists imagined that there was an "RNA World," in which primitive RNA molecules assembled themselves randomly from building blocks in the primordial ooze and accomplished some very simple chemical chores. But these molecules were thought only to be carrying information from DNA to ribosomes. Discovery of catalytic RNAs changed this idea and opened up a wealth of opportunities to allow investigators to modulate gene expression post-transcriptionally using ribozymes and derivatives. In addition to ribozymes, a new RNA-based strategy for regulating gene expression in mammalian cells has recently been described. This strategy is known as RNA interference (RNAi). Although much is known about the mechanisms of RNAi, there lie a number of hurdles that need to be overcome along the applicative path of gene-silencing technology which includes the activation of innate immunity, off-target effects, and in vivo delivery.

Currently, high-throughput sequencing, bioinformatic and biochemical approaches are identifying an increasing number of regulatory RNAs. Unfortunately, our ability to characterize the detailed story of regulatory RNAs is significantly lacking. Extensive research of these RNAs is an emergent field that is unraveling the molecular underpinnings of how RNA fulfills its multitude of roles in sustaining cellular life. The resulting understanding of the physical and chemical processes at the molecular level is critical to our ability to harness RNA for use in biotechnology and human therapy, a prospect that has recently spawned a multibillion-dollar industry.

Nevertheless, RNA research can be daunting, and without a thorough understanding of the challenges and complexities inherent in handling this fragile nucleic acid, forays into the RNA world can be quite frustrating.

In this book, we have made an attempt to bring together the contributions of the leading noncoding RNA researchers to embellish the story of regulatory RNAs and provide a snapshot of the current status of this dynamic field.

The book consisting of 21 chapters offers a comprehensive overview of our current understanding of the regulatory noncoding RNAs, namely, small interfering RNAs (siRNAs), microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), long noncoding RNAs (lncRNAs), small RNAs (sRNAs), etc., and their applications in understanding biological systems and diseases, including therapeutics. This book is divided into three major sections as per its title. The first section "Basics" consists of eight chapters (Chaps. 1–8). The first chapter gives an overview of the entire landscape of noncoding RNAs, mainly highlighting their history and functions with a focus on the current status of research and future perspectives. This is followed by chapters on discovery, biogenesis, evolution, regulatory functions, and molecular mechanisms of different category of noncoding RNAs.

The "Methods" section provides state-of-the-art experimental and computational methodologies for noncoding RNA detection using different techniques and experimental analysis of noncoding RNA regulatory networks in different systems. This part includes Chaps. 9–15 and provides different bioinformatic, high-throughput RNA sequencing, ncRNA-specific microarrays, and biochemical approaches to identify these RNAs as well as protocols for transfection, gene knockout experiments, and regulatory RNA–based cellular reprogramming and pathways in different species. Further, some chapters are devoted to methods and protocols that have been developed by the authors themselves.

The "Applications" section includes Chaps. 16–21, which cover applicative areas of various noncoding RNAs within a biological system. These serve as biomarkers for different diseases like cancer, target cancer stem cells, act as regulators in cell lineage determination, etc. Further, RNAi therapeutics is applied against solid organ malignancies, cellular reprogramming, and stem cell–based regenerative therapy.

We are grateful to our friends and colleagues who have encouraged and supported us in many ways towards preparation of this book. We acknowledge them, with sincere thanks and appreciation. We take this opportunity to thank all the authors who have contributed excellent chapters to this book and the reviewers for their critical comments to improve the quality and integrity of the chapters. Their special effort has made this book a valuable resource for scientists and aspiring research students interested in the intersection of RNA biology and clinical research. We would like to express our sincere appreciation to Sabine Schwarz and Ursula Gramm of Springer Heidelberg for their invitation to initiate this book and their continuing support and commitments in making this book a reality and to other staff members involved in the production of the book.

Bibekanand Mallick and Zhumur Ghosh

Contents

Part I
Basics

Chapter 1
Renaissance of the Regulatory RNAs

Zhumur Ghosh and Bibekanand Mallick

Abstract "Non-coding RNAs (ncRNAs)" originate from various types of regulatory DNA, which lie deep in the wilderness of so-called junk DNA present within the genomes. Far from being humble messengers, a group of ncRNAs are powerful players in how genomes operate and are better termed as "regulatory RNAs". The new regulatory role of RNA began to emerge recently as researchers discovered different classes of regulatory RNA molecules, namely, small interfering RNAs (siRNAs), microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), long noncoding RNAs (lncRNAs), etc. These versatile RNA molecules appear to comprise a hidden layer of internal signals that control various levels of gene expression in physiology and development, including chromatin architecture/epigenetic memory, transcription, RNA splicing, editing, translation, and turnover. RNA regulatory networks may determine most of our complex characteristics, play a significant role in diseases, and constitute an unexplored world of genetic variation both within and between species. In this chapter, we have attempted to provide a snapshot of the entire landscape of these versatile molecules.

Keywords Gene expression • long noncoding RNA • microRNA • noncoding RNA • regenerative therapy • regulatory RNA • ribozymes • RNA world • siRNA

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1.1 Introduction

Beginning of life on earth is one of those *big events* where the prime role is played by the *tiniest ones*. To depict the exact scenario regarding what happened millions of years ago, scientists study how life works now and then trace back. Today's cells keep all-inclusive instruction manual – the DNA under tight wraps in the nucleus. Tiny pores in the wrapping are the only way in or out. When the cell needs directions, the DNA makes a copy of the particular pages required in the form of a short, single strand of ribonucleic acid – the messenger RNA that can leave the nucleus. Outside the nucleus lies the cell's framework – the cytoplasm. Messenger RNA (mRNA) wends its way through the maze looking for the nearest relay station: a ribosome. Ribosomes call in their interpreters: transfer RNA (tRNA). These recognize parts of the mRNA message and give it to the ribosome. Ribosomes get the instructions from DNA to make proteins, which carry out functions in the cell and in the body ranging from digesting the burger you had for lunch to determining your skin color. Ribosomes assemble proteins from building blocks, called amino acids that tRNAs line up in the correct order. Yet another kind of RNA in the ribosome (rRNA) helps move the assembly line along.

Researchers wondered regarding which came first, DNA, RNA, or protein? This classic "chicken-and-egg" problem made it immensely difficult to conceive of any plausible prebiotic chemical pathway to the molecular biological system. It is obvious that the first information molecule must have been able to reproduce itself and carry out tasks similar to those done by proteins today, and this limited the choice. Among the options, RNAs were found to perform numerous functions, which were once thought to be domains of proteins. Their unique properties bagged appreciation of the scientific community and obligated them to revise the tenets of "central dogma". Hence, they imagined an "RNA World," in which primitive RNA molecules assembled themselves randomly from building blocks in the primordial ooze and accomplished some very simple chemical chores. This concept originated in late 1960s and was supported by different groups (Woese 1967; Crick 1968). RNA molecules mainly garnered attention with the discovery of ribozymes – the catalytic RNAs in 1980s (Guerrier-Takada et al. 1983; Kruger et al. 1982). Tom Cech and his group discovered that an intron within a pre-rRNA from *Tetrahymena thermophila* catalyzes its own cleavage (called self-splicing) to form the mature rRNA product. This explained why some RNAs act as natural RNA enzymes with self-splicing activity, which is a favorable prerequisite factor for origin of life on earth (Kruger et al. 1982).

The breakthrough discovery of catalytic RNAs entailed a remarkable increase in knowledge about the folding of RNA molecules and their functional activities. Moving a bit further along the landscape of present day research, the explosion of high-throughput next generation sequencing methods (Mortazavi et al. 2008), large-scale genome sequencing, and genome-wide transcriptome studies (Lao et al. 2009) in various organisms has led to the discovery of the RNAi (RNA interference) phenomenon (A. Fire and G. Mello, Nobel Prize in Medicine or

Physiology, 2006) and the role of noncoding RNAs (ncRNAs) in it, that act as transcriptional and posttranscriptional regulators. Apart from regulating gene expression, these ncRNAs also play a dominating role in maintaining genome stability (Moazed 2009) and have led to novel insights into the biological systems. This "regulatory RNA" field is presently expanding at an unprecedented rate, and exciting new developments will undoubtedly emerge over the next years.

Recently, it has been revealed from deep sequencing data of Encyclopedia of DNA Elements Consortium (ENCODE) transcriptome projects that eukaryotes transcribe up to 90% of their genomes, whose large fraction includes large and short RNAs with no coding ability (Birney et al. 2007). Earlier, there was a belief that more complex organisms would have a greater number of protein-coding genes; however, it is now well established that human and mouse have approximately the same number of genes as that of the microscopic organism, *Caenorhabditis elegans* (Taft et al. 2007). The complexity of cellular functions in advanced organisms and their small percentage of coding genome (~2–3%) was always a tough question to explain their correlation, but not anymore because of the discovery of thousands of different types of ncRNAs in recent years. It is now clear that biological complexity probably correlates to non-protein coding genes, not protein coding genes as thought earlier (Taft et al. 2007).

1.2 The Serendipity

With the discovery of RNAi in 1998 by Andrew Fire, Craig Mello, and colleagues (Fire et al. 1998), the long-believed concept about RNA became complicated. They observed silencing of gene expression by double-stranded RNAs (dsRNAs) in nematodes. This serendipitous phenomenon, termed as RNAi, was discerned when they injected dsRNAs into the *Caenorhabditis elegans* and observed silencing of a gene whose sequence was complementary to that of the dsRNAs. Since then, RNA has become the heart and soul of a scientific study and created a new revolution in the field of biological sciences. This revolution started unnoticed in the late 1980s and early 1990s when plant biologists working with purple petunia were surprised to find that introducing numerous copies of a gene that codes for deep purple flowers led to plants with white or patchy flowers, which was not expected (Napoli et al. 1990; van der Krol et al. 1990). Somehow, the inserted transgenes silenced both themselves and the plants' own "purple-flower" genes. These observations mystified the biologists for a few years but were readily deciphered after the findings by Fire and Mello in 1998. This RNAi phenomenon was originally thought to be confined to exogenous dsRNAs; however, it gradually became clear that genomes of plants and animals encode various types of endogenous dsRNAs, namely, small interfering RNAs (siRNAs), microRNAs (miRNAs), etc. The canonical RNAi pathway in animals has been described in details in Chapter 5. New classes of ncRNAs and more members

of existing classes continue to be elucidated in past years and are yet to be discovered in future.

1.3 Regulatory RNAs to Date

The world of ncRNAs keeps expanding with the advent of new molecular and genomic technologies in recent years. Figure 1.1 depicts the different types of regulatory ncRNAs identified till date.

There have been recent discovery of new ncRNAs sitting adjacent to transcription start sites, e.g., promoter-associated small RNAs (PASRs) (Kapranov et al. 2007), transcription initiation RNAs (tiRNAs) (Taft et al. 2009a), and termini-associated small RNAs (TASRs) located near 3′ end of the genes (Kapranov et al. 2007; Kapranov et al. 2010), aside from identification of other regulatory ncRNAs such as small nucleolar RNAs (snoRNAs) and processed snoRNAs (psnoRNAs) (see Chap. 3), small RNAs (sRNAs) in bacteria (see Chap. 4), long noncoding RNAs (lncRNAs) (see Chap. 6), siRNAs, miRNAs, piRNAs, small modulatory RNAs (smRNAs), tiny noncoding RNAs (tncRNAs), etc. While many of these ncRNAs remain undeciphered at an appreciable level, miRNAs, siRNAs, and

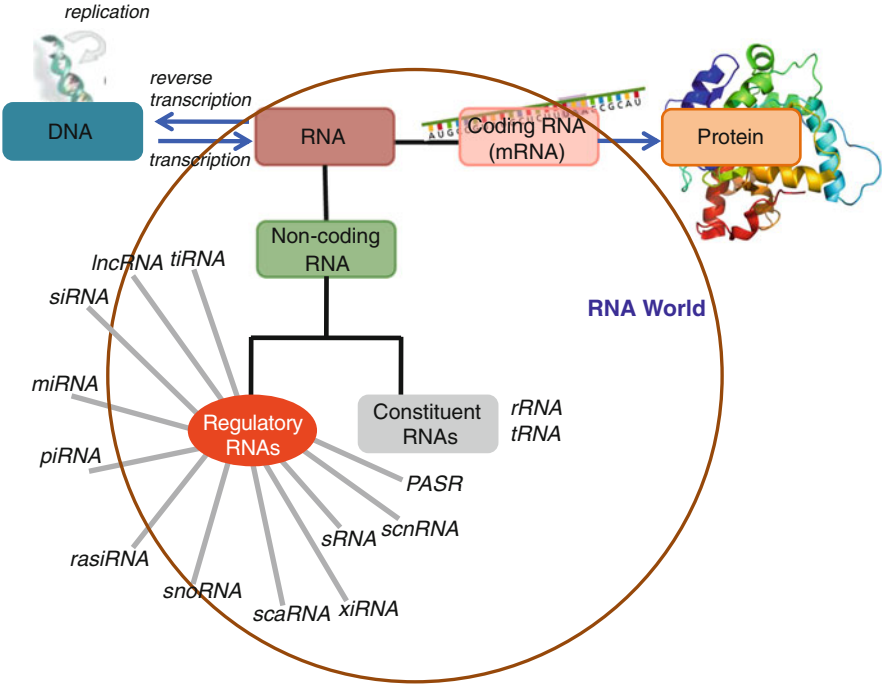

Fig. 1.1 The expanding noncoding RNA landscape

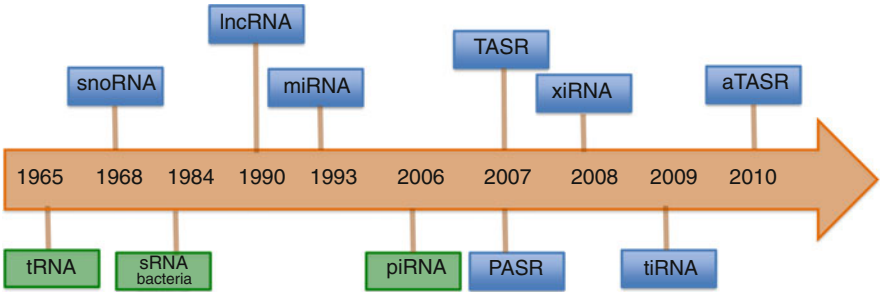

Fig. 1.2 Chronological trajectory of the major discoveries related to RNAs

piRNAs have been most thoroughly investigated to infer their evolution, function, and applications in myriad areas of biological systems. In humans, there are over 1,000 miRNAs, hundreds of siRNAs, and millions of piRNAs, complying with the observation of ncRNAs occupying a substantial portion of the genome. They contribute significantly to complex regulatory systems of a higher organism by coordinating important cellular functions at the transcriptional and/or posttranscriptional level. These regulatory RNAs have expanded the RNA world in due course of time (see Fig. 1.2 for chronological trajectories for major RNA discoveries). And, Table 1.1 provides an overview of various types of regulatory RNAs discovered till date.

1.4 Mini Silencers

Extensive research in the past few years on gene silencing has revealed that the argonaute protein family members are key players in these pathways (Hutvagner and Simard 2008; Peters and Meister 2007) guided by different types of small RNAs. Argonaute proteins are evolutionarily conserved and phylogenetically classified into the AGO subfamily and the PIWI subfamily. AGO proteins, ubiquitously expressed in cells bind to siRNAs and miRNAs and regulate posttranscriptional gene silencing either by destabilization of the mRNA or by translational repression. Although miRNAs and siRNAs were independently discovered, they share a common chemical composition, biogenesis-related events, RISC complex assembly Table 1.2, and mechanism of action (see details in Chaps. 5 and 2). They also differ in their evolutionary conservation process, and possibly they target different genes (Bartel and Bartel 2003). piRNAs are the most recent development in the RNAi field that were first reported in 2006 by four independent studies (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Watanabe et al. 2006) through cloning of small RNAs associated with PIWI proteins. PIWI proteins are mostly expressed in the germlines (Seto et al. 2007) and bind to these novel class of ncRNAs and facilitate silencing of transposons, the mobile genetic elements, and serve diverse functions in germline development and gametogenesis (refer to Chap. 5).

Table 1.1 Overview of different types of regulatory RNAs

ncRNAs	Organisms	Length (nt)	Characteristics/functions	References
siRNAs	Plants, animals, protists, fungi	21–22	Dicer-dependent cleavages of complementary dsRNA duplexes produce siRNAs. siRNAs associate with argonaute proteins and are involved in posttranscriptional gene regulation, transposon control, and viral defense	Hamilton and Baulcombe (1999), Malone and Hannon (2009)
miRNAs	Plants, animals, algae, protists, viruses	18–25	Associate with argonaute proteins and are involved in posttranscriptional gene regulation	Carthew and Sontheimer (2009), Huntzinger and Izaurralde (2011), Czech and Hannon (2011)
tiRNAs (transcription initiation RNAs)	Human, chicken, *Drosophila*	~18–22	Regulation of chromatin modifications and protein recruitment involved in transcription initiation	Taft et al. (2009a)
piRNAs	Metazoans excluding *T. adhaerens*	24–30	Associate with PIWI-clade argonaute proteins and regulate transposon activity and chromatin state and regulate DNA methylation affecting gene expression	Seto et al. (2007), Lin (2007), Thomson and Lin (2009)
snoRNAs	Eukaryotes, archaea	80–200	Guides chemical modifications (methylation, pseudouridylation) of RNAs (such as rRNAs and tRNAs)	Bachellerie et al. (2002), Dieci et al. (2009)
psnoRNAs (processed snoRNAs)	Eukaryotes	20–100	Regulate alternative pre-mRNA splicing, function as miRNA-like regulators of translation	Khanna and Stamm (2010), Taft et al. (2009b)
sRNAs	Bacteria	50–300	Posttranscriptional gene regulation	Repoila and Darfeuille (2009), Waters and Storz (2009)

moRNAs (miRNA-offset RNAs)	Chordate, human	~20	Derived from regions adjacent to pre-miRNAs; function is unknown	Langenberger et al. (2009), Shi et al. (2009)
tel-sRNAs (telomere small RNAs)	Mammals	~24	Derived from G-rich strand of telomeric repeats; probably involved in maintenance of telomeres	Cao et al. (2009)
natsiRNA (natural antisense transcript-derived siRNA)	Plants	21–24	Regulate stress-response genes	Vaucheret (2006); Yoshikawa et al. (2005)
crasiRNAs (centromere repeat-associated small interacting RNAs)	Vertebrates	34–42	Derived from centrosomes; involved in chromatin modifications, and formation and maintenance of centromeric heterochromatin	Carone et al. (2009)
rasiRNA (repeat-associated small interfering RNA)	*Drosophila*, unicellular eukaryote, plants	24–29	Associate with argonatute and PIWI argonaute protein subfamily; maintain heterochromatin structure, silencing of transposons, and retrotransposons in the germline	Gunawardane et al. (2007)
hc-siRNAs (heterochromatin siRNAs)	Plants	~24	Derived from repeat sequences of genomes (transposons, retroelements, centromeric repeats) and involved in DNA and histone methylation	Daxinger et al. (2009)
scnRNAs (small scanRNAs)	Protozoa	~28	Expressed during conjugation and participate in chromatin modifications	Kurth and Mochizuki (2009)
qiRNAs (quelling deficient, QDE2-interacting small RNAs)	Plants	20–21	Mostly originate from ribosomal RNA locus, contribute to DNA damage checkpoints by inhibiting protein synthesis	Lee et al. (2009)
casiRNAs (chromatin-associated siRNAs)	Plants	24	Guide de novo cytosine methylation at the homologous genomic DNA region and transcriptional events	Kasschau et al. (2007)

(continued)

Table 1.1 (continued)

ncRNAs	Organisms	Length (nt)	Characteristics/functions	References
tasiRNAs (trans-acting siRNAs)	Plants	21	Posttranscriptional gene regulation	Vazquez et al. (2004), Allen et al. (2005)
lncRNAs (long (regulatory) noncoding RNAs)	Animals	$> \sim 200$	Epigenetic regulation	Ponting et al. (2009), Mercer et al. (2009), De Lucia and Dean (2011)
xiRNAs (X-inactivation RNAs)	Mammals	25–42	Processed from duplexes of two lncRNAs, Xist and Tsix that are responsible for X chromosome inactivation in placental mammals; probably involved in translational silencing	Kanduri et al. (2009)
PASRs (promoter-associated small RNAs)	Animals	22–200	Located at 5′ end of the genes; may be involved in regulation of gene expression	Kapranov et al. (2007)
TASRs (termini-associated small sRNAs)	Animals	22–200	Located at 3′ boundaries of genes	Kapranov et al. (2007)
non-PASRs	Animals		Located at a site distant from PASRs	Fejes-Toth et al. (2009)
aTASRs (antisense TASRs)	Human	< 200	Found within 50 bp and antisense to 3′ UTR of the transcripts; are closely associated with 3′ end of known RNAs, pointing to the existence of RNA-copying mechanism	Kapranov et al. (2010)

The information provided in this table are up to date as per our knowledge (till the time of drafting of the book). These information are likely to change in due of time because of new research discoveries

Table 1.2 Comparison between three most popular small regulatory RNAs

Features	siRNAs	miRNAs	piRNAs
Organisms	Eukaryotes	Eukaryotes, viruses	Worm, zebra fish, mammals
Origin	Endogenous and Exogenous: transposons, viruses, DNA heterochromatin	Endogenous	Endogenous
Length	21–22	18–25	24–30
Nature of precursors	dsRNA	dsRNA	ssRNA
Genomic location	Dispersed throughout	Dispersed throughout	Clustered
Site of biogenesis	Cytoplasm/nucleus	Nucleus/cytoplasm	Not clearly known
Argonaute	AGO1–AGO4	AGO2	PIWI/aubergine, AGO3
Site of expression	All tissues	All tissues	Germlines
Type of transcripts	Polycistronic	Polycistronic/ monocistronic	Polycistronic
Phylogenetic conservation	Rarely conserved	Highly conserved	Not conserved

The information provided in this table are up to date as per our knowledge (till the time of drafting of the book). These information are likely to change in due of time because of new research discoveries

piRNAs are found in the testes and ovaries of zebra fish and *Drosophila* as well as in the testes of mammals. In the germline, these small RNAs ensure genomic stability by silencing endogenous selfish genetic elements (retrotransposons and repetitive sequences). piRNA biogenesis is driven by two distinct processes that have been revealed by deep sequencing and genetic studies (Siomi et al. 2010). Majority of unique piRNAs are derived from transposon-rich heterochromatic clusters (Brennecke et al. 2007; Yin and Lin 2007). There is a "ping-pong" amplification cycle which is needed to amplify siRNA triggers in plants, nematodes, and yeast (Verdel et al. 2009) (see Chap. 2). The ping-pong model was developed from observations in *Drosophila*, but a similar mechanism appears to function in other animal groups (Aravin et al. 2007; Houwing et al. 2007; Grimson et al. 2008; Palakodeti et al. 2008; Lau et al. 2009).

A considerable fraction of the piRNAs isolated to date map to transposon-encoding regions (although this is highly variable from species to species) (Girard and Hannon 2008), and piRNA mutations lead to massive transposon over-expression. piRNA–PIWI complexes are therefore assumed to directly control transposon activity. piRNAs bound to PIWI proteins direct homology-dependent target cleavage *in vitro*, suggesting that transposons are silenced through posttranscriptional transcript destruction (Gunawardane et al. 2007; Saito et al. 2006; Nishida et al. 2007). piRNAs bound to Aub and AGO3 direct homology dependent cleavage of mature transposon transcripts after export from the nucleus. Mutations in piRNA pathway genes disrupt germline development, often producing complex and poorly understood phenotypes that are difficult to directly associate with transposon targets of the pathway (see Chap. 5). There are also evidences,

which show that piRNAs might also have a role in regulating translation (Grivna et al. 2006; Unhavaithaya et al. 2009). These ncRNAs are also assumed to play a significant role in regulating gene expression, which might be restricted to specific tissues or developmental stages. Majority of the piRNAs map to the unannotated regions of the genome in poriferans, cnidarians, worm, and mouse and only a limited set match transposons and other repeats (Aravin et al. 2006; Girard et al. 2006; Grimson et al. 2008; Batista et al. 2008; Ruby et al. 2006) which supports this hypothesis. All these hints toward the biological function for this novel class of small RNAs well beyond transposons and germline development.

1.5 Exploring the Genomic Dark Matter

In recent years, novel strategies – both computational and experimental – have been undertaken to identify a great number of novel ncRNA candidates in various model organisms from *Escherichia coli* to *Homo sapiens* (Storz 2002; Washietl et al. 2005; Huttenhofer et al. 2001; Wassarman et al. 2001). These findings demonstrated that the number of ncRNAs in genomes of model organisms is much higher than it had been anticipated.

Among the different experimental strategies for identifying novel ncRNAs, RNA sequencing is one of the most powerful and widely adopted approaches and relies on the generation of specialized cDNA libraries, e.g., RNP libraries (see details in Chap. 9). Other methods include microarrays for identifying ncRNAs expressed under a given experimental condition (see Chap. 12) and/or ncRNAs of various sizes in a single experiment employing hybrid LNA/DNA microarrays (see details in Chap. 9), "genomic SELEX" to select ncRNA candidates from the sequence space represented by the genome of an organism of interest, or targeted deep sequencing of classes of RNA with distinct 5′ and 3′ ends or affinity for specific proteins after extraction with immunoprecipitation (see Chap. 10). Apart from such biochemical methods, bioinformatics tools are also employed to identify various types of ncRNAs from different species and model organisms (Washietl et al. 2005; Vogel and Sharma 2005; Eddy 2002). These bioinformatic tools are often based on sequence, secondary structure, and thermodynamic identities, and/or conservation features of ncRNAs revealed through comparative genomics approaches. For comprehensive understanding of the principles and methods for prediction of small RNAs among bacteria and their targets refer to chapter 11 for biocomputational approaches, and chapter 14 for experimental approaches. The long ncRNAs represent another major unexplored component of genomes of great potential biological importance (see Chap. 8), but they are not properly acknowledged and explored unlike other small RNAs (Carninci and Hayashizaki 2007). Moreover, lncRNAs surprisingly have no significant homology identified across each lncRNA in their sequences and mechanisms of function, unlike other ncRNAs such as miRNAs. These raise questions regarding diversity in their functions and

origins. Therefore, many methods, both computational and experimental have come up in these years to identify and characterize lncRNAs (refer to Chap. 13) and make comprehensive catalogs of these ncRNAs for better understanding of their functions in gene regulation and human diseases.

However, without a clue to their biological functions, the newly identified ncRNA molecules raise the burning questions: what are the functions of all of these RNA transcripts? Or, if they are not functional, why does the cell devote its resources to producing them? Hence, next to "novel approaches" for identifying ncRNAs in different organisms comes the novel methods preferably high-throughput methods (Willingham et al. 2005; Krutzfeldt et al. 2005) to understand their biological roles in those organisms.

1.6 ncRNAs: A Password to Future Personalized Therapy

Continual discoveries of ncRNAs have changed the landscape of human genetics and molecular biology. Over the past 10 years, it has become clear that ncRNAs are involved in all developmental processes (see Chap. 7), including stem cell and germline maintenance, development and differentiation, and when dysfunctional, underpin disease (Lee and Calin 2011; Qureshi and Mehler 2011). Several classes of ncRNAs, such as siRNAs, miRNAs, piRNAs, snoRNAs, etc., are implicated in different diseases, namely, cancer, heart diseases, immune disorders, and neurodegenerative disorders (see Chap. 18) and metabolic diseases, etc. (Galasso et al. 2010; Taft et al. 2010).

ncRNAs also play a dominant role towards shaping the epigenetic program in human embryonic stem cells and adult cells (Lunyak and Rosenfeld 2008). This has opened up the avenue to understand how cells remember their own fates and hence can improve regenerative medicine in several ways. Specific ncRNAs can be used as markers to track and predict when cells are acquiring or forgetting specific cell fates (see Chap. 17). For instance, it may be possible to learn from the pattern of ncRNAs that an embryonic stem cell is ready to become cardiac cells, which can be used to treat a patient with cardiac hypertrophy. Further, beyond tracking cell fate, ncRNAs may be used for direct manipulation of stem or adult cell fates. They can be used for reprogramming pluripotent stem cells into desired cell types (see Chap. 15). While these potential applications are far in the future, we believe that better knowledge of this new level of gene regulation will lead to more facile and efficient manipulation of cell fates for regenerative medicine in future.

Moreover, siRNAs have become not only an exciting new tool in molecular biology but also the next frontier in molecular therapeutic applications. In this volume, we have described the types of choices that must be made in the development of siRNA therapeutics, the features of the siRNA molecule that are important for maximizing silencing activity, how to design delivery vehicles to transport siRNAs to their intended location, and examples of ongoing clinical trials utilizing siRNA therapeutics to treat solid tumors, acute kidney failures, and some of the

acute and dreadful viral infections (see Chap. 19). Furthermore, it has been observed that some cellular pathways are altered in cancer stem cells (CSC), and these preferentially offer targets for RNAi therapy against cancers (see Chap. 16). RNAi provides a unique opportunity to silence cancer-causing stem cell genes at the pretranslation level, which is otherwise not possible with conventional therapies such as cytotoxic chemotherapy, small molecule inhibitors, or monoclonal antibodies.

Since ncRNAs are linked to pathological conditions and, in particular, disease development and progression, ncRNAs might become useful biomarkers for diagnostic purposes. For example, miRNAs have been found to be associated with disease prognosis, survival, and mortality in biopsies (Schetter et al. 2009; Bloomston et al. 2007). Their expression levels can be determined by in situ hybridization and microarray, e.g., on a tumor section and its normal adjacent counterparts (see Chap. 21). Major challenge lies in translating the molecular signatures determined in the laboratory to the clinical setting.

The fundamental roles of ncRNAs in development, differentiation, and malignancy suggest that these classes of molecules are potential targets for novel therapeutics. Antisense oligonucleotide approaches used for inhibition, and siRNA-like technologies used for replacement are currently being explored for therapeutic modulation of miRNAs. Several approaches are currently adopted to silence ncRNAs. Table 1.3 enlists the different approaches for the purpose, which has been mostly applied to miRNAs till today.

Table 1.3 Approaches employing ncRNAs in therapeutic applications

Approaches	Name of the tools/methods	Applications	References
Inhibition of mature miRNAs	microRNA sponges	Silence oncomiR family	Ebert et al. (2007)
	2′-Ome AMOs	Silence oncomiR	Krutzfeldt et al. (2005)
	2′-MOE AMOs	Silence oncomiR	Weiler et al. (2006)
	LNA-antagomir	Silence oncomiR	Elmen et al. (2008a)
Manipulation of miRNA precursor	amiRNAs	Silencing of target genes involved in metastasis	Liang et al. (2007) Zhang et al. (2006)
Inhibition of pri-miRNA	AMOs (RNase H-based)	Silence polycistronic clusters of miRNAs	Wu et al. (2004)
Replacement of mature miRNAs	Pre-miRNA-like shRNAs	Restore tumor suppressor miRNAs	Brummelkamp et al. (2002)
	Double-stranded miRNA mimetics	Restore tumor suppressor miRNAs	Tsuda et al. (2006)
Designing small oligonucleotides with perfect complementary to the seed	Target protectors	Inhibit functions of oncomiR	Choi et al. (2007)

AMOs anti-miRNA oligonucleotides, *2′-Ome* 2′-O-methyl, *2′-MOE* 2′-O methoxyethyl, *LNA* locked nucleic acid, *oncomiR* oncogenic miRNAs, *amiRNAs* artificial miRNAs

Specific knockdown of miRNAs by anti-miRNA oligonucleotides (AMOs), double-strand miRNA mimetics, and overexpression of miRNA duplexes have been conducted *in vitro* and *in vivo*. Inhibition of specific miRNAs in mouse model has been performed by antagomirs. Also RNase H-based AMOs have been found useful for targeting polycistronic pri-miRNAs, like the miR-17–92 cluster (Wu et al. 2004). Specific dose-dependent silencing of miR-122 has been performed by systemic administration of 16-nucleotide unconjugated locked nucleic acid (LNA)-AMO which is complementary to the 5′ end of miR-122 (Elmen et al. 2008b). Another de novo engineered ncRNA inhibitors are "miRNA sponges" which inhibit miRNAs with a complementary heptameric seed, such that a single sponge can be used to block an entire miRNA family with the same seed. Inhibition of Drosha, Dicer, or any other components in the maturation pathway is another method for therapeutic targeting of ncRNAs. This method however might be difficult to be made specific in its therapeutic effect. Moreover, artificial miRNAs (amiRNAs) are recently developed miRNA-based tools to silence endogenous genes. These are created from an endogenous miRNA precursor by exchanging the miRNA/miRNA sequence of it with a sequence designed to match the target gene of interest (see Chap. 20).

An alternative therapeutic strategy of replacement of defective/absent RNA effectors is needed if there is a loss in the activity of ncRNAs in the diseased/affected cells. Lentiviral delivery of short hairpin RNAs is one of the systems for the delivery of shRNA constructs designed to mimic the pri-miRNA by including the miRNA flanking sequence into the shRNA stem (Chang et al. 2006; Zeng et al. 2005). Further, there has been activation of tumor suppressor miRNAs, such as miR-127, by chromatin-modifying drugs which can inhibit tumor growth through downregulation of their target oncogenes (Grunweller and Hartmann 2007).

All these highlight the clinical potential of ncRNAs as biomarkers for diagnosis, prognosis, and prediction of therapeutic outcome.

1.7 Future Perspectives

The possibility of self-replicating ribozymes emerging from pools of random polynucleotides and surviving in a prebiotic soup has put forth these RNAs to be a challenging molecule, which leads us to an "RNA world." The logical order of events begins with prebiotic chemistry and ending with DNA/protein-based life. The present challenge lies in decoding the genomic dark matter. Further, the absolute number of protein-coding genes encoded by a genome is essentially static across all animals from simple nematodes to humans (Taft et al. 2007), which hints for additional genetic elements that must be involved in the development of the increasingly complex cellular, physiological, and neurological systems. Noncoding RNAs are the likely candidates, which can resolve such discrepancy within the genomic content and illuminate on the genomic dark matter, as they are adaptively

plastic, capable of regulating processes both broadly and sequence-specifically, and are now known to be components of nearly all cellular and developmental systems.

It is becoming clear that a comprehensive understanding of human biology must include both small and large noncoding RNAs. With new systems biology approaches, and in-depth investigation of other important players and their interactions, we may see an emerging integration of RNA-dependent regulatory networks into normal cell physiology. It is perhaps only through inclusion of these elements in the biomedical research agenda along with studies to determine the mechanistic basis of the causative variations (identified by genome-wide association studies), that complex human diseases will be completely deciphered.

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