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Non-Coding RNAs

Edited by Lütfi Tutar, Sümer Aras and Esen Tutar

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Meet the editors

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Contents

Preface

Noncoding RNAs (ncRNAs) have made a huge impact on RNA biology. ncRNAs play important roles in regulating gene expression in animals, plants, and various human diseases. This book provides an overview of current knowledge on ncRNA research by dealing with miRNA- and ncRNA-related human diseases, plant miRNA markers, and the roles of lncRNAs in cancer and epigenetics. The book starts with a brief introductory chapter on ncRNAs. The second chapter discusses the pathogenetic role of miR-335-5p in gastrointestinal tumors. The third chapter focuses on miRNA expression in smoking-induced periodontitis. The fourth and fifth chapters describe the role of herpesvirus-encoded miRNAs in avian diseases and the role of three miRNA families in the progression of skin aging. The sixth chapter covers miRNA-based markers in plants and their application in plant genotyping. The seventh chapter elaborates the role of ncRNAs, especially circRNAs, miRNAs, mRNAs, and their interactions, in brain cells. The last two chapters review lncRNAs as hallmarks of cancer with clinical applications and their functions as epigenetic regulators. Overall, the book content provides a unique perspective to scientists in the field.

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Chapter 1

Introductory Chapter: Noncoding RNAs—A Brief Overview

Sümer Aras, Esen Tutar and Lütfi Tutar

1. Introduction

Noncoding RNAs (ncRNAs) are an attractive research field to prompt extensive genome-wide transcriptional efforts by different international initiatives such as the Encyclopedia of DNA Elements (ENCODEs) [1] and the Functional Annotation of the Mammalian Genome (FANTOM) [2]. Existence of ncRNAs is ubiquitous to all three domains of life, but they play different roles according to type of its RNA family [3, 4]. Dysfunction of ncRNAs may lead to various human diseases from tumorigenesis to neurological, cardiovascular, and developmental disorders [5]. Hence, ncRNAs have become a hot topic in molecular genetic and epigenetic research.

Findings of Human Genome Project have disclosed that approximately 1.5% of human genome is comprised of protein encoding genes. On the other hand, the majority of the human genome is transcribed and yields ncRNAs. Noncoding RNAs, which are not translated as peptides or proteins, may be categorized as housekeeping noncoding RNAs and regulatory noncoding RNAs. Noncoding RNAs may be grouped into two major classes based on their size: small noncoding RNAs (sncRNAs) are shorter than 200 nucleotides (nts) in length and long noncoding RNAs (lncRNAs) are longer than 200 nts. Albeit these RNAs are named as noncoding, some lncRNAs code for small bioactive peptides [6, 7].

SncRNAs accorporate functional RNAs including r-RNAs, snRNAs, and t-RNAs, which play important roles in transcriptional and translational regulations. Furthermore, sncRNAs also contain regulatory RNAs, which play roles in gene expression such as P-element-induced wimpy testis (PIWI) interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and microRNAs (miRNAs). Per contra, lncRNAs represent a large group of noncoding regulatory RNAs. The lncRNAs are divided according to their mode of action, such as natural antisense transcripts (NATs), intergenic (lincRNAs), intronic lncRNAs, circular RNAs (circRNAs), promoter-associated long RNAs (pRNAs), and enhancer RNAs (eRNAs).

2. miRNAs and siRNAs

siRNAs and miRNAs are 19–24 nts in size and silent transcription of genes via inducing mRNA degradation or translational repression. Generally, protein-coding genes are negatively regulated by a single miRNA or multiple miRNAs [8]. While miRNAs originated from pri-miRNAs, source of siRNAs is double-stranded RNAs. Moreover, miRNAs potentially play important roles in biological processes in a cell such as cell differentiation, cell proliferation, cell death, and development by inducing mRNA degradation or translational repression. Dysregulation of miRNAs leads to several human diseases including cancer, cardiovascular, and neurodegenerative diseases [9].

3. piRNAs

piRNAs are 21–35 nts long and originated from long single-chain precursor transcripts. Their main functions are transposon repression, DNA methylation, silencing transposable elements, regulating gene expression, and fighting with viral infections. PIWI proteins are guided by piRNAs to cleave target RNA, promote heterochromatin assembly, and methylate DNA [10]. Up- and downregulated expressions of piRNAs in several cancer types and Alzheimer's disease suggest that piRNAs take part in several human diseases [11].

4. lncRNAs

lncRNAs are more than 200 nts long and originated in multiple ways and may be transcribed from both noncoding DNA by RNA polymerase II and protein coding (e.g., H19 and TUG1). LncRNA genes are more abundant than short ncRNAs and outnumber protein-coding genes. They mainly function in genomic imprinting and X-chromosome inactivation. Furthermore, they take roles in gene regulation, chromatin remodeling, cancer cell invasion, and metastasis and cell differentiation by acting as *cis*- or *trans*-regulators in biological processes [12]. Moreover, function of lncRNAs linked to some human diseases including hepatocellular carcinoma, Alzheimer's disease, and diabetes [13].

5. Future perspectives

Mounting evidence and recent discoveries of novel short and long regulatory noncoding RNA classes revealed remarkable complexity of RNA-guided regulation on biological processes in a cell. There is a complex problem between RNA regulatory network and protein-based regulatory mechanisms. Noncoding RNA regulatory network is a challenging process to analyze and untwist. For example, notwithstanding their size, both lncRNAs and miRNAs play regulatory roles on protein-coding genes at post-transcriptional repression, but also lncRNAs may act as miRNA sponges and may degrade regulatory effect on mRNA [14]. It is known that dysregulation of ncRNAs may stir up several diseases including cancer and neurodegenerative diseases. Hence, further development of bioinformatics, genome scanning, and biochemical techniques is required to illuminate detailed functions and interactions of ncRNAs. It is clear that further research on ncRNAs will change our understanding about the nature of genome composition by rummaging previous dogmas.

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Chapter 2

MicroRNA-335-5p and Gastrointestinal Tumors

Pablo M. Santoro, Alejandra Sandoval-Bórquez and Alejandro H. Corvalan

Abstract

Noncoding genomics, i.e., microRNAs and long coding RNAs (lncRNA), is an emerging topic in gastrointestinal tumors. In particular, the coordinate deregulation of miRNA-335-5p across these tumors and its potential clinical applications is an example of this scenario. This chapter discusses the pathogenetic role of miRNA-335-5p in esophageal, gastric, colon, liver, gallbladder, and pancreatic tumors. This pathogenetic role is examined in the context of the competing endogenous network, the language through lncRNA that reduce the quantity of miRNA available to target mRNA. The translational application of miRNA-335-5p, through the aberrant methylation of the promoter region of MEST—its host gene—as a potential biomarker for noninvasive detection of gastric cancer, is also discussed.

Keywords: ncRNA, miRNA-335-5p, gastrointestinal tumors, gastric cancer, competing endogenous, CERNA, DNA methylation, biomarkers

1. Introduction

Gastrointestinal tumors (i.e., esophagus, stomach, colon, liver, gallbladder, and pancreas) are among the most common cancers by incidence and mortality in males and females worldwide [1]. Furthermore, projections of global mortality and disease burden indicate that new cases and deaths from these tumors will increase by 2030 [2]. Given this scenario, understanding of the molecular basis of gastrointestinal tumors is essential to the development of novel strategies for diagnosis and disease treatment. Large genomic studies focusing on protein-coding regions have identified multiple of genes recurrently mutated in gastrointestinal and other human neoplasms [3]. However, molecular classifications based on coding genes do not fully capture the clinical heterogeneity found in gastrointestinal tumors [4]. This observation indicates that other segments of the genome might also contribute to the emerging complexities observed in the development and progression of gastrointestinal neoplasms. In this chapter, we describe recent advances in our understanding of noncoding genome in gastrointestinal cancer. In particular, we will focus on miRNA-335-5p, since not only has it been found to be critically involved in myriad tumors but it has also proved to be a potential biomarker for noninvasive diagnosis of cancer and for the treatment of preneoplastic conditions [5, 6].

2. Noncoding genomics

The traditional view of the unidirectional flow (i.e., DNA-RNA-protein) of genomic data has been reclassified as multidirectional, based on the fact that even though 80% of DNA is transcribed into RNA, only 2% ultimately represent the coding genes which are translated into protein [7]. Therefore, the majority of RNA is defined as noncoding RNA (ncRNA) which in turn includes a wide range of RNA families such as those involved in the translation and splicing of messenger RNA (mRNA) as well as those associated with the modification of ribosomal RNA [7]. ncRNA also plays an essential role in all multiple biological functions, i.e., cell proliferation, apoptosis, cell migration and invasion, and cell differentiation being involved in each of the cancer hallmarks as well [8]. Based on the size of its sequence, ncRNA can be divided into short (~20–200 nucleotides; nt) and long ncRNA (200 to ~100,000 nt) [9].

2.1 Short noncoding RNAs

Short ncRNAs (sncRNAs) are represented by P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and microRNAs (miRNAs). piRNAs (24–32 nt) are specialized for repression of mobile and other genetic elements in germ line cells (e.g., LINE1 piRNAs and piR-823) [10]. piRNAs and PIWI have been found deregulated in a tissue-specific manner in a variety of neoplasms, opening novel opportunities to diagnosis and treatment of disease [10]. siRNAs regulate posttranscriptional gene silencing and the defense against pathogen nucleic acids (e.g., L1-specific siRNA and oocyte endo-siRNAs) [11]. Therefore, they seem to have great potential in disease treatment, especially as promising epigenetic therapy through the silencing of cancer-related genes [12].

miRNAs represent the largest group of short noncoding RNAs, highly conserved and involved in the posttranscriptional regulation of gene expression in multicellular organisms [13]. miRNAs were discovered in the 1990s while studying fetal development of *Caenorhabditis elegans* [14]. To date, more than 30,000 miRNAs have been found in over 200 species [15]. In humans, the latest miRNA database miRBase release (Release 22.1) contains 2588 annotated mature miRNAs [15]. It is estimated that 60% of coding genes may be regulated by miRNAs. miRNAs have been found deregulated in a tissue-specific manner in human neoplasms, offering novel opportunities for diagnostic assessment and disease treatment [16].

Functional studies have confirmed critical roles of miRNAs in development and disease, particularly in cancer [17]. miRNAs can act as tumor suppressors or oncogenes, and miRNA mimics have shown promise in preclinical and early stages of clinical development [17]. miRNAs reflect the developmental lineage and differentiation state of the tumors being mostly downregulated compared with normal counterpart tissues [18]. Particularly, gastrointestinal tumors cluster together reflecting their common derivation from embryonic endoderm [18]. miRNA-335-5p is among the most frequently deregulated miRNAs in gastrointestinal tumors.

2.2 miRNA-335-5p structure and regulation of its expression

Although initially described in developmental biology, as differentially expressed and maternally imprinted during mouse and human lung development [19], later studies have shown that miRNA-335-5p is extensively deregulated in human tumors [20]. miRNA-335-5p is a transcript located on chromosome 7q32.2, in the second intron of the mesoderm-specific transcript homolog (MEST)

gene [21], which encodes 17 different mRNAs [22]. In humans, the mature sequence of miRNA-335-5p forward strand, miRNA-335-5p, corresponds to 16 -UCAAGAGCAAUAACGAAAAAUGU-38 (http://www.mirbase.org, accession: MIMAT0000765, ID: hsa-miR-335-5p) [23]. The strong correlation between the expression of miRNA-335-5p and its host gene MEST suggests that the mechanism responsible for silencing miRNA-335-5p expression should be the promoter methylation of MEST [5, 24, 25] (**Figure 1**). The promoter of MEST gene contains three CpG islands upstream of transcriptional start site [26]. The treatment with 5-aza-2′-deoxycytidine (5-aza-dCyd) restores the expression levels of miRNA-335-5p in hepatocellular and gastric cancer [24, 27]. Furthermore, an inverse correlation between expression levels of miRNA-335-5p and its methylation status was revealed in these cancer tissues [24, 27].

2.3 miRNA-335-5p and gastrointestinal malignancies

The coordinated deregulation of miRNA-335-5p across the gastrointestinal tract neoplasms implied the relevant role of this microRNA in these tumors. Esophageal adenocarcinoma (EAC) is one of the fastest rising incidences of cancers with a dismal prognosis [28]. EAC is the final stage of Barrett esophagus (BE), an adaptive response to chronic gastroesophageal reflux in which the squamous epithelium of the esophagus is replaced by glandular columnar epithelium [28]. By combining multidimensional genomic measurements from the TCGA, Xi and Zhang [29] proposed a genomic signature of five differentially expressed miRNAs including miRNA-335-5p that can be applied for novel diagnostic approaches and disease treatment. Gastric cancer (GC) remains the fifth cause in cancer incidence and the third cause of death by neoplasms worldwide [1]. GC is a highly heterogeneous disease with unique ethnogeographical associations [30–32]. Profiling studies have not identified miRNA-335-5p as part of any miRNA signatures with clinical significance. However, gene-specific approaches suggest that miRNA-335-5p is downregulated in GC and clinically associated with advanced TNM stage and worse prognosis [5, 33]. Functional studies have shown that exogenous expression of miRNA-335-5p deregulated many biological cell processes such as cell cycle, proliferation, apoptosis, migration, invasion, and metastasis [5, 25, 33, 34] (**Figure 2**).

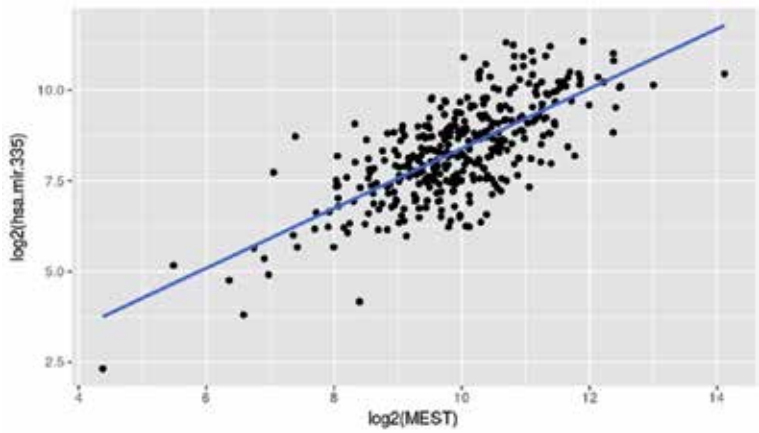

Figure 1.
Linear regression model (blue line) using RNAseq data from 368 tumor samples from the stomach adenocarcinoma from The Cancer Genome Atlas (TCGA) consortium (taken from Sandoval-Bórquez et al. [5] with permission).

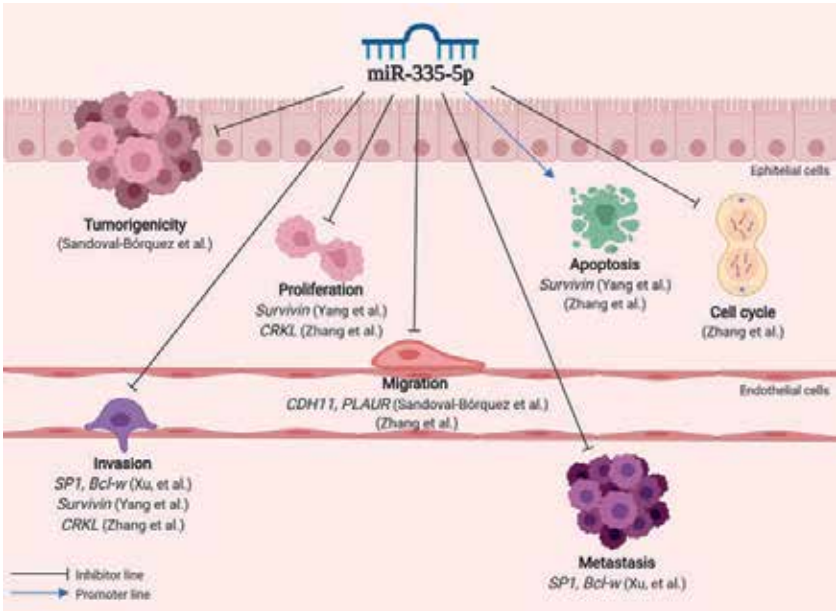

Figure 2.
Cellular processes by which miRNA-335-5p contributes to their regulation though of different target genes in gastric cancer cell lines. miRNA-335-5p inhibits tumorigenicity, cell cycle, proliferation, apoptosis, migration, invasion, and metastasis [5, 25, 33, 34]. Abbreviations: CRKL, V-crk avian sarcoma virus CT10 oncogene homolog-like; CDH11, cadherin 11; PLAUR, plasminogen activator urokinase receptor; SP1, specificity protein 1; BCL-w, BCL2-like 2 (taken from Sandoval-Bórquez et al. [5] with permission).

Of note, upregulation of miRNA-335-5p might be associated with tumor recurrence, the major factor of treatment failure in this disease [35].

Colorectal carcinoma (CRC) is the first cause of death by cancer in developed countries [1]. In this tumor downregulation of miRNA-335-5p has been associated with microsatellite instability (MSI) [36] and ability to discriminate between non-serrated and serrated adenomas [37]. Even though it has been described as upregulated in tumor samples relative to normal mucosa, functional studies have shown that its overexpression inhibits invasion and metastasis in CRC cell lines [38, 39]. The number of deaths for hepatocellular carcinoma (HCC) is nearly equal to the worldwide annual incidence of newly diagnosed cases [40]. Almost 80% of HCC is attributed to chronic hepatitides B and C which evolve to cirrhotic/fibrotic liver and ultimately HCC [41]. Screening of multiple miRNAs identified miRNA-335-5p among unique seven miRNA signatures that could be associated with the development HBV-related HCC [42]. Gallbladder carcinoma (GBC) is an infrequent but highly lethal biliary tract tumor mostly associated with the presence of gallstones and chronic inflammation [43]. Tumor suppressor and cancer-prone miRNAs have been identified in GBC including the downregulation of miRNA-335-5p, which together with other microRNAs produces a signature clinically associated with prognosis and prediction of treatment response [44]. Gene-specific analysis in paired tumor and normal tissues also confirms downregulation of miRNA-335-5p in association with histologic grade, stage, presence of metastases, and poor survival [45].

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy, and because of the late presentation, as few as 20% of patients are candidates for curative treatment. Ectopic expression of miRNA-335-5p in pancreatic cancer cell lines significantly suppressed cell growth by inhibiting c-Met [46]. Other malignancies in which miRNA-335-5p is deregulated have been comprehensively described Luo et al. [20].

2.4 Long noncoding RNAs and the competing endogenous network of miRNA-335-5p in gastrointestinal malignancies

Long ncRNAs, which span from 200 to ~100,000 nt, make up the largest portion of the human noncoding transcriptome [47]. lncRNAs are essentially regulatory molecules implicated in multiple cellular processes in a tissue-specific manner [48]. Multiple reports have compiled the emerging role of the deregulated expression of lncRNAs in human tumors, and based on tissue-specific transcription, this novel class of genes holds strong potential as biomarkers and new therapeutic opportunities in cancer [49–52]. lncRNAs reduce the quantity of miRNA available to target mRNA (i.e., "sponges"), through the miRNA response elements (MREs) contained within the lncRNA and the 3′UTR of mRNA, which serve as miRNA-binding sites [53]. Based on the relevance of these interactions, a new language has been proposed—the competing endogenous RNA (CERNA)- [52]. In the case of miRNA-335-5p, few CERNA networks have been described, which indicate that this is an emerging area of research in gastrointestinal tumors. As shown in **Table 1**, the lncRNA nuclear-enriched abundant transcript 1 (NEAT1) facilitates Sora resistance in HCC cells via negative regulation of miRNA-335/c-Met/Akt pathway that suppresses apoptosis [54]. The misato family member 2 (MSTO2P) has shown a high expression in parallel to a significantly low expression of miRNA-335-5p in clinical samples as well as in vitro [55]. As of this publication, no mRNA targets for this network have been described. Nuclear-enriched abundant transcript 1 (NEAT) is also upregulated in GC and promotes proliferation, migration, and invasion via targeting the miRNA-335-5p/ROCK1 axis [56]. The zinc finger E-box binding homeobox 1 antisense RNA 1 (ZEB1-AS1) has shown to be critical for the proliferation and invasion of GC cells by regulating miRNA-335-5p [57]. However, the mRNAs associated with this CERNA network are currently unknown.

Further characterization of CERNA networks associated with the deregulation of miRNA-335-5p could be taken from the comprehensive evaluation of genes involved in metastasis and tumor invasion pathways after exogenous miRNA-335-5p expression [5]. Through this experiment up to 19 out of 62 (30.6%) genes were significantly increased [5]. Of note, miRNA-335-5p can target several messenger RNAs, and deregulation of miRNA-335-5p can effectively affect multiple signaling pathways leading to metastasis and tumor invasion [58]. In this scenario, ingenuity pathway analysis (IPA) narrowed the field to nine upregulated genes (CDH11, COL4A2, CTGF, CTSK, MMP7, PDGFA, PLAUR, TIMP1, and TIMP2) (**Figure 3**). Most of these upregulated genes belong to intracellular signaling pathways in cancer such as PI3K-Akt (COL4A2, MYC, PDGFA, SPP1), proteoglycans (HIF1A, MYC, PLAUR, TGFB1), and Hippo (CTGF, MYC, NF2, TGFB1). Among these genes, PLAUR significantly increased mRNA levels after knockdown of miRNA-335-5p expression in GC cells. PLAUR is a membrane-bound glycoprotein with a GPI anchor that encodes the receptor of urokinase-type plasminogen activator and binds and activates PLAU [59]. This activated serine protease converts plasminogen to plasmin, degrading all components of the extracellular matrix and promoting invasion and metastasis [60]. Furthermore, PLAUR has signaling properties through interactions with membrane-bound integrins, which are able to affect migration and cell proliferation [61]. In GC, the overexpression of PLAUR has been reported to be closely related to cell invasion and metastasis [62, 63]. In vitro analysis also showed a significant increase of PLAUR expression in miRNA-335-5p knockdown cells, and, consequently, cells overexpressing miRNA-335-5p exhibited a low level of PLAUR. Accordingly, elevated levels of PLAUR were observed in tumor tissues when compared with their paired non-tumor mucosa [5]. Another relevant gene overexpressed after exogenous miRNA-335-5p expression was

lncRNA name	ID	Abbreviation	Target coding gene	Topic	Validation	Reference
Nuclear-enriched abundant transcript 1	ENSG00000245532	NEAT1	c-Met	Hepatocellular carcinoma	In vitro/in vivo	Chen and Xia [54]
Misato family member 2, pseudogene	ENSG00000203761	MSTO2P	Unknown	Gastric cancer/metastasis	Clinical/in vitro	Li et al. [55]
Nuclear-enriched abundant transcript 1	ENSG00000245532	NEAT1	ROCK1	Gastric cancer	Clinical/in vitro	Wang et al. [56]
ZEB1 antisense RNA 1	ENSG00000237036	ZEB1-AS1	Unknown	Gastric cancer	Clinical/in vitro/in vivo	Zhang et al. [3, 57]

Table 1.
Competing endogenous RNA (CERNA) associated to miR-335-5p.

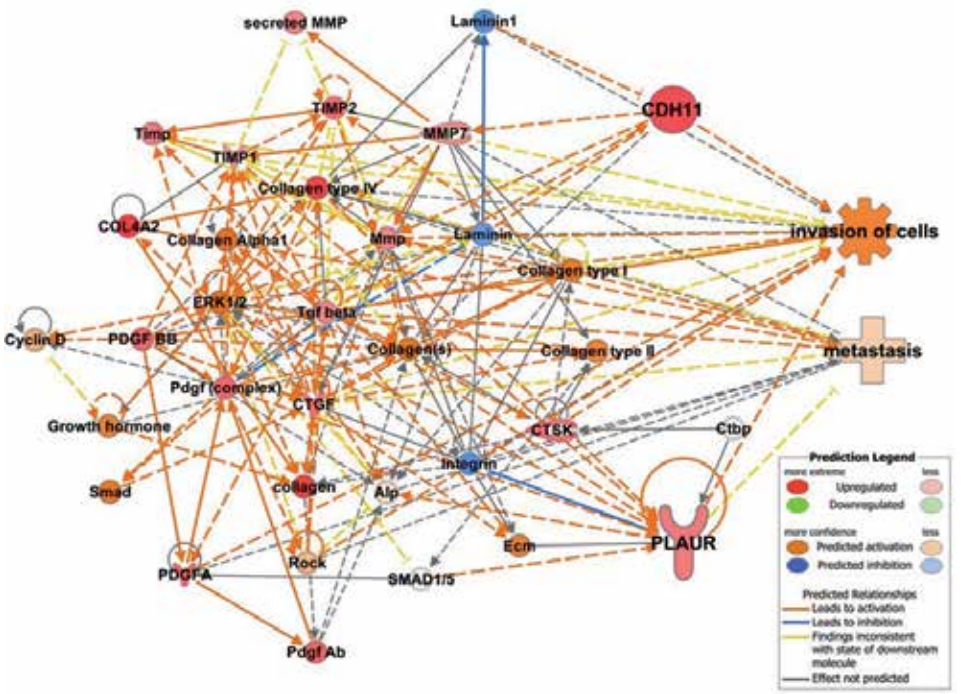

Figure 3.
*Ingenuity pathway analysis (IPA) for network enrichment analysis identified metastasis and invasion downstream genes of miRNA-335. A network of nine significantly overexpressed (red) genes during miRNA-335 inhibition. The MAP tool shows activation and inhibition of neighboring genes and predicts activation of metastasis and invasion of cells in silico. *p < 0.05 (taken from Sandoval-Bórquez et al. [5] with permission).*

CDH11, which encodes a type II classical cadherin, an integral membrane protein that mediates calcium-dependent cell-cell adhesion [64]. CDH11 has been reported as deregulated in various tumor types, suggesting a role in metastasis and tumor invasion [65, 66], and its overexpression was found in advanced cases of gastric cancer [67]. Binding experiments demonstrate the presence of direct targeting of miRNA-335-5p in the CDH11 gene [5]. Interestingly, preliminary data of potential targets of miRNA-335-5p in association with PLAUR and/or CDH11 reveal 15,898 new lncRNAs or 27,688 transcripts for further exploration of the role of CERNA in metastasis and tumor invasion pathways.

2.5 Translational applications of miRNA-335-5p

Methylated cell-free DNA in plasma has emerged as a potential biomarker for diagnosis, prognosis, and prediction of treatment response in gastrointestinal tumors [24, 68]. In addition, several studies have shown that downregulation of miRNAs is associated with DNA methylation of the promoter region of its host genes [69].

Envisaging the clinical application of the downregulation of miRNA-335-5p in GC, Sandoval-Bórquez et al., [5] expand to plasma the reported inverse correlation between the expression of miRNA-335-5p and aberrant promoter methylation of its host gene (MEST) in tissues and cell lines [24] (**Figure 4A**). Furthermore, these authors demonstrated that this aberrant hypermethylation could be a surrogate biomarker for noninvasive diagnosis of GC since it was significantly found in GC cases in comparison with healthy donors ($p = 0.029$, Pearson's correlation) (**Figure 4B**).

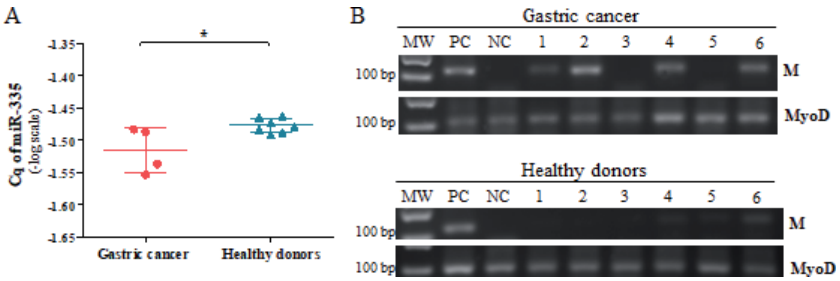

Figure 4.
Potential clinical application as noninvasive detection of GC by the downregulation of miRNA-335-5p. In (A) expression of miRNA-335-5p in plasma from GC patients and healthy donors by Cq of miRNA-335-5p, data were transformed to logarithmic values (–log), and results indicate the mean ± SD. In (B) methylation-specific PCR of the promoter region of MEST gene in plasma from GC patients and healthy donors, MyoD was used as a control of DNA conversion. MW, weight marker; M, PCR product with primers specific for methylated promoter region of MEST, host gene of miRNA-335-5p; PC, positive control of methylation (methylated GC cell line); NC, negative control of methylation (peripheral blood lymphocytes) (taken from Sandoval-Bórquez et al. [5] with permission).

3. Final conclusions

In this review, we summarize the role of miRNA-335-5p in gastrointestinal tumors with a focus on GC. We also explored the role of miRNA-335-5p in noncoding and coding gene networks and its downstream signaling pathways involved in the biological effects of tumor growth, invasion, and metastasis. This evidence supports the potential use of miRNA-335-5p in noninvasive diagnosis as well as therapeutic prospects in gastrointestinal cancers.

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Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Chapter 3

Role of MicroRNA in Smoking–Induced Periodontitis

Herman S. Cheung, Carlos Carballosa and Jordan Greenberg

Abstract

Stem cells participate in tissue restoration and therapies. The oral cavity, the site of smoking exposure, contains stem cells which are involved in the development, maintenance, and repair of oral tissues. By residing in the oral cavity, stem cells are exposed to and susceptible to the effects of smoking. Periodontitis has been associated with increased incidences of other illnesses such as cardiovascular diseases. We hypothesis that smoking suppression of stem cell potentials by miRNAs is a separate and independent pathway than the pathway via the nicotine receptor activation. Smoking perturbs miRNA expression, resulting in decreased stem cell regeneration potential that delays healing of periodontitis.

Keywords: periodontal ligament stem cells, cigarette smoking, e-cigarette vapor, regenerative potential

1. Introduction

Cigarette smoking (CS) is one of the leading causes of preventable death in the world. Despite the smoking cessation attempts in the **United States** (**US**), 15.1% of US adults are still active smokers [1]. Cigarette smoke exposure increases the risk of various cancers and systemic diseases [2] in smokers; however, smoking is a difficult habit to quit due to the addictive property of nicotine. Nicotine replacement therapies (NRT) help smokers overcome their nicotine addiction through gradually decreasing nicotine dose delivery regimens. **Electronic cigarettes** (**ECs**), marketed as a "safer alternative" to cigarette smoking, have not been fully regulated by the FDA and are still believed to cause major health concerns due to the concentrated delivery of toxic chemicals like nicotine, among others [3, 4]. Additionally, due to a lack of regulation, labeling and dosage of nicotine can vary widely among EC products. In the United States alone, EC use has doubled among US adults and tripled among adolescents within the last 5 years [5, 6].

Internalization of smoking- and vaping-related toxins occurs primarily through the respiratory system. For specific compounds like nicotine, the subsequent absorption into specific tissues is largely dependent on tissue pH [7]. Nicotine is a weak base (pKa of 8.0) and is more readily absorbed in slightly basic conditions where it is less "ionized" [7–9]. The oral cavity, the initial site of smoke exposure, is a slightly acidic environment. Cigarette smoke is more alkaline than originally thought [7], thereby improving oral nicotine absorption. Electronic cigarette (EC) liquids, on the other hand, are a slightly more basic pH [8]. Therefore, nicotine delivered from these devices is more readily absorbed in the mouth. Nicotine in

the smoker's saliva, which traps ionized nicotine and maintains elevated levels of exposure in the mouth, is almost 87 times higher than in the blood plasma [10–12].

1.1 Periodontal ligament stem cells (PDLSCs)

Periodontal ligament stem cells (PDLSCs) are an adult stem cell population that reside in the periodontal ligament and give rise to tooth-supporting structures such as the alveolar bone, periodontal ligament, and cementum [13, 14]. In addition to their regenerative capabilities, PDLSCs can be easily isolated following natural tooth loss or routine dental procedures [13]. A recent clinical trial using autologous PDLSCs as a therapeutic approach for the regeneration of periodontal bone defects confirmed the safety of this approach for human use [15]. Stem cells have proven to be effective for the treatment of a variety of injuries and diseases. However, compromising stem cell function, through infectious or genetic diseases, can lead to ineffective clinical outcomes; therefore these conditions are included as exclusion criteria in patient recruitment for stem cell-based therapies. Environmental risk factors such as cigarette smoking and nicotine use can also compromise stem cell function leading to inefficacious outcomes [16–19]. This suggests that they deserve consideration as exclusion criteria [18, 19].

1.2 Nicotinic acetylcholine receptor

Although the effects of nicotine exposure on human health are known, its implication in regard to stem cell biology and function remains greatly unknown. Nicotinic acetylcholine receptor (nAChRs) are one of the mediators of the nicotinic effect. Once internalized and in the blood stream, nicotine is free to complex with nAChRs. These transmembrane, ligand-gated ion channels are the main mediators behind nicotine's cellular effects [20]. Nicotine, a nAChR agonist, activates the receptor, which causes an opening of the transmembrane ionic channel and a subsequent influx of extracellular ions [22, 23]. Although all nAChRs serve the same basic purpose (i.e., transmembrane ion flux), the downstream implications initiated by receptor activation are dependent on the specific combination of subunits used to compose each nAChR. To date, 16 unique subunit varieties have been identified in the mammalian species [20–22]. Functional nAChRs are composed of five subunits and classified as either homopentameric, if subunits are identical, or heteropentameric, if subunits are nonidentical [20]. The homopentameric α7 nAChR has recently been identified on PDLSCs. In the presence of nicotine, these cells experience an increase in apoptosis and a decrease in migratory and osteogenic potential [23–27]. Interestingly, these effects were only partially inhibited (~40–50%) in cells pretreated with mecamylamine, a non-specific nAChR antagonist, and alpha bungarotoxin (aBTX), an α7-specific nAChR antagonist [26, 27]. Others have shown that similar nicotinic effects of PDLSC regeneration potential by decreasing proliferation, migration, and osteogenic differentiation capabilities in vitro are mediated through agonist-induced activation of α7 nicotinic acetylcholine receptors (nAChRs) present on PDLSCs [26, 27]. However, the detrimental effects of nicotine were still partially observed even after the deactivation of these receptors [27], suggesting that there may be additional mediators behind these effects. This data suggests that additional mechanisms are likely to exist as evident by the only partial reversal of nicotinic effects in the presence of nAChR antagonists.

1.3 MicroRNAs (miRNAs)

miRNAs are 22-nucleotide-long small, noncoding RNAs which may alter gene expression by base pairing with complementary mRNA [28, 29]. These

miRNAs are expressed throughout the body, including in muscular and skeletal tissues, and have been shown to affect cell viability, cell differentiation, and even organ development by downregulating the genes associated with these biological processes. Each miRNA can target several genes, and therefore upregulation of a single strand can affect various biological processes.

2. Biological effect of nicotine

We have published studies regarding the in vitro and in vivo biological and physiological effects of nicotine and CS on mesenchymal stem cells (MSCs) and PDLSCs. While investigating the effects of nicotine on the mechanical properties of MSCs, we concluded that nicotine significantly increases the stem cell membrane of Young's modulus (i.e., stiffness) in a dose-dependent manner [30]. We predicted that this increase in stem cell stiffness would impair the ability of the stem cells to respond to mechanical stimuli, therefore hindering mechano-induction and ultimately stem cell differentiation potential. In addition, we predicted that the loss of membrane compliance would delay MSC migration from stem cell niches.

To investigate these consequences, we evaluated the effects of nicotine exposure on the three processes critical for effective stem cell regeneration potential: proliferation, migration, and differentiation [20]. In vitro exposure to nicotine significantly reduced the proliferation and migration potential of both MSC and PDLSCs [20]. MSC and PDLSC proliferation decreased significantly when culturing cells in media containing 1 μM of nicotine. In fact, by day 5, both nicotine-exposed groups showed greater than a twofold decrease in the total number of cells, indicating the loss of proliferative potential while exposed to nicotine. Mesenchymal stem cells (MSC) and PDLSCs exposed to nicotine also traveled shorter distances compared to non-treated cells. The distance traveled by the nicotine-treated MSC was significantly shorter than that by the control MSC. The average speed of MSC migration was also slower in the nicotine-treated group than in the control group. Analogously, the nicotine-treated PDLSC migrated less than the control PDLSC. Furthermore, the nicotine-treated PDLSC also moved slower than the control PDLSC. Nicotine critically affected the differentiation potential of MSCs and PDLSCs [18, 19]. Under conditioned media stimulation, MSC and PDLSC differentiated into osteoblasts, and their calcium deposition increased. However, the osteogenic differentiation medium supplemented with nicotine reduced calcium deposition as evident by a decrease in MSC and PDLSC alkaline phosphatase activity. Altogether, these findings suggest that nicotine can inhibit the differentiation of osteoblasts from MSC or PDLSC.

To understand the molecular mechanism of the nicotine-induced effect, we also analyzed the global miRNA expression patterns of nicotine-treated PDLSC by microarray. A total of 225 miRNAs were differentially expressed at a twofold difference between nicotine-treated PDLSC when compared with the control [20]. The miRNA expression profiles of the control and 1.0 μM nicotine-treated groups were differentially clustered and separated from each other by either principle component analysis or hierarchical clustering. Compared to the control group, 16 miRNAs were differentially expressed in the 1.0 μM nicotine group ($P_{corr} < 0.05$ and fold change >2). All of these miRNAs showed dose-dependent changes from 0.5 to 1.0 μM nicotine. The top 10 highly expressed miRNAs were selected for validation and showed a similar expression change to the microarray results. Interestingly, five miRNAs related to osteogenesis (hsa-miR-29b, hsa-miR-30d, hsa-miR-137, hsa-miR-424, and hsa-miR-1274a) showed $P_{corr} < 0.05$.

We confirmed these results also occur in vivo and showed that PDLSCs isolated from cigarette smokers showed similar trends in proliferation, migration, and

differentiation potentials and miRNA expression [21]. The proliferation of PDLSC from smokers was significantly slower than those of non-smokers by 2.53-fold at day 5 ($p < 0.01$) and 2.88-fold at day 7 ($p < 0.05$). In cell migration analysis, conducted by scratch wound assay, smoker PDLSCs moved slower than the non-smoker PDLSCs. The reduction of scratch wound area by smoker PDLSC was smaller than that by non-smoker PDLSC at 12 hours (20.07 +/− 3.78% versus 25.92 +/− 4.00%, $p < 0.05$) and at 24 hours (60.10 +/− 8.55% versus 72.27 +/− 5.90%, $p < 0.05$), suggesting that CS reduces the migration ability of PDLSC. Cigarette smoking (CS) also inhibited osteogenic differentiation as seen by a reduction in alkaline phosphatase and Alizarin S Red and chondrocyte differentiation as indicated by the blue stain of acidic polysaccharides [21].

We also examined the expression of the top 10 differentially expressed miRNAs identified in our previous report. Interestingly, the miRNA profile of cigarette smokers showed similar trends in expression for the majority of the miRNAs. A miRNA-gene target list was subsequently generated for two of the similarly upregulated miRNAs, hsa-miR-1305 (629 target genes) and hsa-miR-18b (202 target genes), using TargetScan in the GeneSpring software (Agilent). **We used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) to perform gene ontology analysis of the gene lists. We identified smoking-associated miRNAs (hsa-miR-1305 and hsa-miR-18b) might target the genes involved in cell cycle, cell projection, cell junction, and cytoskeleton. This suggested that cigarette smoking would account for the negative influence on PDLSC proliferation and migration potential** [21].

It is important to note that these effects persisted given that PDLSCs from smokers underwent numerous cell passages after isolation (i.e., four and eight passages, splitting cells 1:3) and that all experiments using these cells were conducted in the absence of exogenous nicotine [21]. Accordingly, we concluded that these miRNAs, and not the nicotine receptors, play an important role in an inhibitory effect on stem cell regenerative potential and the long-term deteriorative effects of CS on stem cells.

Others have shown that similar nicotinic effects of PDLSC regeneration potential by decreasing proliferation, migration, and osteogenic differentiation capabilities in vitro are mediated through agonist-induced activation of α7 nAChRs present on PDLSCs [26, 27]. However, the detrimental effects of nicotine were still partially observed even after the deactivation of these receptors [27], suggesting that there may be additional mediators behind these effects. These results hint at a possible mechanism for the nicotine-induced effects on PDLSC regeneration potential that involves both nAChRs and miRNA. A link between the nicotinic effect and the miRNA expression has yet to be fully determined; however, there does appear to be a correlation between the two.

Electronic cigarettes (ECs) are marketed as a "safer" alternative to cigarette smoking due to the significant reduction in toxic chemical exposure. Experienced EC users, however, are still capable of achieving cigarette-like levels of exposure to vapor compounds like nicotine, especially in the oral cavity—the initial site of exposure. Nicotine alone has been shown to significantly impact the osteogenic differentiation potential of PDLSCs [16, 17], but its effect in tandem with additional EC vapor (ECV) compounds remains unknown. PDLSCs were cultured in osteogenic media supplemented with EC vapor extract. EC vapor (ECV) was generated by VitroCell's VC1 automated vaping robot using an eVic EC filled with 50%/50% (w/v) PG/VG, 0 or 36 mg/ml nicotine, and non-flavored e-liquid. Nicotine vapor content was determined to a final, physiological concentration of 1 or 10 μM nicotine.

EC condition media containing 10 μM reduced osteogenic differentiation. In comparison, CS blocks mineralization significantly more than EC in a

dose—response fashion. The greatly enhanced inhibitory effect of CS may be due to the 7000 other chemicals besides nicotine. miRNA microarray analysis showed that 1 μM nicotine ECV treatments upregulated miRNA (i.e., hsa-miR-29b (4.15-fold, $p < 0.05$), hsa-miR-424 (2.10-fold, $p < 0.05$), and hsa-miR-30d (3.18-fold, $p < 0.05$)) targeted genes related to osteogenesis. After 7 days of induction, high concentrations of nicotine ECV suppressed the gene expression of early osteogenic differentiation markers RUNX2, ALPL, and COL1A1 by >25%. Nicotine ECV also suppressed mineralized nodule formation after 21 days in a dose-dependent manner. In comparison, cigarette smoking is tenfold inhibitory than ESC. This may be attributed to over 7000 other toxic chemicals in CS. Our pilot study is the first to investigate the potential effects of ECV by influencing the osteogenic differentiation potential of PDLSCs. A further understanding of the consequences of ECV on adult stem cell health is imperative given the rapid rise of EC use and routine use of postnatal stem cells in regenerative applications [31–33].

3. Conclusion

In conclusion, stem cells are effective for the treatment of a variety of injuries and diseases. However, compromising stem cell function, through infectious or genetic diseases, can lead to ineffective clinical outcomes. Literature reports suggested that smoking effects of stem cell regeneration potential by decreasing proliferation, migration, and osteogenic differentiation capabilities in vitro are mediated through agonist-induced activation of α7 nAChRs present on PDLSCs [26, 27].

Our work focuses on the determination of the potential harmful effect of smoking on the regenerative capabilities of stem cells and the mediators behind these effects. We showed that smoking suppression of stem cell potentials by miRNAs is a separate and independent pathway than the pathway via the α7 nAChR activation. Smoking perturbs miRNA expression, resulting in decreased PDLSC regeneration potential that delays healing of smoking-induced periodontitis.

This contribution will be significant because it will provide much needed data regarding smoking as well as consequences of vaping on oral cell/tissue health. Moreover, the data gathered as part of this work has the potential to be used in future commercial applications such as the development of a prescreening method or an early detection system for destructive oral diseases such as periodontitis.

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Chapter 4

Role of Virus-Encoded microRNAs in Avian Viral Diseases

Venugopal Nair and Yongxiu Yao

Abstract

To survive in the host cells, viruses have to adapt various strategies, which include the modulation of microRNA (miRNA) pathway through virus-encoded miRNAs to modulate the host cellular environment. It has been shown that several avian viruses, mostly herpesviruses, encode a number of miRNAs. These include 26 miRNAs encoded by the highly oncogenic Marek's disease virus-1, 36 miRNAs encoded by avirulent Marek's disease virus-2, 28 miRNAs by herpesvirus of turkeys, 10 miRNAs by infectious laryngotracheitis virus, 41 miRNAs by duck enteritis virus, and 2 miRNAs by avian leukosis virus subgroup J. Although locations of some of the miRNAs are conserved within the repeat regions of the genomes among some of the antigenic and phylogenetic closely related herpesviruses, there are no sequence conservation of miRNAs encoded by different avian herpesviruses. Moreover, some of the virus-encoded miRNAs have the same seed sequence as host miRNAs serve as functional orthologs. For example, mdv1-miR-M4-5p, a functional ortholog of gga-miR-155, is critical for the Marek's disease virus in inducing tumors. In this review, we describe the advances in our understanding on the role of the herpesvirus-encoded miRNAs in avian diseases. Additionally, we also describe the potential association of avian leukosis virus subgroup J encoded E (XSR) miRNA in the induction of myeloid tumors in certain genetically distinct chicken lines.

Keywords: avian viruses, microRNAs, MDV, DEV, ILTV and ALV

1. Introduction

MicroRNAs (miRNAs) are small RNA molecules of ~22-nucleotide that profoundly affect gene expression by targeting the 3'UTR (untranslated region) of the targeted mRNA (messenger RNA). Ever since the first discovery of miRNA in *Caenorhabditis elegans* [1], the identification of miRNAs, of which some are evolutionarily conserved [2–4], has proceeded at a quick pace. It is well known now that miRNAs are key regulators of gene expression in many species including mammals, plants, flies, worms, as well as in a number of viruses. Out of 38,589 miRNAs identified so far, 530 are encoded by viruses (www.miRbase.org). Most of the viral miRNAs are encoded by DNA viruses, with members of the family *Herpesviridae* encoding for the vast majority suggesting the importance of miRNA-mediated gene regulation in the biology of herpesvirus infections. Long-term survival as latent infections in different cell types are characteristic feature of virus-host interactions in herpesviruses. This requires sophisticated survival strategy to avoid detection by the innate and adaptive immune mechanisms of the host. Herpesviruses achieve this using a variety of mechanisms through translational

control, epigenetic control of viral/host gene expression, and restricted gene expression [5, 6]. The miRNAs, with the small size along with the ability for specific repression of multiple transcript targets, make them ideal tools for herpesviruses to reshape the gene expression to favor viral replication in an infected cell. Hence, it is of no surprise that herpesviruses encode 91% of virus-encoded miRNAs identified so far. The number of miRNAs encoded by different herpesviruses varies, i.e., 5 miRNAs in Bovine herpesvirus 5 and 70 in Rhesus lymphocryptovirus (miRBase V22.1). In addition to herpesviruses, some of other virus families also encode miRNAs [7, 8] including miRNAs encoded by the human Torque Teno Virus [9]. Although retroviruses have not been widely documented to exploit the miRNA pathway [10], demonstration of E (XSR) miRNA encoded by avian leukosis virus subgroup J (ALV-J) using the canonical miRNA biogenesis pathway [11], and a conserved cluster of RNA polymerase III (pol III)-transcribed miRNAs from the bovine leukemia virus (BLV) genome [12, 13] showed that retroviruses also exploit the miRNA pathway.

2. miRNA-encoding avian viruses and associated diseases

Avian herpesviruses are a major group of pathogens affecting different species of poultry. All of the pathogenic avian herpesviruses belong to the subfamily *Alphaherpesvirinae*. These include infectious laryngotracheitis virus (ILTV, Gallid herpesvirus) in the *Iltovirus* genus, pathogenic Marek's disease virus-1 (MDV-1, Gallid herpesvirus 2) in *Mardivirus* genus, and unassigned duck enteritis virus (DEV). ILTV induces laryngotracheitis, a contagious respiratory tract infection that results in severe losses in egg production and high mortality in infected poultry flocks. Although the safety of vaccine strains has been questionable, live attenuated vaccines are used to control this disease [14]. *Mardivirus* genus is classified into three closely related, but distinct groups including the pathogenic Marek's disease virus-1 (MDV-1, Gallid herpesvirus 2), attenuated Marek's disease virus-2 (MDV-2, Gallid herpesvirus 3), and the antigenically related herpesvirus of turkey (HVT, Meleagrid herpesvirus 1). Based on their pathogenicity, MDV-1 is further grouped into different pathotypes named as virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv + MDV) [12]. Marek's disease (MD) is an immunosuppressive, neurological, and oncogenic disease caused by highly contagious MDV-1. MD that is widespread in the poultry population around the world causes estimated economic losses of US$ 2000 million annually [15]. Although, controlled by the use of vaccines such as nonpathogenic HVT and MDV-2 vaccines or live attenuated MDV-1 strains [16], there is concern on the continued evolution of the virus toward greater virulence, challenging the sustainability of Marek's disease vaccination strategy [17, 18]. DEV induces acute disease in waterfowl species with high mortality [19, 20].

Avian leukosis viruses (ALVs) are members of *Alpharetrovirus* genus of the *Retroviridae* family. ALVs cause neoplastic diseases and other reproduction problems with enormous economic losses in the global poultry industry. According to their mode of transmission, the ALVs are classified as either endogenous (ALV-E) or exogenous viruses. Based on viral envelope interference, host range, and cross-neutralization patterns, exogenous ALVs from chickens have been further divided into different subgroups (A, B, C, D, and J) [21]. ALV-J was first described in the UK in the late 1980s [22] and has been primarily associated with myeloid leukosis in meat-type chickens. ALV-J caused more serious damage than all other subgroups worldwide. ALV-induced disease particularly by ALV-J is still widespread in poultry population in China and causes huge economic losses [23].

3. Identification of miRNAs encoded by avian viruses

The majority of viral miRNAs had initially been identified by a protocol involving size fractionation of small RNA, ligation of linkers, reverse transcription, concatamerization, cloning followed by Sanger sequencing [7]. The bioinformatics approaches to identify miRNA-encoding loci in viral genome based on commonalities in the predicated secondary structures of pre-miRNAs have also been developed. Exploring the small RNAs libraries with a higher degree of reliability and unprecedented depth has become possible with the advent of massively parallel sequencing technologies. We and others have reported the identification of miRNAs encoded by a number of avian herpesviruses. These include 14 miRNAs (26 mature sequences) encoded by MDV-1 [24, 25], 18 miRNAs (36 mature sequences) encoded by MDV-2 [26, 27], 17 miRNAs (28 mature sequences) encoded by HVT [27, 28], 7 miRNAs (10 mature sequences) encoded by ILTV [27, 29], 24 miRNAs (33 mature sequences) encoded by DEV [30], and 1 miRNA (2 mature sequences) encoded by ALV-J [11] (**Figure 1**).

3.1 MDV-1 miRNAs

High throughput sequencing of small RNA libraries from highly virulent RB1B strain infected chicken embryo fibroblast (CEF) has led to the identification of the first MDV-1-encoded miRNAs in 2006 [24]. Subsequently, more MDV-1 miRNAs were discovered by analyzing small RNA library of MSB-1, a MDV-1 cell line established from an MDV-induced spleen lymphoma [25, 31]. A total of 14 precursor sequences producing 26 mature miRNAs have been identified from the MDV-1 genome (www.miRbase.org; **Figure 1A**). The MDV-1 miRNAs are clustered in three separate genomic loci: cluster 1 (Meq-cluster), cluster 2 (Mid-cluster) flank the Meq oncogene, and cluster 3 (LAT-cluster) lies in the region encoding the latency-associated transcripts (LATs) [24, 25, 31] (**Figure 1A**). All three MDV-1 miRNA clusters are in the repeat regions of the viral genome. Although differing in virulence, the miRNA sequences are highly conserved among 23 different MDV-1 strains [31, 32]. Despite this, the expression level of Meq-cluster miRNAs is higher in vv+ MDV-induced lymphomas than vvMDV strain-induced lymphomas. One polymorphism in the miRNA promoter region has been proposed to be responsible for this differential expression. On the other hand, there is no difference on the level of the LAT-cluster miRNAs expression [31, 33], implying that miRNAs from Meq-cluster may play a more significant role in MD oncogenesis. Indeed, the significantly decreased oncogenicity of the virus with the deletion of the Meq-cluster miRNAs has proved this hypothesis [34, 35]. mdv1-miR-M4-5p, a functional ortholog of gga-miR-155 and a member of Meq-cluster miRNA, is the most highly expressed viral miRNA in tumors, representing over 70% of MDV miRNA sequencing reads [31]. Similar to the role of gga-miR-155 in lymphoid malignancies, mdv1-miR-M4-5p was shown to play a key role in MDV-1-induced oncogenesis [34, 35]. The promoter prmiRM9M4, corresponding to the 1300-bp immediately upstream from the first Meq-cluster miRNA mdv1-miR-M9, has been shown to drive the transcription of both Meq-cluster and Mid-cluster miRNAs with two distinct transcriptional models during different infection phases [33]. Indeed, this promoter has been shown to be active during MDV-1 latency by both DNA hypomethylation and active histone marks [36], confirming its transcriptional activity. In contrast, the transcription of LAT-clustered miRNAs is driven by a p53-dependent promoter, which contains at least two 60-bp tandem repeats harboring a p53-response element but has no consensus core promoter element [37].

Studies on correlation of miRNA expression signature with MDV-transformation have been undertaken. Microarray analysis has been carried

out to determine the global miRNA expression profiles in seven distinct MDV-transformed cell lines [38]. Compared to uninfected splenocytes and CD4+ T-cells or non-MDV avian viral transformed cell lines, these profiles revealed that the expression of a set of host miRNAs is altered in the MDV-transformed cell lines. The downregulated host miRNAs in the MDV-transformed cell lines include miR-26a, miR-150, miR-223, miR-155, and miR-451. The differential expression of these host miRNAs may contribute to MDV pathogenesis, considering the link of several mammalian homologs of these miRNAs with cancer development [39]. For example, miR-26a with tumor suppressor function is downregulated in a number of avian lymphoma cells. miR-26a has been shown to regulate interleukin 2 (IL-2) expression [40]. It is well known that IL-2 regulates T-cell proliferation, suggesting that downregulation of miR-26a and the upregulation of IL-2 expression could be a conserved mechanism in avian viral transformation. Taken together, analysis of the functional targets of the differentially expressed miRNAs would contribute toward better understanding of the molecular pathways of MD oncogenicity.

3.2 MDV-2 miRNAs

MSB-1 is a MDV-transformed lymphoblastoid cell line, which is co-infected with both BC-1 strain of MDV-1 and HPRS24 strain of MDV-2 [41]. About 17 novel MDV-2 miRNAs were identified by analyzing small RNA library from MSB-1 [26]. Of these, 16 are expressed in the same direction and clustered in a 4.2-kb long repeat region that encodes R-LORF2 to R-LORF5, suggesting that they may be derived from a common primary transcript. The single miRNA outside the cluster was located within the C-terminal region of the ICP4 homolog in the short repeat region. Despite lacking sequence homology, the relative genomic positions of miRNA clusters in MDV-1 and MDV-2 are conserved by locating within the repeat regions of the viral genome. Subsequently, an additional miRNA mdv2-miR-M32 has been reported by high throughput sequencing of small RNAs from MSB-1 cells [27]. Uniquely, all 18 MDV-2 encoded miRNA precursors give rise to 2 mature forms resulting in 36 mature MDV-2 miRNAs, representing both strands of the duplex (www.miRbase.org; **Figure 1A**). Two separate studies on sequencing of MSB-1 small RNA library showed that MDV-2 miRNAs accounted for 10 and 13% of the sequencing reads, respectively [25, 27].

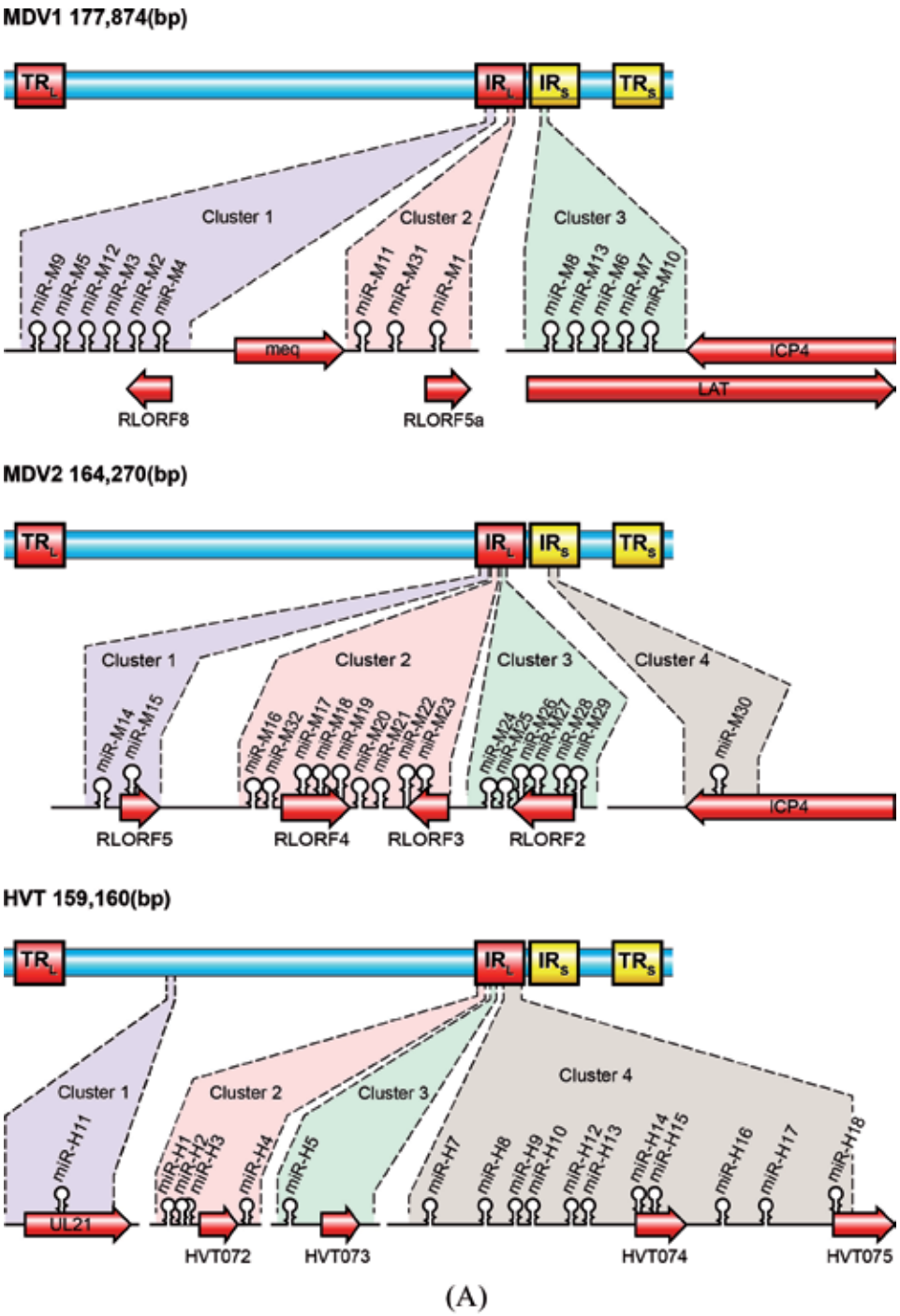
3.3 HVT miRNAs

HVT-encoded miRNAs were identified from HVT-infected CEF using both traditional cloning and sequencing of a small RNA library [28] and high throughput sequencing technology [27]. About 17 precursor miRNAs producing 28 mature miRNAs (www.miRbase.org; **Figure 1A**) have been identified in the HVT genome. Except for miR-H11, which is located within an intron of UL21 in the UL region, all other HVT miRNAs are located in the long-repeat regions, demonstrating some degree of positional conservation with MDV-1 and MDV-2. Interestingly, 10 HVT miRNAs were located in a region containing two tandem repeats, and small sequence variations were observed following multiple sequence alignment of the miRNA precursors, suggesting evolution by duplication. This represents the first example of evolution of virus-encoded miRNAs by duplication.

3.4 ILTV-miRNAs

A total of seven ILTV precursor miRNAs producing 10 mature miRNAs from ILTV-infected chicken embryo kidney (CEK) cells and leghorn male hepatoma

(LMH) cell line have been identified by deep sequencing of small RNA populations [27, 29] (www.miRbase.org; **Figure 1B**). Four of the miRNAs (iltv-miR-I1-I4), which are not associated with any annotated ORFs were located at the extreme terminus of the ILTV genome. miR-I5 and miR-I6, the two most highly expressed miRNAs, are located in the repeat regions within ICP4. The iltv-miR-I7 was mapped in the replication origin (oriL) of the palindrome stem loop sequence. Out of seven



(A)

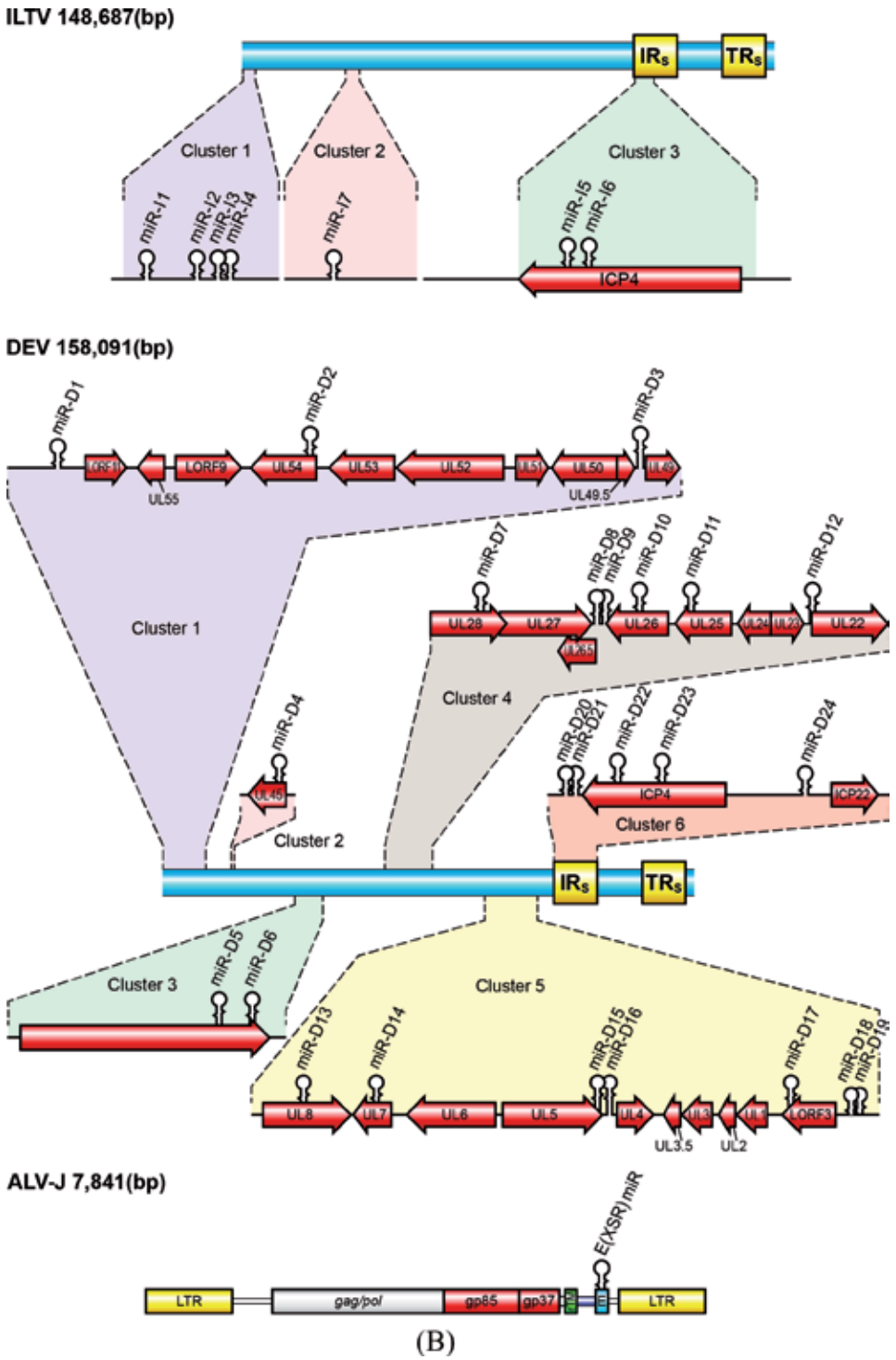


Figure 1.
Diagrammatic representation of the viral genomes showing the positions of miRNAs of MDV1, MDV2, HVT (A) and ILTV, DEV and ALV-J (B). Position and orientation of selected transcripts are shown. The genome size (base pairs) of viruses is shown on the top-left corner. Adapted from Yao et al., [92].

miRNAs, only three (iltv-miR-I3, -I5, -I6) have been confirmed by Northern hybridizations although the expression was confirmed for all by the end point PCR using small RNA libraries generated from ILTV-infected CEK.

3.5 DEV miRNAs

About 33 mature miRNAs derived from 24 pre-miRNA sequences within the DEV genome have been identified by deep sequencing of small RNAs from DEV vaccine strain-infected CEF cultures [30] (www.miRbase.org; **Figure 1B**). Compared to the location of most Mardivirus-encoded miRNAs which are at the repeat regions, genomic positions of DEV-encoded miRNAs are unique. Five out of six miRNA clusters from both the coding and non-coding regions of the 158,091-bp viral genome were encoded within the unique long region. Similar to miRNA-offset RNAs, two miRNA precursors, DEV miR-D18 and miR-D19, overlapped with each other although only the dev-miR-D18-3p was functional in reporter assays. In addition, 12 putative DEV miRNAs have been reported by computational approach [42], none of these miRNAs overlapped with the 24 DEV miRNAs described above [30]. In a recent report, 39 mature viral miRNAs have been identified from a Chinese virulent strain of DEV infected duck embryo fibroblast cells [43]. Of those, only 13 miRNA sequences and 22 "seed sequences" of miRNA were identical with the previously reported DEV-miRNAs encoded by vaccine strain [30]. About eight additional viral miRNAs were detected and confirmed by stem-loop RT-qPCR [43].

3.6 ALV-J miRNAs

One of the distinct features of HPRS-103 sequence, the ALV-J prototype virus, is the presence of E (also called XSR) element, a distinct hairpin stem-loop structure in the 3′ untranslated region. Indeed, the expression of miRNA from this hairpin-like structure of E element has been demonstrated in ALV-J-infected/transformed cells [11]. E (XSR) miRNA, a small RNA population encoded from within the E (XSR) element, was identified using deep sequencing approach on ALV-J-transformed cell line IAH30 [11] (**Figure 1B**). E (XSR) miRNA is suggested to play a major role in ALV-J pathogenesis and neoplastic transformation as it accounted for a quarter of the sequences of the IAH30 "miRNAome." Although the E element per se is not absolutely essential for ALV-J induced tumor, the E element does contribute to the oncogenicity in certain genetic lines of chicken when the oncogenicity of viruses derived from the parental and E element deleted HPRS-103 viruses was compared [44]. The hypothesis of polymorphisms, particularly those in any potential E (XSR) miRNA target sites could account for differential susceptibility phenotypes among these lines can be investigated by comparison of the genomes of these lines.

4. Viral orthologs of host miRNAs

Two classes of virus-encoded miRNAs are grouped: viral specific miRNAs and orthologs of host miRNAs. Similar to some viral regulatory proteins, several viral miRNAs have evolved to mimic host effectors. The "seed" region of a miRNA (~nucleotides 2–8 at the 5′ end) plays a key role in directing RISC to its mRNA targets. Binding with perfect seed complementary to the target transcript takes ~60% of regulation by a particular miRNA [45]. Some of viral miRNAs share seed sequences with host miRNAs and at least three viruses: BLV, MDV-1, and Kaposi's Sarcoma-associated Herpesvirus (KSHV) have been shown to regulate transcripts via the same target docking sites as their counterpart host miRNAs [46–48]. Such viral miRNAs could potentially regulate hundreds of transcripts having target sites for a particular host miRNA. Such regulatory networks could affect specific functions such as inhibiting apoptosis.

KSHV and MDV-1 express two distinct miRNAs, which are functional ortholog of host miR-155, a conserved cellular miRNA that is required for the rapid expansion of B and T cells after antigenic stimulation and highly expressed in activated myeloid and lymphoid cells [31, 46–48]. Interestingly, in both MDV-1 and KSHV-induced tumors, there is downregulation of endogenous levels of miR-155 [38, 47, 49], although the mechanisms for such downregulation is not fully understood. It is intriguing to know why these two viruses chose to express their own miR-155 orthologs, while suppressing the host-encoded miRNA with potentially identical functions. The direct role of miR-155 on oncogenesis [50, 51] and induction of cancer [47, 52] have been shown by a number of studies. Furthermore, it has been shown that upregulation of miR-155 is associated with lymphocyte transformation by reticuloendotheliosis virus strain T (REV-T) [53, 54] and EBV [55]. It is therefore striking that mdv1-miR-M4-5p is highly expressed in MDV-1 transformed cells derived from MDV-1-induced T-cell lymphomas [31, 46]. Moreover, lymphoma induction in infected birds is greatly reduced with seed region mutagenesis or deletion of mdv1-miR-M4-5p, suggesting the importance of mdv1-miR-M4-5p in the induction of tumors [34, 35]. The fact that transformation ability of the miR-M4-deleted MDV-1 virus is partly restored by introduction of chicken miR-155, suggests that viral miRNAs can play a major role in enhancing the oncogenic potential of a herpesvirus *in vivo*. However, the oncogenicity of vvMDV strain GX0101 with mdv1-miR-M4 deletion has significantly decreased but was not totally abolished [35]. This report, coupled with the finding that HVT with mdv1-miR-M4 inserted in the genome failed to induce tumors [32], suggested that other factors are also required for viral transformation. Moreover, our recent studies have shown that mdv1-miR-M4 is not essential for maintaining the proliferation of transformed cell lines suggesting that it has probably a more significant role in the initiation of neoplastic transformation [56].

miR-H14-3p, one of the HVT-encoded miRNAs, showed close sequence identity with perfect match of the 21/23 nucleotides including identical seed sequence to the chicken gga-miR-221, suggesting that it is a virus-encoded ortholog [27]. Indeed, the proposed ortholog status of the two miRNAs are supported by the evidence that HVT-miR-H14-3p also modulates the expression of p27Kip1 (cyclin-dependent kinase inhibitor 1B), a known target of miR-221 [57]. Compared to other known viral miRNA orthologs, where only the seed sequences are identical, the full length mature miRNA sequences of miR-H14-3p and gga-miR-221 are almost identical, strongly suggesting that hvt-miR-H14-3p is most likely to have been acquired from the host genome. This is the first example of virus-encoded miRNA showing such close and extended sequence identity with a host miRNA. Furthermore, partial sequence conservation has been observed between the gga-miR-221 locus on chromosome 1 of the chicken genome and the downstream flanking region of hvt-miR-H14-3p in the HVT genome, suggesting hvt-miR-H14-3p is "pirated" by the virus from the host, despite of the fact that herpesviruses have frequently pirated and subverted host genes to their own purposes. Interestingly, p27Kip1, the target shared by the two miRNAs, is a regulator of the cell cycle G1 to S phase transition. MDV-1-induced tumorigenesis may also involve a similar mechanism as miR-221-mediated repression of p27 in cancer progression [58–60]. In order to support viral genome replication and to promote growth of infected cells for additional viral production, the downregulation of p27 could move the cell cycle to the S phase [32]. The sequence homology is also observed between mdv1-miR-M31 and miR-221 in the seed region [31], although it is limited to the minimal miRNA seed region at nucleotide positions 2–7. Although targeting of p27 by miR-M31-3p has not been experimentally validated, the finding that miR-M31 deleted virus reduced the mortality and gross tumor incidence of infected chickens significantly has implied that miR-M31 may act as potential oncogene by targeting p27 [61].

gga-miR-29 is known to function both as an oncogene and a tumor suppressor depending on the context [62]. Seed sequence homology has been observed between mdv2-miR-M21 and miR-29 suggesting the potential functional ortholog between the two miRNAs. In fact, blv-miR-B4, a BLV-encoded miRNA, has been shown to be a functional ortholog of host miR-29 [12]. Furthermore, RLCV-encoded miR-rL1-6-3p and EBV-encoded miR-BART1-3p also share seed sequence with miR-29. Thus, a picture is emerging wherein several virus-encoded miRNAs are likely to target host pathways [63].

It is estimated that ~8% of avian virus-encoded miRNAs possess identical heptameric seed sequence with host miRNAs, thus could potentially act as functional orthologs [7]. However, based on low abundance, untested biogenesis, and unknown functional relevance, it is not clear, whether all of the currently annotated viral or host miRNAs are bona fide miRNAs, thus some seed matches between host and viral miRNAs could arise by chance. Therefore, any proposed functional orthologs between the viral and host miRNAs require further experimental validation.

5. Target identification of avian herpesvirus miRNAs

Although over 500 virus-encoded miRNAs have been identified, an in depth functional study is lacking for most. However, it is clear that virus-encoded miRNAs can target both cellular and viral mRNAs and this has been confirmed by several studies analyzing the mRNA targetome of the virus-infected cells [64]. The best characterized viral miRNA functions in supporting viral replication include regulating the latent-lytic switch; evading the immune response; promoting cell survival, proliferation, and/or differentiation. All of these functions should be particularly important during persistent infections. As viral miRNAs and proteins work synergistically to promote a cellular environment favorable to the completion of the viral life cycle, modulation of the host cell environment is achieved by multiple and partly redundant mechanisms. The identified targets of avian herpesvirus-encoded miRNAs are summarized in **Table 1**.

5.1 Viral targets of viral miRNAs

Compared to identification of cellular targets, identifying viral targets of viral miRNAs is more straightforward as viral genomes encode fewer candidate mRNAs. Known examples of viral targets include transcripts, which are antisense to the viral miRNA precursor and transcripts elsewhere with imperfect matches. Perhaps, the former one is the most straightforward examples of determining viral miRNA function as the perfect matching between the miRNA and its target would be predicted to result in a siRNA-like cleavage of the target mRNA if both are co-expressed. For example, iltv-miR-I5 that is antisense to ICP4 cleaves ICP4 mRNA [65]. ICP4, an immediate early viral transactivator, plays a key role in the induction of lytic replication. The ICP4 targeting by viral miRNAs is thought to mediate lytic/latent switch and render the latent state more robust [66]. Apart from iltv-miR-I5, iltv-miR-I6 also maps antisense to the ICP4 gene. However, reporter assay on inhibition of luciferase activity by iltv-miR-I6 was not significant [65]. It has been proposed that this is due to the blockage of accessibility to the binding region following in silico folding of RNA containing the targets for iltv-miR-I5 and iltv-miR-I6. This is consistent with the previous finding that target RNA folding is essential for the efficacy of designed siRNAs [67, 68]. Sequences antisense to known miRNA stem-loop structures may fold into stem-loop structures themselves at an increased propensity.

Virus	miRNAs	Targets	Proposed function
MDV-1	mdv1-mir-M3	Smad2	Anti-apoptotic [32]
	mdv1-mir-M4-5p	PU.1, CEBPB, HIVEP2, BCL2L13, PDCD6, GPM6B, RREB1, c-Myb, MAP3K7IP2, C1orf109, CSNK1A1, LATS2, MAP3K14, NR1D2, RORA, RPS6KA3, WEE1, ZCHSD2, JARID2, PBEF1, RAP2A	Mimics cellular mir-155 [33–35] in regulating apoptosis, proliferation, immune tolerance and tumorigenesis.
		LTBP1	Anti-apoptotic [36]
		hnRNPAB	Promote proliferation [37]
		UL28	Impair MDV morphogenesis/maintain latency [31]
	mdv1-mir-M4-3p	UL32	Impair MDV morphogenesis/maintain latency [31]
	mdv1-mir-M7-5p	ICP4 and ICP27	Establish and/or maintain latency [28]
	mdv1-mir-M11	Meq	Tumor suppressor [38]
	mdv1-mir-M2 mdv1-mir-M3 mdv1-mir-M4 mdv1-mir-M12	R-LORF8	Lymphocyte growth [39]
	MDV1-miR-M2-3p MDV1-miR-M9-5p	IL-18	Restricting innate antiviral immunity [53]
MDV-2	MDV2-miR-M18-5p MDV2-miR-M18-3p MDV2-miR-M26-5p MDV2-miR-M28-3p MDV2-miR-M30-3p		
	mdv2-mir-M24 mdv2-mir-M25 mdv2-mir-M26 mdv2-mir-M27 mdv2-mir-M28 mdv2-mir-M29	R-LORF2	Lymphocyte growth [39]
HVT	hvt-mir-H5	ICP4	Establish and/or maintain latency [40]
ALV-J	E(XSR)miRNA		Potential role in oncogenesis

Table 1.
Avian virus encoded miRNAs and proposed functions highlighted in this review.

This strategy could allow a virus to express both the viral gene and encode miRNAs antisense to mRNA transcripts lying on the other strand.

Analysis of miRNA binding clusters on the MDV-1 genome by PAR-CLIP identified very strong binding clusters near the 5′ end of the genome. These clusters essentially are antisense to the MDV-1 miRNAs miR-M2, miR-M3, miR-M4, and miR-M12, which are members of Meq-cluster, adjacent to each other, highly expressed and all located antisense to an MDV-1 mRNA encoding the viral R-LORF8 protein [64]. Analysis of indicator constructs in co-transfected 293 T cells or transduced MSB-1 cells confirmed that R-LORF8 is indeed targeted by all four miRNAs. Interestingly, expression of R-LORF8 gene in a form lacking the viral miRNA binding sites caused a substantial reduction in MSB-1 cell growth, suggesting that dysregulated expression of R-LORF8 can exert a deleterious effect in MDV-1-transformed T cells. The molecular basis for this effect is not clear as the function of R-LORF8 in the viral life cycle is currently unknown. The same pattern is observed in MDV-2, where viral miRNAs (miR-M24, M25, M26, M27, M28, and M29) are transcribed from the DNA strand located antisense to R-LORF2. In addition, there are also some other miRNAs encoded by MDV-1, MDV-2, HVT, and DEV, which are antisense to certain viral transcripts [24–28], the possible regulatory roles of those miRNAs against their antisense mRNA transcripts require to be verified individually.

As described above, mdv1-miR-M4-5p is a functional ortholog of cellular miR-155. mdv1-miR-M4-5p is also the first avian herpesvirus miRNA known to target both viral and cellular mRNAs. In addition to the cellular targets shared with miR-155, mdv1-miR-M4-5p and mdv1-miR-M4-3p also target viral UL28 and UL32 proteins, respectively. This provided the first evidence of late viral gene targeting by herpesviral miRNA [69]. Rather than 3′UTR, both target sequences are located in the coding region. UL28 and UL32 homologs, which are required for the cleavage and packaging of virion DNA in human herpesvirus 1 (HHV-1) have been found in all subfamilies of mammalian and avian herpesviruses. However, the role of UL28 and UL32 in MDV-1 packaging has not been demonstrated. As UL28 and UL32 are involved in the later stages of MDV replication, it is possible that mdv1-miR-M4 contributes in maintaining MDV-1 latency by downregulating the production of UL28 and UL32 and impairing late MDV morphogenesis and reactivation.

Following the observation of the increased viral pathogenicity or oncogenicity of infected chickens when mdv1-miR-M11 is deleted from Mid-cluster, the potential mechanisms mediated by miR-M11 has been investigated [61]. Meq gene has been predicated as candidate target gene by bioinformatics approach with RNAhybrid. Indeed, Meq as miR-M11 target has been confirmed by dual luciferase assay and qRT-PCR showing downregulation of Meq transcript level following virus infection. It has been suggested that the viral miRNAs in the Meq- or Mid-clusters cooperate with each other for establishing, maintaining latency, and/or triggering tumorigenesis.

ICP4 and ICP27, two MDV immediate-early (IE) genes, have been identified as potential targets for mdv1-miR-M7-5p by both bioinformatics prediction and subsequent experimental validation [70]. Indeed, this is reflected by a negative correlation between an increase in ICP27 expression and the decreased expression of mdv1-miR-M7-5p during virus reactivation. This is consistent with the early finding that miR-M7-5p is at extremely low levels in MDV-infected CEF but highly expressed in MSB-1 cells [25]. MDV miRNAs produced from LAT-cluster may contribute to the latency by targeting two IE genes. These findings further support the view that herpesvirus miRNAs play a key role in controlling the lytic/latent switch during infection [71, 72].

5.2 Cellular targets of viral miRNAs

Viruses such as herpesviruses with latent infection need to keep the host cells alive long enough. Thus, viral miRNAs can promote virus replication through prolonging cell survival and evading immune recognition. Among avian virus-encoded miRNAs, the targets of mdv1-miR-M4-5p are most extensively studied due to its critical role in virus induced oncogenesis. As viral orthologs of miR-155, both kshv-miR-K12-11 and mdv1-miR-M4 potentially target the same group of transcription factors as gga-miR-155 including Pu.1, CEBPβ, HIVEP2, BCL2L13, PDCD6, MAP3K7IP2, GPM6B, RREB1, and c-Myb [46, 69]. Subsequent analysis of >1000 cellular mRNAs targeted by MDV-1 miRNAs in MSB-1 cells using photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) resulted in the identification of 73 mRNA 3′UTR targets for miR-M4-5p, of which nine (C1orf103, CSNK1A1, LATS2, MAP3K14, MYB, NR1D2, RORA, RPS6KA3, and WEE1) had previously been identified as targets for miR-155 or kshv-miR-K12-11 in EBV-transformed human B cells and KSHV-transformed human B cells, respectively [64]. Indicator analysis of these 3′ UTRs gave data consistent with the hypothesis that these mRNAs are indeed targets for both miR-M4 in MSB1 and miR-155 in human B-cell lymphomas. Four additional mRNA targets (*FCHSD2, JARID2, PBEF1*, and RAP2A) were also conserved between miR-M4 in MSB-1 and

miR-155 in EBV-transformed B cells. Among these mRNA targets, JARID2, a cell cycle regulator and part of a histone methyltransferase complex, has been shown to promote apoptosis and decrease cell survival when ectopically expressed [53]. *WEE1* encodes a kinase that blocks cell-cycle progression and has been associated with inflammation and cancer [73]. MYB, a transcription factor, is involved in the regulation of hematopoiesis and tumorigenesis. Another interesting shared gene target for miR-155/miR-M4/miR-K12-11 is large tumor suppressor (LATS), which inhibits cell proliferation and promotes apoptosis by inhibiting YAP transcriptional activity through Hippo pathway. Being evolutionarily conserved from avian to mammal, loss of LATS expression is observed in many human cancers, including acute lymphoblastic leukemia [74]. These observations suggest a potential selective advantage for MDV-1 in inducing downregulation of LATS. Taken together, these observations provide additional evidence for the impact of miR-155 and its orthologs on pathways regulating lymphocyte activation, differentiation, apoptosis, proliferation, immune tolerance, and tumorigenesis [7].

In addition to the candidate mRNA targets described above, latent TGF-β binding protein 1 (*LTBP1*) has also been identified as bona fide host mRNA targets for miR-M4 [75]. Inhibition of *LTBP1* expression by miR-M4-5p induced a significant decrease of TGF-β1 secretion and activation, with suppression of TGF- β signaling and upregulation of c-Myc expression. Interestingly, miR-155 has been shown to suppress TGF-β signaling through targeting SMAD2 and SMAD5 in human diseases [76, 77]. The KSHV-encoded miR-K12-11 also inhibits TGF-β signaling through downregulation of SMAD5 [78]. Thus, these findings indicate that dysregulation of the TGF-β signaling pathway by miR-155 and its viral orthologs may be a common feature shared by oncogenic herpesviruses. Interestingly, MDV1-encoded miRNA miR-M3 also suppress TGF-β signaling through targeting host gene SMAD2, and has been shown to suppress drug-induced apoptosis in cell culture [79], thus adding another layer of confidence to the conclusion that suppression of TGF-β signaling pathway is indeed involved in MDV-induced oncogenesis.

Another identified cellular target of miR-M4 is chicken heterogeneous nuclear ribonucleoprotein AB (hnRNPAB). HnRNPAB, a member of the hnRNP family proteins, plays important roles in both normal biological processes and cancer development. Downregulation of hnRNPAB expression promotes proliferation of both CEF and chicken fibroblast cell line DF-1 [80]. Hence, downregulation of hnRNPAB by miR-M4-5p may be one of the important strategies for MDV-1 to trigger the development of MD lymphomas.

PAR-CLIP identified a list of 1104 cellular mRNAs targeted by MDV-1 miRNAs and a list of 1183 mRNAs targeted by MDV-2 miRNAs in MSB1, of which 419 mRNA targets were shared. Analysis of those cellular mRNAs identified several that were targeted by five or more different viral miRNAs in their 3′ UTRs, suggestive of a possibly important role in restricting innate antiviral immunity. IL-18, a proinflammatory cytokine induced upon infection by several different viruses, is able to stimulate IFN-γ production from T cells [81]. Surprisingly, chicken IL-18 gene contains seven viral miRNA target sites in its 3′ UTR [64]. Indeed, MSB1 cell growth is highly sensitive to inhibition by ectopic IL-18 expression. Whether this is due to induction of chicken IFN-γ expression remains to be determined. Thus, in addition to express viral interleukin-8 (vIL-8), which is required for disease progression and tumor development, this data suggest a second way in which MDV manipulates the host cell immune response.

Taken together, all of the data above suggests that latent/oncogenic viruses may proactively create a cellular environment beneficial to viral latency and oncogenesis through viral miRNA targeting cellular factors involved in antiviral processes including apoptosis. Surely, more targets of miRNAs encoded by avian viruses will

be identified with the advances in high-throughput technologies. We should be able to understand the role played by these small and highly effective modulators of gene expression once more targets of viral miRNAs are discovered and an integrated approach of demonstrating the functions and molecular pathways is developed.

6. Role of viral miRNAs in pathogenesis

Despite, several possible cancer-related genes have been identified as their targets of viral miRNAs, the direct *in vivo* role of those miRNAs such as KSHV-encoded miRNAs in neoplastic diseases is difficult to demonstrate in the absence of suitable models. However, excellent disease models of infection by MDV in natural avian host allow examination of the oncogenic potential of virus-encoded miRNAs. As described above, it has been shown that the deletion of the Meq-clustered miRNAs from the viral genome by BAC mutagenesis abolished the oncogenicity of the virus. miR-M4 is essential for the virus in inducing tumor as the mutant virus with miR-M4 deletion or seed sequence mutation almost lost its oncogenicity, providing evidence for a direct *in vivo* role of a viral miRNA in tumor induction [34]. In a separate study, deletions of the Meq-cluster or miR-M4 alone from the viral genome of vvMDV strain GX0101 strongly decreased its oncogenicity [35]. Subsequent work demonstrated that except for mdv1-miR-M4, most of the other Meq-clustered miRNAs also play critical roles in MDV oncogenesis as both mortality and gross tumor incidence of birds infected with the mutant viruses have reduced significantly for the corresponding virus with single miRNA deletion [82]. These studies provide further information for understanding the molecular determinants that trigger the development of tumors by oncogenic MDVs.

Having demonstrated the critical roles of Meq-clustered miRNAs in MD pathogenesis and/or tumorigenesis, the role of miRNAs in Mid-cluster, which is transcribed by the same promoter as the Meq-cluster in the latent phase of the infection has been investigated by infection of miRNA deleted virus in animal model [61]. The role of miR-M31-3p acting as oncogene and miR-M11-5p acting as tumor suppressor in MD lymphomagenesis have been proposed based on the decreased mortality and gross tumor incidence by miR-M31 deletion mutant and increased viral pathogenicity or oncogenicity of infected chickens when miR-M11 is deleted.

7. Conclusions

Recent advances in sequencing technology have led to the identification of a number of miRNAs encoded by avian viruses. Given their ability to target cellular and viral transcripts, and the lack of immune response, miRNAs represent an ideal mechanism of gene regulation during viral infection, latency, and persistence. Similar to host miRNAs, the determination of biologically relevant activities of the reported viral miRNAs is the next step forward. Although the function of most viral miRNAs is to be determined, evidence so far does support the view that viral miRNAs are key regulators in virus biology. By targeting key viral lytic genes directly or modulating cellular regulatory pathways indirectly, the virus-encoded miRNAs could contribute significantly toward switching between lytic and latent infections, thereby regulating viral pathogenesis *in vivo*. Whether a small or large number of critical miRNA-target interactions have functional significance is a key question to be answered.

It is crucial to define the miRNA targets for both understanding observed phenotypes and providing clues to their functional role. Genome wide biochemical screening are becoming more powerful approaches for the generation of reliable

and accurate target information of viral miRNAs although target identification using target prediction software provided an initial strategy. Several techniques such as RIP-CHIP (RNA-binding protein immunoprecipitation microarray), HITS-CLIP (high-throughput sequencing cross-linking and immunoprecipitation), PAR-CLIP and proteomics analysis have all contributed large amount of data on potential targets of virus-encoded miRNAs [47, 83–89] although only PAR-CLIP has been used for avian herpesvirus miRNA targetome identification [64]. Cross-linking, ligation and sequencing of hybrids (CLASH) technology, another biochemical screen for miRNA targets, promises to generate the most accurate target information to date, leading the way in the generation of high confidence target datasets which will be invaluable for future studies [90, 91]. These new technologies allow unprecedented and largely unbiased views into miRNAs-mediated regulation of gene expression in virus-infected cells. Undoubtedly, further studies using different approaches and technologies are required toward the clear definition of miRNAs targetome and their functional relevance in viral infection, latency, reactivation, and pathogenesis.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

MicroRNAs in the Functional Defects of Skin Aging

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Abstract

Humankind has always been intrigued by death, as illustrated by the eternal quest for the fountain of youth. Aging is a relentless biological process slowly progressing as life cycle proceeds. Indeed, aging traduces an accumulation of physiological changes over time that render organisms more likely to die. Thus, despite our mastery of advanced technologies and robust medical knowledge, defining the molecular basis of aging to control lifespan is still currently one of the greatest challenges in biology. In mammals, the skin is the ultimate multitasker vital organ, protecting organisms from the world they live in. As a preferential interface with the environment, the skin is reflecting the internal physiological balances. The maintenance of these balances, called homeostasis, depends on the concurrent assimilation of diversified signals at the cellular level. MicroRNAs (miRNAs) are noncoding RNAs that regulate gene expression by mRNAs degradation or translational repression. Their relatively recent discovery in 2000 provided new insights into the understanding of the gene regulatory networks. In this chapter, we focused on the role of three miRNA families, namely miR-30, miR-200, and miR-181, playing a key role in the progression of the skin aging process, with particular input in mechanistic considerations related to autophagy, oxidative stress, and mitochondrial homeostasis.

Keywords: skin, microRNA, epidermis, keratinocyte, fibroblast, aging, autophagy, oxidative stress, mitochondria, miR-30, miR-200, miR-181

1. Introduction

Skin, the largest vital organ in the body, is made of three distinct layers from the top to the depth: the epidermis, which is a fine layer of epithelial keratinized cells called keratinocytes; the dermis consisting of fibroblasts in an intracellular matrix with various additional structures such as hear follicles, sweat glands, nerve endings, and capillaries; and the profound subcutaneous tissue called hypodermis. As a physical barrier between the body and the environment, the skin is affected by both intrinsic and extrinsic aging. Intrinsic or chronological aging is a natural continuous dynamic process that normally begins in the mid-1920s. During this inexorable process, the skin undergoes a physiological deterioration characterized by skin atrophy, increased physical and immunological vulnerability, with a reduced capacity of tissue repair in case of wounding. More precisely, intrinsic aging is leading to a 10–50% thinning of the epidermis, the flattening of the dermal-epidermal junction, an atrophy of the dermis with disorganization of the collagen and elastic fibers, a reduction of the microvasculature, and a loose of adipose tissue [1]. The

thinning of the epidermis and the reduction of the skin regeneration capacities are mainly linked to the dysfunctions of the epidermal stem cell compartment, which progressively lose its capacity to generate progenitor cells that are able to ensure the physiological renewal of the epidermis or to sustain wound repair [2].

Skin is also constantly exposed to environmental insults such as ionizing or UV radiations, chemicals, or climatic variations [3]. UV exposure is the main player in extrinsic skin aging and leads to phenotypic changes named photoaging. The photo-aged skin is characterized by a thickening of the epidermis with abnormal keratinocytes differentiation, an accumulation of abnormal elastic tissues (called solar elastosis) with a disorganization and degradation of the collagen fibers in the dermis, an abnormal pigmentation, and an activation of the immune response [4]. Skin is affected by both UV-A and UV-B radiations. UV-B rays are mainly limited to the superficial epidermal part of the skin and directly induce DNA lesions such as cyclobutane dimers and 6–4 photoproducts in exposed cells, leading to keratinocytes senescence, apoptosis, or carcinogenesis [5]. UV-A rays penetrate deeper into the dermis and induce DNA, protein, and lipid damages through the generation of reactive oxygen species (ROS), which in turn activate MAP-kinase p38, JNK, and ERK pathways with induction of the AP1 transcription factor resulting in the expression of the MMP1, -3, and -9 responsible for extracellular matrix degradation [6]. ROS also oxidate cellular components including proteins, lipids, DNA, and RNA, with altered metabolism and further damages.

ROS are also produced during the chronological aging process mainly through mitochondrial activity of the electron transport chain and this is the basis of the free radical theory of aging [7]. It states that mutations acquired in mitochondrial DNA (mtDNA) during life can disrupt metabolisms in the mitochondria and increase ROS. In the skin, mtDNA mutations accumulate with age and UV stress can accelerate this damage. Mitochondrial dysfunctions specifically contribute to skin aging phenotype especially through abnormal pigmentation and hair graying and loss [8]. More precisely, mitochondria play a pleiotropic role in pigmentation by modulating the melanin production through interacting with melanosomes [9]. Moreover, increased oxidative stress linked to mitochondrial dysfunction is observed in aged melanocytes and hair follicle epithelium [10].

Elimination of damaged mitochondria seems then to be a safety mechanism preserving cellular function, tissue homeostasis and organismal soundness. Selective mitochondrial autophagy, named mitophagy, has been described to ensure this function. Autophagy is a cellular quality control mechanism preliminary nonselective playing an essential role in cells for bulk proteins and organelle recycling. Mitophagy modulates the turnover of mitochondria under equilibrium conditions and adjusts the number of mitochondria according to the cellular needs. Increasing evidence suggest that impairment of mitophagy is involved in aging and age-related diseases [11] but the genetics and epigenetics mechanisms modulating mitophagy during aging remain to be better understood.

Multiple epigenetic changes are considered as reliable hallmarks of tissue aging such as modification of DNA methylation motifs, histone post-translational modifications, and modulation of noncoding RNA expression [12]. The latter is an emerging scientific domain in which an incredibly expanding number of studies have been published over the last decade, highlighting day after day the key roles of long noncoding RNAs, circular noncoding RNAs, and microRNAs (miRNAs) in the control of physiologic balances. For example, the stem cell function is governed by numerous factors such as growth factors, cellular metabolism, mediators of inflammation, extracellular matrix, interaction with niche cells, and so on. It has been clearly described that the imbalance between stem cells renewal and commitment can give rise to deleterious effects leading to pathologies or accelerated aging [13, 14]. Recent single-cell analyses

from multiple tissues, including epidermis, revealed a clonal heterogeneity of gene expression level among a defined cell population [15–17], thus reflecting a distinct fluctuating transcriptome in individual cells which governs cell fate. Thus, cell fate decisions rely on the integration of dynamic regulatory networks of gene expression and these fluctuating transcriptomes are likely to be under the control of noncoding RNAs, which cooperate with each other and are co-regulated.

Isolated human primary keratinocytes are a valuable model for studying epidermal aging as they retain features of the tissue they are extracted from. Recently, we took advantage of this model to identify miRNAs modulated with chronological aging through a genome-wide expression analysis of cell extracted from skin biopsies of healthy infants (3–6 years old), young adults (20–40 years old), and aged adults (60–71 years old). This microarray screening allowed us to identify 60 miRNAs significantly modulated ($P < 0.05$, fold change >1.5) between at least two of the three sample groups analyzed [18]. Most of them were differentially expressed between the youngest group and the two adult groups. Considering that physiological aging starts as early as 20 years old and that our cell samples were prepared from photo-protected skin areas, one can speculate that constant modulation of miRNAs expression as soon as 20 years could constitute an epigenetic signature of intrinsic chronological aging. Thus, according to our miRNome analysis during aging, this signature would be constituted by the overexpression of miR-181d-5p, miR-1972, miR-200c-5p, miR-30a-3p, miR-30a-5p, miR-30c-2-3p, miR-30c-5p, miR-365a-5p, miR-4298, miR-6812-5p, and miR-6831-5p and the underexpression of miR-4443. Among these miRNAs of the epidermis aging signature, no mechanistic studies are referenced in the literature for miR-1972, miR-4298, miR-6812-5p, and miR-6831-5p, thus limiting any biological interpretation. Furthermore, if few studies are published to date for miR-365 and miR-4443, they mostly come from cancerology studies, as it is traditionally the case for miRNA studies. Considering the debate on the relevance of the cancer cell lines as reliable mechanistic models [19–21], we decided here to focus on data obtained from pathological and physiological models excluding the cancer field, as much as we can. Consequently, we will focus here our attention on miRNA members from three different families. Interestingly enough, the miR-30 family is highly represented in this signature, and a recurrent biological pathway targeted by miR-30 is autophagy. In addition, many members of the miR-200 family have been associated to oxidative damage. Finally, the miR-181 family is progressively enriched with overexpression of additional members (miR-181a-2-3p and miR-181b-5p) with elderly. Several published studies converge toward a control of mitochondrial homeostasis by miR-181. As described earlier, the autophagic flux, the response to oxidative stress and the maintenance of functional mitochondria are all affected with skin aging and thus constitute cellular processes of particular interest regulated by miRNAs.

2. The miR-30 family in the control of the autophagic flux

The miR-30 family is composed of six members (miR-30a, miR-30b, miR-30c-1, miR-30c-2, miR-30d, and miR-30e) transcribed from three clusters of two genes located on human chromosome 1 (miR-30c and miR-30e), chromosome 6 (miR-30a and miR-30c-2), and chromosome 8 (miR-30b and miR-30d) [22]. Each gene is able to produce two mature miRNA sequences, the 3p and 5p strands (**Table 1**), with various abundances. We specifically observed an induction of miR-30a-3p, miR-30a-5p, miR-30c-2-3p, and miR-30c-5p in aged human skin [18]. In our miRNome analysis, the miR-30a is the most overexpressed miRNA with aging, with a 4- to 6-fold increase depending on the mature strand. For a long time, mir-30a-5p has been associated with the regulation of autophagy in various cancer cells [24, 25]

microRNA family	Mature sequences (*homo sapiens*)	Sequence alignments (ClustalW)	Lentgh (nt)	Identity (%)
miR-30	miR-30a-5p	UGUAAACAUCCUCGACUGGAAG--	22	100.00
	miR-30b-5p	UGUAAACAUCCUACACU--CAGCU	22	85.00
	miR-30c-1-5p	UGUAAACAUCCUACACUCUCAGC-	23	77.27
	miR-30c-2-5p	UGUAAACAUCCUACACUCUCAGC-	23	77.27
	miR-30d-5p	UGUAAACAUCCCCGACUGGAAG--	22	95.45
	miR-30e-5p	UGUAAACAUCCUUGACUGGAAG--	22	95.45
	miR-30-5p consensus	********** *** **		
	miR-30a-3p*	CUUUCAGUCGGAUGUUUGCAGC	22	100.00
	miR-30b-3p*	CUGGGAGGUGGAUGUUUACUUC	22	63.64
	miR-30c-1-3p*	CUGGGAGAGGGUUGUUUACUCC	22	59.09
	miR-30c-2-3p*	CUGGGAGAAGGCUGUUUACUCU	22	54.55
	miR-30d-3p*	CUUUCAGUCAGAUGUUUGCUGC	22	90.91
	miR-30e-3p*	CUUUCAGUCGGAUGUUUACAGC	22	95.45
	miR-30-3p consensus	** ** * ***** *		
miR-200	miR-200a-5p*	CAUCUUACCGGACAGUGCUGGA	22	100.00
	miR-200b-5p*	CAUCUUACUGGGCAGCAUUGGA	22	77.27
	miR-200c-5p*	CGUCUUACCCAGCAGUGUUUGG	22	68.18
	miR-141-5p*	CAUCUUCCAGUACAGUGUUGGA	22	81.82
	miR-429-5p*	N/A	-	-
	miR-200-5p consensus	* **** * *** * *		
	miR-200a-3p	UAACACUGUCUGGUAACGAUGU-	22	100.00
	miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA-	22	81.82
	miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA	23	77.27
	miR-141-3p	UAACACUGUCUGGUAAAGAUGG-	22	90.91
	miR-429-3p	UAAUACUGUCUGGUAAAACCGU-	22	77.27
	miR-200-3p consensus	*** **** * ***** *		
miR-181	miR-181a-1-5p	AACAUUCAACGCUGUCGGUGAGU	23	100.00
	miR-181a-2-5p	AACAUUCAACGCUGUCGGUGAGU	23	100.00
	miR-181b-1-5p	AACAUUCAUUGCUGUCGGUGGGU	23	86.96
	miR-181b-2-5p	AACAUUCAUUGCUGUCGGUGGGU	23	86.96
	miR-181c-5p	AACAUUCAAC-CUGUCGGUGAGU	22	100.00
	miR-181d-5p	AACAUUCAUUGUUGUCGGUGGGU	23	82.61
	miR-181-5p consensus	******** ******** **		
	miR-181a-1-3p*	-ACCAUCGACCGUUGAUUGUACC	22	100.00
	miR-181a-2-3p*	-ACCACUGACCGUUGACUGUACC	22	86.36
	miR-181b-1-3p*	-CUCACUGAACAAUGAAUGCAA-	21	52.38
	miR-181b-2-3p*	-CUCACUGAUCAAUGAAUGCA--	20	55.00
	miR-181c-3p*	AACCAUCGACCGUUGAGUGGAC-	22	90.48
	miR-181d-3p*	--CCACCGGGGGAUGAAUGUCAC	21	61.90
	miR-181-3p consensus	** * *** **		

Multiple sequence alignment of miRNA mature 5p or 3p strands was done for each family using Clustal Omega program [23]. Mature sequences with an asterisk (*) correspond to the passenger strand, whereas the seed sequences in the guide strand are indicated in bold. The identity between multiple miRNA strands is expressed as relative to the first miRNA for each guide or passenger strand within a family. Stars (*) are aligned with conserved nucleotides among the different members of either guide or passenger strands for each family.

Table 1.
Mature sequences of miRNAs from miR-30, miR-200, and miR-181 families.

and, more recently, in other several types of normal cells such as cardiomyocytes [26, 27], endothelial cells [28–30], thymocytes [31], lens epithelial cells [32], or hepatic stellate cells [33].

Autophagy is well-conserved catabolic process across phyla that directs the degradation of either bulk or selective cellular components. Molecular

mechanisms governing autophagy in mammals has been extensively reviewed [34]. Briefly, the process is regulated by a core machinery involving a step-by-step interaction of multiple molecular partners called autophagy-related (ATG) proteins. The initiation step is under the control of the protein kinase ULK1, which phosphorylates Beclin-1 (BECN1) on S14, thus boosting the activity of the VPS34-P150 complexes that induce the nucleation of the autophagophore. Subsequent phagophore extension requires first the intervention of ATG5-ATG12-ATG16L complexes. The closure of the autophagosome relies then on the activity of the ATG4-ATG3-ATG7 complexes that convert the inactive microtubule-associated protein LC3-I into the active LC3-II form by conjugation with phosphatidylethanolamine. Finally, LC3-II allows the autophagosomes to fuse with lysosomes to form autolysosomes where all contents are enzymatically digested (**Figure 1**).

Plethora of microRNAs have been shown to modulate the different proteins involved at each step of the autophagic process [35–37]. Although the six members of the miR-30 family have distinct mature sequences, the seed sequence is perfectly

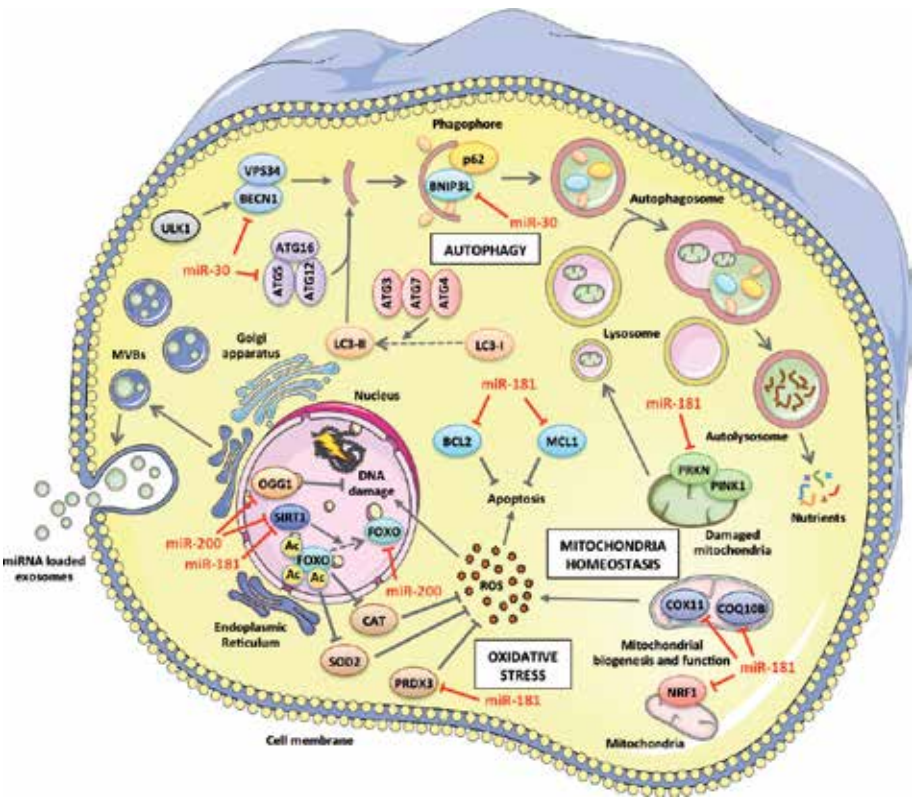

Figure 1.
MiRNAs regulate multiple gene regulatory networks implicated in skin aging. Members of the miR-30, miR-181, and miR-200 families are overexpressed with skin aging and regulate critical cellular processes such as autophagy, oxidative stress, and mitochondria homeostasis. Autophagy is regulated by the interaction of multiple molecular partners called autophagy-related (ATG) proteins and leads to the enzymatic digestion of autophagosome content. Oxidative stress is characterized by an accumulation of reactive oxygen species (ROS), which disturbs cellular homeostasis and leads to DNA damage and apoptosis. Mitochondria homeostasis relies on an effective biogenesis and a proper elimination of compromised mitochondria. MiR-30, miR-181, and miR-200 directly or indirectly target multiple key proteins implicated in these different processes. Fusion between multivesicular bodies (MVBs) and cell membrane allows the liberation of exosomes and their miRNA cargo in the extracellular compartment. Red inhibitory arcs symbolize miRNA inhibitory effect by inducing mRNA decay or translation inhibition. Gray inhibition arcs indicate inhibition effect on proteins or processes. Continuous plain gray arrows represent activation effect on protein or process. Continuous straight gray arrows represent intracellular dynamics. Dotted straight gray arrows represent protein post-translational modifications.

conserved (**Table 1**). Thus, it is not surprising that other members of the miR-30 family have been involved as well in the negative control of autophagy, such as miR-30b in vascular smooth muscle cell [38], miR-30c in neurons [39], miR-30d in astrocytes [40], and miR-30e in cardiomyocytes [41]. The different members of the miR-30 family have been primarily associated to the targeting of *BECN1* [24–33]. Importantly, this regulation of *BECN1* by miR-30a has been established *in vivo* as well [30, 39, 41, 42]. *ATG5* is another recurrent downstream target of all the members of the miR-30 family [43–52]. Finally, *BNIP3L* (*aka NIX*) is an additional factors of the autophagy pathway negatively controlled by both miR-30c [53] and miR-30d [50, 51], whereas miR-30d also decreases the luciferase activity of reporter plasmids carrying the 3′UTR of *ATG2B* or *ATG12* [50, 51].

The functional link between these miRNAs and the mechanisms of skin aging is not clarified yet; however, the decline in effectiveness of autophagy is clearly one of the hallmarks of aging [54]. This has been observed in diverse organisms from nematodes to rats, including human cells, and this is accompanied in part by a downregulation of *ATG5* and *BECN1* [55], which are notorious targets of the miR-30 family members. Accordingly, members of the miR-30 family have been shown to be induced in senescent cells, including aged keratinocytes [18, 56]. Apparent conflicting data exist in the literature as a recent study comparing gene expression from young (9–18 years old, average 12.7) and aged dermal fibroblasts (50–94 years old, average 67) revealed by RNA-seq analysis that the major autophagy-modulating genes (*BECN1*, *MAP1LC3B*, *ATG5*, *ATG7*, *ULK1*, *PIK3C3*, *mTOR*) were not differentially expressed [57]. However, the downregulation of mRNA target expression by miRNA binding in the 3′UTR occurs in two manners, either through mRNA decay or translation inhibition [58, 59]. Thus, the measure of mRNA levels does not simply reflect the final activity of the protein, and for example, miR-30a has been preferentially associated to *BECN1* translation inhibition rather than mRNA decay in endothelial cells [29, 30]. Indeed, in the same study, even though the mRNA levels of multiple effectors of autophagy were not downregulated with aging in dermal fibroblasts, excessive residual autophagic bodies were found in these cells, thus exposing an impaired autophagic flux in aged skin [57]. This is consistent with a previous report showing a nearly 80% reduction in the autophagic flux, as determined by RT-qPCR and immunocytofluorescence analysis of LC3B expression in synchronized aged normal human skin fibroblasts and compared to young fibroblasts [60]. Likewise, accumulation of autophagic vacuoles containing debris and deformed mitochondria was found in both senescent human keratinocytes and aged dermal fibroblasts by transmission electron microscopy analysis [61, 62].

In fibroblasts, the defect in the autophagic flux was identified at the final degradation step of the autophagolysosome and was correlated with weakened turnover of dermal extracellular proteins, possibly leading to a collapse of the dermis structure and skin fragility [62]. In keratinocytes, the autophagic process, especially the nucleophagy, plays a key role in the control of the terminal differentiation [63, 64]. In addition, it has been demonstrated that *Becn1* also plays a crucial role for skin development in mice [65]. Indeed, conditional knockout of *Becn1* in the epidermis layer results in mice having stiff and shiny skin with extensive water loss and death within a day after birth. The silencing of BECN1 in human keratinocytes is associated with a considerable drop in expression of the keratins 1 and 10 (KRT1 and KRT10) together with puncta formation of the integrin alpha 6 (ITGA6), suggesting a failure in the normal endosomal trafficking. Since the skin phenotype is not observed in *ATG5* or *ATG14* KO mice, the authors suggested that BECN1 is required for normal mouse skin development through the regulation of the endocytic pathway but autonomously from the autophagy pathway. However, an ATG5/ATG7-independent alternative autophagy has been described earlier [66–68], and

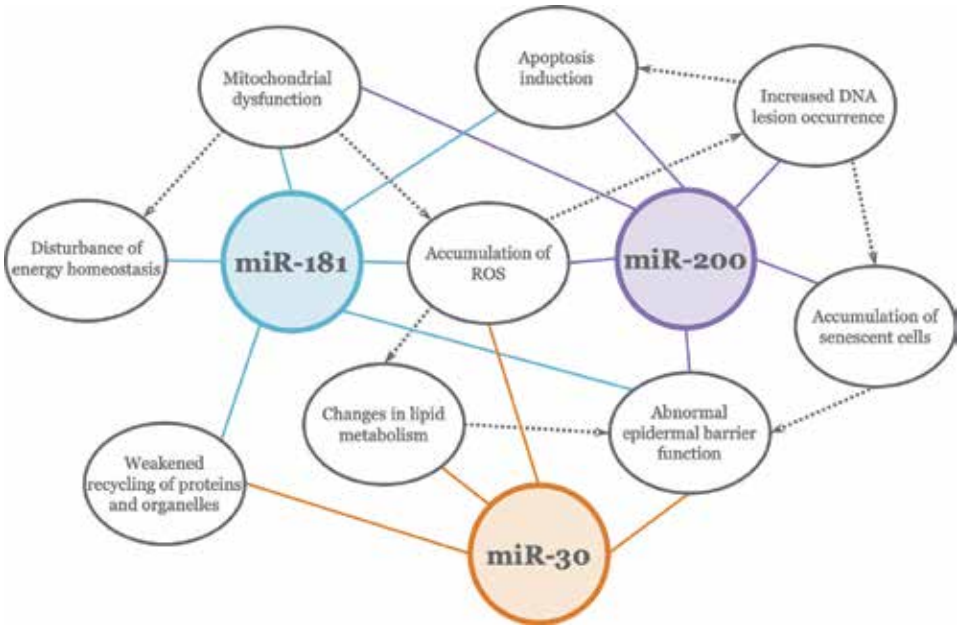

Figure 2.
Features of skin aging resulting of combined miRNA action. Overexpression of miR-30, miR-181, and miR-200 family members recapitulates many of the skin aging features. Each trait is directly regulated by one, two, or three of the families presented here. Some of the alterations related to miRNA regulation will also indirectly contribute to the exacerbation of the other traits. Colored lines represent direct effect of the corresponding miRNA. Dotted gray arrows symbolize indirect action of one feature on another.

thus, we cannot totally exclude the direct contribution of the autophagy pathway in normal skin development.

The importance of the multiple autophagy pathways in skin function has been recently reviewed elsewhere [69]. The causative link between miR-30 members' induction, autophagy reduction, and tissue aging still needs to be demonstrated especially in skin for miR-30a. Toward this aim, we already showed that a reduced epidermal differentiation is correlated with an abnormal barrier function in an organotypic skin models prepared with keratinocytes artificially overexpressing miR-30a [18]. Finally, the impairment of the various modes of autophagy (aggrephagy, lipophagy and mitophagy) leads to the accumulation of protein aggregates, to the aberrant handling of lipid droplets causing changes in lipid metabolism, and to the accumulation of dysfunctional mitochondria responsible for ROS production, which are all some features of aged tissue (**Figure 2**), including skin [70]. Interestingly, the upregulation of miR-30b and miR-30d (clustered at the same locus) significantly increases by about twofold in retinal epithelial cells treated with a sublethal dose of H_2O_2, a potent inducer of ROS [71]. All of these converging data from the literature strongly suggest that the negative regulation of the autophagic pathway by miR-30 could be an important mechanism contributing to tissue aging.

3. The miR-200 family in the control of the oxidative balance

Oxidative stress results from an imbalance between the production of free radicals and their molecular scavengers aiming at the restoration of the redox equilibrium in the cell. At moderate levels, ROS induce biochemical modifications of lipids, proteins, and DNA and thus take part in the signaling cascades controlling cellular processes such as differentiation or trafficking of intracellular vesicles. However,

a sustained excessive concentration of ROS will disturb the cellular homeostasis and eventually induces cell death or senescence. Every day, parts of the skin are directly exposed to solar radiations that induce the production of ROS. Since keratinocytes are exposed to both UV-A and UV-B, they display a powerful antioxidant system and an efficient DNA repair machinery compared to dermal fibroblasts.

Since the epidermis turnover takes about a month, even a little decline in this ROS scavenging mechanism will contrariwise strongly affect keratinocytes homeostasis and leads to a progressive accumulation of senescent cells that no longer participate in the regenerative process. Indeed, the steady-state ROS levels were found to be 2.6-fold higher in primary human keratinocytes from old donors (60–82 years) compared to young ones (2–45 years) [72]. This was correlated with a particular increase in 8-hydroxy-2′-deoxyguanosine (8-OH-dG) residues, a highly mutagenic DNA lesion leading to the transversion of GC to TA upon replication by a DNA polymerase [72, 73].

Consistently, a previous report has shown that about 3% of proliferating human keratinocytes contain these oxidized guanines versus 19% in senescent human keratinocytes [74]. The 8-oxoguanine DNA glycosylase (OGG1) is a key enzyme coordinating the removal of 8-OH-dG lesions by catalyzing the first step of the repair process (**Figure 1**), and its expression level is significantly decreased with aging in keratinocytes [72]. *In silico* analysis indicated that *OGG1* was a potential target of miR-33a and miR-200a, with respectively two putative seed sequences and one putative seed sequence in its 3′UTR. In primary keratinocytes from human elderly donors, only miR-200a was strongly upregulated, whereas miR-33a was downregulated, suggesting that only miR-200a was responsible for *OGG1* lower expression [72]. This prediction was confirmed as overexpression of miR-200a-3p mimics by transient transfection reduced both endogenous *OGG1* expression and luciferase activity derived from a reporter plasmid exhibiting the *OGG1* 3′UTR sequence. Concomitantly with OGG1 downregulation, a significant increase of the senescence marker CDKN2A (*aka* P16INK4) was observed, thus showing a direct link between the redox balance and senescence in aged keratinocytes (**Figure 2**).

To note, we have not observed a differential expression of miR-200a-3p in our own miRNA sequencing between keratinocytes from young (3–6 years), adult (20–40 years), and elderly (60–71 years) human skin samples [18]. This difference may come from the differential segregation of young and old keratinocytes in the two studies and/or from the anatomical location of the biopsies as sun-exposed or photo-protected areas would present a different pattern of expression. Nevertheless, the miR-200 family is composed of five members, including miR-141, miR-200a, miR-200b, miR-200c, and miR-429 (**Table 1**), and we effectively detected a significant twofold increase in miR-200c-5p levels in both adult and aged keratinocytes as compared to the young cells from photo-protected skin biopsies [18]. The functional significance of the elevated level of expression of the so-called passenger strand still needs to be depicted. Recently, a study on miR-122 has opened up a new perspective in the miRNA field as it showed that the passenger strand is not always an innocent bystander but could also cooperates with the guide strand to achieve the same function through different mechanisms [75]. In agreement with our miRNome analysis in aged keratinocytes, miR-200c-3p was increased by about threefold in aged skin fibroblasts (65–80 years) as compared to young ones (4–6 years), isolated from skin biopsies protected from sun exposition [76]. Thus, overexpression of miR-200 family members cannot be solely interrelated to photo-aging but also to intrinsic chronological aging.

Interestingly, the existence of a crosstalk between the miR-200 family and oxidative stress has been investigated in other physiological contexts. Oxidative stress induced by H_2O_2 treatment in normal liver cells or normal endothelial cells

triggers progressive overexpression of all of the five members, with discrepancy among them [77, 78]. Even if the five members of the miR-200 family are divided into two clusters, namely miR-200a/miR-200b/miR-429 on chromosome 1 and miR-200c/miR-141 on chromosome 12, it has been reported that the promoters of both miR-200 clusters comprise TP53-binding sites and that all of the three transcription factors TP53/TP63/TP73 are able to activate the transcription of the miR-200 family members [77–80]. MiR-200c-3p has been shown to target *SIRT1*, *FOXO1,* and *ZEB1*, all of them being downmodulated in aged skin cells [76, 78, 81]. A complex regulatory loop exists between *SIRT1* and *FOXO1* as the latter is a direct target of the deacetylase SIRT1, and at the same time, FOXO transcription factors are regulating *SIRT1* expression. MiR-200c-3p directly targets both *SIRT1* and *FOXO1* (**Figure 1**) [76]. Thus, by decreasing SIRT1 level, FOXO transcription factors become hyperacetylated, which in turn provokes their detachment from *SIRT1* promoter, thus further decreasing *SIRT1* expression [82]. FOXO1 hyperacetylation similarly decreases the expression of the ROS scavenger catalase (CAT) and superoxide dismutase 2 (SOD2) [83, 84].

Another consequence of miR-200c-induced *SIRT1* downregulation is the increased acetylation of TP53, a post-translational modification associated to apoptosis induction via a *TP53* transcription-independent pathway [85]. The overexpression of miR-200c-3p effectively enhances apoptotic DNA fragmentation and increases the percentage of senescent cells together with overexpression of CDKN1A (*aka* p21 WAF1/CIP1) [78]. Finally, miR-200c is also targeting the transcription factor ZEB1, which has been recently associated to ROS-induced senescence in human dermal fibroblast [86]. In physiological conditions, ZEB1 positively regulates the expression of the DNA methyltransferase *DNMT1* that methylates CpG islands in the *TP53* promoter, thus decreasing the transcription rate. In this study, the authors found that *ZEB1* expression is strongly repressed by elevated ROS levels but they are still interrogating the mechanistic relationship between ROS and *ZEB1* expression. One can speculate that miR-200c-3p overexpression during oxidative stress is one part of the answer.

Moreover, miR-200a-3p is regulating the ROS-stress response signaling by targeting the MAP kinase p38 alpha (*aka* MAPK14), which normally activates the expression of *NRF2* (*aka NFE2L2*). NRF2 is a well-known master regulator of adaptive protection against oxidative stress in cells and especially in keratinocytes [87, 88]. Indeed, a gradient of *Nrf2* expression was spotted in the murine epidermis, with higher levels of Nrf2 in the suprabasal differentiated cells and lower levels in the proliferating basal cells [89]. The gradient of *Nrf2* expression and activity is crucial for long-term epidermis homeostasis. In one hand, high concentrations of Nrf2 will establish a safeguard for suprabasal keratinocytes daily assaulted by pollutants and radiations, thus maintaining the skin functional integrity. On the other hand, low concentrations of Nrf2 will preferentially orient basal transit amplifying keratinocytes toward apoptosis under stress conditions, which is imperative for the elimination of mutated stem/progenitor cells and potential malignant transformation. Furthermore, it has been demonstrated that NRF2 also improves human keratinocyte differentiation *in vitro* by increasing the expression of Keratin-10 and Loricrin, even if the underlying mechanism has not been addressed yet [90]. Finally, the inhibition of MAPK14 signaling targeted by miR-200a-3p triggers a lack of NRF2, which will directly affect keratinocyte differentiation together with an accumulation of ROS and the generation of mitochondrial injury resulting in cell death [77, 90]. A defective keratinocyte differentiation program and an increased keratinocyte apoptosis are two hallmarks of epidermis aging that may fit with the consequences of miR-200 overexpression (**Figure 2**).

4. The miR-181 family in the control of mitochondria homeostasis

In our microarray approach aiming at identifying modulated miRNA with epidermis aging, we found out that three members of the miR-181 family were significantly upregulated, namely miR-181-a (fold change 1.61), miR-181-b (fold change 1.54), and miR-181-d (fold change 2.40). Two of which were previously associated with keratinocytes replicative senescence: miR-181a (fold change 1.30) and miR-181b (fold change 1.38) and with human skin aging as well, although this latter result was not statistically significant in this particular study [81]. Additionally, miR-181a was also found to be tightly related to human dermal fibroblasts senescence [91], making it a consistent miRNA associated with skin aging. The miR-181a and miR-181b are two intronic clustered miRNAs existing in double copies on chromosome 1 (miR-181a-1 and miR-181b-1) and on chromosome 9 (miR-181a-2 and miR-181b-2), whereas the miR-181c and miR-181d constitute a third cluster on chromosome 19 (**Table 1**).

Multiple targets have been identified for the miR-181, including SIRT1, a key regulator of cell survival in the context of oxidative stress. The essential crosstalk between oxidative stress and SIRT1 has been fully reviewed elsewhere [92]. As discussed previously, SIRT1 deacetylates the FOXO transcription factors and subsequently stimulates the expression of antioxidants. In addition, the SIRT1-FOXO axis is also involved in autophagy induction. SIRT1 promotes the activation of FOXO transcription factors that positively regulate the expression of several autophagy-related genes such as *ULK1*, *MAP1LC3A/B*, *GABARAPL1*, *ATG12*, and *BNIP3* [93–97]. Moreover, SIRT1 directly deacetylates the proteins ATG5, ATG7, and ATG8, thus controlling the dynamic of protein interaction and assembly requisite in the progression of the autophagic flux [98]. The importance of SIRT1 in regulating the autophagic flux was also demonstrated *in vivo* with a knockout mouse model. Indeed, the $Sirt1^{-/-}$ mice partially resemble the $Atg5^{-/-}$ mice, including the accumulation of damaged organelles and notably atypically shaped mitochondria, disturbance in energy homeostasis, and early perinatal mortality [98].

The disruption of the mitochondrial function is also retrieved when miR-181 are overexpressed, independently of *Sirt1* expression. Indeed, miR-181 additionally targets several members of the BCL2 family: BCL2 and MCL1, two major antiapoptotic effectors, and to a lesser extent the proapoptotic effector BIM (**Figure 1**) [99]. BCL2 is the most famous member of the family, and it has a role in almost all the main pathways governing cell aging. First, BCL2 promotes longevity by favoring the antiapoptotic signaling [100]. Second, BCL2 has an antioxidant function as it relocates glutathione to the mitochondrial membrane [101]. Third, BCL2 inhibits starvation-induced autophagy both *in vitro* and *in vivo* by binding to BECN1. Importantly, only BCL2 proteins localized at the endoplasmic reticulum present an inhibitory effect on starvation-induced autophagy, whereas BCL2 proteins localized at the mitochondrial membrane do not play a role in this process [102]. Likewise, MCL1 has been shown to regulate the balance between apoptosis and autophagy under stress conditions [103]. Thus, the miR-181 family seems to finely control the cell fate by favoring the cell death via apoptosis over the cell survival through autophagy within oxidative environment.

Remarkably, consistent enriched expression of miR-181 is found in mitochondria across different cell models even though these miRNAs are not encoded in the mitochondrial genome but come from the nucleus [104, 105]. The precise subcellular localization of particular miRNAs at the mitochondria led to the classification of miRNAs such as "mitomiRs," a group of approximately 60 members [106]. The miR-181a is one of the most consistent—if not the most consistent—mitomiR. Since mitochondria play a key role in the aging process, it is reasonable

to assume that mitomiRs disrupt gene regulatory networks eventually contributing to tissue decline with aging. Very recently, a seminal study has demonstrated that miR-181a/b is controlling a group of elemental genes for mitochondrial biogenesis and function [107]. NRF1, a master regulator of mitochondrial biogenesis; the cytochrome *c* oxidase assembly protein COX11 and the coenzyme Q-binding protein COQ10B, two actors of the mitochondrial respiratory chain assembly; and the thioredoxin-dependent peroxide reductase PRDX3, another potent ROS scavenger, are newly validated direct targets of miR-181a/b (**Figure 1**). In accordance, the inactivation of miR-181a/b stimulates both the mitochondrial biogenesis and activity in a knock out mouse model.

In order to keep up with redox equilibrium, the cell has to maintain a perfectly tuned balance between mitochondrial biogenesis and its recycling. The elimination of defective mitochondria is mediated by the BCL2-related outer membrane protein BNIP3L/NIX, which contains a conserved LC3-binding motif and acts as a receptor for addressing damaged mitochondria to autophagosomes, which then deliver the organelle to lysosomes for degradation and recycling. A recent *in vitro* study has shown that transient transfection of miR-181a decreases the colocalization of mitochondria with lysosomes after drug-induced mitochondria depolarization [108]. Under stress conditions, the depolarization of the mitochondria switches the localization of the PTEN-induced serine/threonine kinase 1 (PINK1) from the inner membrane to the outer membrane where it quickly accumulates, flagging the damaged organelle for elimination. From the outer mitochondrial membrane, PINK1 phosphorylates the cytosolic Parkin (PRKN) ubiquitin ligase, which in turn is recruited to the mitochondria (**Figure 1**). The addition of ubiquitin chains on several outer membrane mitochondrial proteins establishes a signal for the selective autophagic removal of the mitochondria, a process called mitophagy [109]. Interestingly enough, the TargetScan prediction algorithm shows no conserved putative binding site in the 3′UTR sequence of *PINK1* mRNA, whereas only one putative miR-181 binding site is present in the 3′UTR sequence of *PRKN*. The direct targeting of *PRKN* by miR-181a was demonstrated *in vitro* both at the mRNA level and the protein level [108]. This inhibition of *PRKN* by miR-181a was further associated to a substantial inhibition of the entire mitophagy process.

Accumulating compromised mitochondria will ultimately lead to the failure of the respiratory chain to produce ATP and will alongside generate even more ROS affecting in cascade the global cell homeostasis. A positive feedback loop may exist between miR-181 and oxidative stress since a recent study has shown that H_2O_2 was able to boost the expression of miR-181a, probably through the activation of the NF-κB signaling [110]. Altogether, these data show that miR-181 overexpression observed with skin aging would exert a deleterious effect by simultaneously preventing mitochondrial turnover and overactivation of cell death through apoptosis (**Figure 2**). However, it is to be noted that *miR-181a/b*$^{-/-}$ mouse model shows normal lifespan, with no apparent skin defects and thus cannot solely recapitulate the aging process [111].

5. Circulating miRNAs in the spreading of the aging message

MiRNAs have been found in all biological fluids such as blood, saliva, urine, or breast milk. These circulating miRNAs are very stable as they are protected from RNAse degradation thanks to a packaging into extracellular vesicles (EVs) made of a lipid bilayer [112, 113] or through complexing with carrier proteins [114, 115]. Indeed, an important part of circulating miRNAs is bound to the argonaute (AGO) proteins and is released in the extracellular compartment after cell death [116].

As AGO proteins are very stable in the presence of both RNAses and proteases, conjugated miRNAs are secured [117]. EVs are classically divided into three different groups: (1) apoptotic bodies, with a diameter comprised between 1 and 5 μm, (2) microvesicles, formed by direct budding of the plasma membrane and ranging in size from 0.1 to 1 μm, and (3) exosomes, the smaller EVs derived from an endosomal origin with a diameter ranging between 30 and 150 nm (**Figure 1**). They represent a new class of paracrine factors mediating cell-to-cell communication [118]. They transfer a complex signal to more or less distant recipient cells through their composite cargo, including proteins, mRNAs, lipids, noncoding RNAs and particularly miRNAs, thus modulating their behavior [112, 118, 119]. Interestingly, miRNAs enclosed in exosomes do not necessarily reflect their relative abundance in the parent cell, indicating that the exosomal miRNA loading occurs through a selective sorting [120, 121]. This allows a controlled released of particular messages to the recipient cells depending on the biological context. The intercellular communication via circulating miRNAs is likely to be involved in aging and age-related diseases. It was recently observed that human senescent dermal fibroblasts, which progressively accumulate in aging tissues [122], release more exosomes than proliferating cells [123]. In accordance, unpublished data from our group suggest that aged keratinocytes also secrete more exosomes as compared to young keratinocytes.

It is now well admitted that exosomes from senescent cells and their miRNA cargo are part of the senescent-associated secretory phenotype (SASP) [123]. Indeed, most of the miRNAs contained in exosomes are predicted to silence proapoptotic pathways and so could be involved in the propagation of senescent cells in tissue, thus greatly contributing to the aging process [122]. In parallel with an increased secretion of exosomes, two recent studies have demonstrated that immune cells are more capable of exosome uptake by internalization in older people [124, 125]. Even if the molecular mechanisms behind these observations are not understood yet, the decreased clearance of senescent cells by the innate immune system is clearly another factor enforcing tissue aging [126].

Specific circulating miRNAs have already been associated with different age-related diseases. For example, a concordance has been noticed between miR-29-3p increase in exosomes released by bone marrow mesenchymal stem cells and aging [127]. This miR-29-3p increase leads to insulin resistance in adipocytes, myocytes, and hepatocytes by downregulation of SIRT1 protein level. Insulin resistance is often developing in elderly type 2 diabetes patients. This suggests a significant role of exosomal miRNAs in aging-associated insulin resistance and represents a new therapeutic target. Additionally, circulating miR-34a in plasma is also increased during aging. This increase is even more important in age-related hearing loss patients and has been correlated with a decrease of diverse miR-34a target expression (*SIRT1*, *BCL2* and *E2F3*) in both plasma and hearing-related tissues [128]. Besides, circulating level of miR-130b was found raised with obesity, another metabolic disorder that accelerates the rate of aging by contributing to the accumulation of the pro-oxidative advanced glycation end-products and therefore shortening life span [129, 130]. The miR-130b directly regulates the expression of the master epidermis transcription factor ΔNp63, a predominant isoform of TP63, which controls the skin stem cell maintenance and longevity and which expression is decreased with skin aging [81, 131]. Interestingly, the miR-181 clustered genes, which expressions are increased with aging, are all negatively regulated by ΔNp63. If miR-130b is slightly increased with keratinocyte replicative senescence, its expression does not change in aged skin biopsies [18, 81], suggesting that the negative modulation of ΔNp63 with aging may come from exosomal release of miR-130b. Thus, the dissemination of local high concentration of miR-130b from other altered skin or body compartments with aging could lead to the increased expression of miR-181 family

members in the epidermis through miR-130b-dependent inhibition of the ΔNp63 action at the genomic loci.

In skin, few things are known concerning implication of circulating miRNAs in aging. However, several studies have demonstrated age-related changes of circulating miRNAs expression level in biological fluids [128, 132], including a decrease of miR-181a, miR-200c, and miR-30b in serum of older individuals [133, 134], one representative from each miRNA family that we focused on here. The observation that these miRNAs are increased in aged human primary keratinocytes compared to young cells [18] but decreased in the serum of elderly people well illustrates the fact that the proportion of miRNAs released by cells does not necessarily reflect the variations in parent cells. In another pathological context, the acute myocardial infarction, miR-30a, is enriched in exosome from patient serum [135]. *In vitro*, miR-30a enrichment in exosomes released by cardiomyocytes is repeated during hypoxia and leads to a reduction of autophagy in cardiomyocytes. To date, very few studies have focused on modulation of exosomes content in miRNA in aged skin cells. A recent study demonstrates that miR-23a-3p, which is enriched in exosomes released by senescent fibroblasts, has an impact on skin homeostasis. Indeed, miR-23a-3p seems to improve the migration of keratinocytes on a scratch closure assay *in vitro* and impairs keratinocytes differentiation [136]. In addition, another study has demonstrated that exosomes, and more specifically miRNA cargo, released by keratinocytes after UV-B exposure influence the activity of melanocytes [119]. The UV-B irradiation changes the exosome composition and lead to the modulation of skin pigmentation by multiple pathways.

Taken together, all of these emerging data exemplify the theory that circulating miRNAs and particularly miRNA exosome cargo play a crucial role in cell-to-cell communication. They are implicated in multiple physiological and pathological mechanisms, including aging and age-related diseases. As they are present in all the biological fluids, and more interestingly in blood, they could be used as biomarkers for various human diseases that limit lifespan. Moreover, the capability of exosomes to transfer information and affect the behavior of distant cells is very interesting for the development of new therapies. In skin context, the exosomes of the different cell types and their roles in skin homeostasis are not really described for the moment. It could be also interesting to consider if some miRNAs implicated in the complementary aging processes, as the ones we described here, are present in exosomes derived from skin cells and how they could affect some gene regulatory networks in recipient cells.

6. Conclusion

Many miRNAs have been already described in the skin to be involved in either keratinocytes or fibroblasts senescence [137]. However, cellular senescence is only one parameter contributing to tissue aging. In this chapter, we described how miRNAs could drive tissue decline with aging, by regulating complex gene regulatory networks with a special focus on autophagy, oxidative stress, and mitochondria homeostasis. Indeed, since one miRNA is targeting multiple effectors at the same time, a dynamic buffering of inter-related pathways will ultimately tip the balance toward a cell fate or another. Here, we tend to demonstrate that some miRNAs are consistently found to be indirectly or directly associated to diverse mechanisms of aging, namely the members of the miR-30, miR-200 and miR-181 family. Interestingly, the three cellular processes detailed in here are closely nested. Autophagy is crucial to remove havocs due to oxidative stress, including damaged mitochondria. Mitochondria are also a direct source of ROS, so maintaining the

homeostasis of this particular organelle ensures a good oxidative equilibrium. One particular finding when we gather different studies from the literature is the fact that the three miRNA families described here, miR-30, miR-200, and miR-181, are all upregulated following an oxidative stress. Since all of them could also worsen this oxidative stress by acting on different pathways, it is not clear what is the cause and what is the consequence so far. Finally, miRNAs are clearly exported out of the parent cell and could possibly diffuse into the whole body. According to all of the emerging data exposed in this chapter, it appears quite clear that circulating miRNAs have a central role in the propagation of functional defects associated to tissue aging. A central fundamental question still remains to date: What is driving in a first place the modulation of miRNAs expression with aging?

Conflict of interest

The authors declare no conflict of interest.

Notes

All of the artworks used in **Figure 1** were adapted from the illustration bank of Servier Medical Art (https://smart.servier.com) provided by Les Laboratoires Servier under a Creative Commons Attribution 3.0 Unported License.

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Chapter 6

MicroRNA-Based Markers in Plant Genome Response to Abiotic Stress and Their Application in Plant Genotyping

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Abstract

The high conservation of miRNA sequences provided an opportunity to develop an effective type of markers that is useful not only for genetic diversity study but also as potential biomarkers in plant stress responses. The fundamental potential of miRNA-based markers relies on the primer design based on the sequences of mature miRNAs, which are part of the step-loop structures. The advantages of this marker system include high polymorphism, reproducibility, and transferability across species. The abundance of mature miRNAs, which is linked to the expression of *MIRNA* genes, varies greatly among miRNAs, tissue types, or developmental stages, indicating the spatially and temporally regulated expression patterns of plant miRNAs. The results confirm the significance, reliability, and the position of miRNA-based markers as stress-sensitive biomarkers, indicating their potential in a wide range of applications of agricultural research.

Keywords: molecular markers, miRNAs, genotyping, stress-biomarkers

1. Introduction

Systematic documentation and evaluation of plant genome based on molecular markers that capture variability at the level of DNA or its protein products is essential for plant genetic resources management. These markers complement the morphological, agronomic, and other characteristics necessary to classify and identify plant genotypes within the species. Their importance and benefits are significant both for research and breeding, and for practice [1, 2]. The study and comparison of molecular information of individual organisms involves the search for DNA polymorphisms [3]. Polymorphisms are differences in DNA sequence caused by mutations. Molecular techniques in which polymorphisms can be visualized without sequencing are called molecular marker techniques [4]. A molecular marker is a specific fragment of DNA that can be identified within the whole genome and that is transmitted to the next generation following the standard rules of inheritance (Mendel's Laws). Marker may be located in or close to a gene or in noncoding regions.

The presence of polymorphisms between individuals will lead to a different pattern of markers after electrophoresis. These patterns are comparable with a "fingerprint"; therefore, these techniques are referred to as fingerprinting techniques.

These patterns reveal the DNA polymorphisms between the studied individuals. The more the individuals are related, the more their fingerprint will match. The level of polymorphisms in a group of individuals reveals the genetic diversity within this group [5].

There are different types of molecular markers. Molecular markers can be divided into two groups (a) biochemical markers, which detect variation at the gene product level such as changes in proteins and amino acids and (b) molecular markers, which detect variation at the DNA level such as nucleotide changes: deletion, duplication, inversion, and/or insertion. DNA markers are based on hybridization or PCR (amplification of DNA) [3]. The multiplex ratio is defined as the amount of markers generated with one single reaction (e.g., one PCR, one hybridization reaction). The obtained pattern can be simple (one or a few bands, low multiplex ratio) or complex (a high number of bands, high multiplex ratio).

Molecular marker methods are either dominant or co-dominant. Using co-dominant marker techniques, the different genotype combinations can be distinguished from each other at the study locus (or multiple loci). This means that homozygous (two identical alleles at a certain DNA locus) and heterozygous (two different alleles at a certain DNA locus) individuals will be identifiable. In the case of a dominant technique, it is not possible to detect the alleles that are present at a certain locus/loci, so homozygotes are not distinguishable from heterozygotes [6, 7].

The high conservation of microRNA sequences provided an opportunity to develop miRNA-based marker system referred to as stable, polymorphic, functional, and transferable genotyping technique.

2. The role of miRNAs in plant genome response to abiotic stress

The plant organism has to cope with the environmental stress in natural and agricultural conditions. The genetic background of the plant organism allows it to adapt and defend itself through different mechanisms at a molecular level.

RNA interference represents the plant immune and defense system. It is a conserved mechanism induced by double-stranded RNA (dsRNA) or hairpin-structured RNA (hpRNA). One of the modules of RNA interference is provided by the microRNA (miRNA) molecules [8–10], which are capable to form double-stranded hairpin-like structures referred to as pre-miRNA. These small molecules have significant regulatory potential in the genetic and epigenetic control of gene expression. They are one of the key players in plant genome response to abiotic and biotic stress factor(s). Especially, deeply conserved miRNA families are integral components of many regulatory networks in plant organism [11, 12].

In general, the function of miRNAs molecules in plant organisms is defined as regulatory in the following processes:

- plant growth and development
- leaf morphology and plant polarity
- root formation
- processes of transition from embryogenic to vegetative phase
- flowering time, formation of flower organs, and reproduction
- defense mechanisms through transferring of signaling molecules.

Plant adaptation mechanism requires complex modifications of gene expression machinery at the transcriptional and posttranscriptional level. Detailed studies of posttranscriptional gene regulation allow identifying stress-responsive miRNAs, which are differentially regulated under various stress factors. In plants, various abiotic stress-regulated miRNAs have been identified and characterized [13–17].

Certain families of miRNAs are either under or over-expressed, or new types of miRNAs can be synthesized under stress [13, 18]. Regulation of target genes expression by miRNA molecules is mediated by hybridization between miRNA sequences and their nascent reverse complementary sequences of mRNAs, which leads to their degradation or translational repression [19–21]. Because of their mode of action, they are generally referred to as negative regulators of gene expression.

Plant adaptation mechanism to environmental conditions includes minimization of their growth rates and reorganizing their resources. The primary focus of the adjustment is cell cycle, cell division, and cell wall constitution [22]; it means developmental processes regulated by conserved families of microRNAs.

3. MicroRNA-based markers

Genomic conservation of miRNA sequences and especially the stem-loop region of precursor molecules of miRNA (pre-miRNA) provided an opportunity to develop a novel type of molecular markers.

MicroRNA-based genotyping technique as a novel type of marker system was published in 2013 by authors Fu et al. [23]. Since then, this system has been applied to genotyping applications of *Setaria italica* and in related grass species [24]. The structure analysis of miRNA genes revealed that repetitive sequences are part of them, which led to development of miRNA-based microsatellite markers in *Oryza sativa* [25, 26] and *Medicago truncatula* and related legume species [27]. Given the origin of sequences of this type of markers, they can be considered as functional markers at the DNA levels [23, 24, 28].

The attributes of miRNA-based markers [23, 24] are as follows:

- good stability due to a direct PCR-based marker system
- improved reproducibility and sequence specificity due to high annealing temperature (more than 60°C) and the use of "touchdown" PCR approach
- relatively high polymorphism because of possible random combinations of primers
- putative functionality due to their polymorphism nature and the ability to predict phenotypes controlled by miRNAs
- cross-genera transferability potential because of the conservation level of miRNAs between species and the way of deriving markers from the consensus sequences of miRNAs.

3.1 MicroRNA-based marker assay

Following subsection provides the approach of microRNA-based marker assay applied in our laboratory. As referred in **Table 1,** the following procedure has been applied in several plant species for different research purposes.

The genomic DNA isolation protocol is based on the type of plant biological material (in terms of secondary metabolite content or other aspects). The primers for the miRNA-based markers are designed according to the mature or precursors

PCR component	Concentration	Final concentration
PCR buffer KCl, $(NH_4)_2SO_4$, 20 mmol × dm^{-3} $MgCl_2$	10×	1×
dNTP mix	2.5 mmol × dm^{-3}	0.8 mmol × dm^{-3}
Primer forward	100 µmol × dm^{-3}	10 pmol × dm^{-3}
Primer reverse	100 µmol × dm^{-3}	10 pmol × dm^{-3}
Taq polymerase	5 U	2 U

Table 1.
Protocol of miRNA-based marker assay.

Amplification process	Temperature	Time period	Number of cycles
Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	30 s	5 cycles
Annealing	64°C (with temperature reduction of 1°C per cycle)	45 s	
Polymerization	72°C	60 s	
Denaturation	94°C	30 s	30 cycles
Annealing	60°C	45 s	
Polymerization	72°C	60 s	
Final polymerization	72°C	10 min	

Table 2.
Amplification protocol of miRNA-based markers.

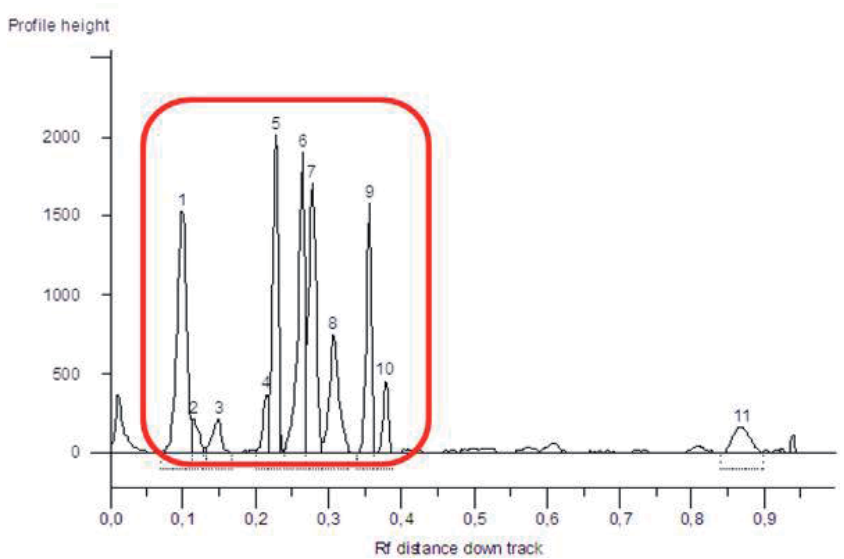

Figure 1.
Representative profile of amplified miRNA loci analyzed by GeneTool software (Syngene).

sequences (pre-miRNAs) available on the miRBase database (http://www.mirbase.org, version 22), taking into account the primer design approach of published methodology [23, 24]. The primers are combined as follows: (a) forward and reverse primers of the same type, (b) forward and reverse primers in random combinations or (c) specific forward primer and universal reverse primer [29].

The amplification protocol has originated from methodologies [23, 24] and was modified [30] (**Tables 1** and **2**). The total volume of PCR was 20 μl and the DNA concentration was 70 ng μl^{-1}. Amplification products are separated on 15% TBE-urea polyacrylamide (PAGE) gels, running in 1× TBE running buffer at constant power 180 V, 30 mA for 90 min. The gels are stained with PAGE GelRed™ Nucleic Acid Gel stain and are visualized on G-Box Syngene electrophoresis documentation system. For the recording of loci number and their position, as well as the identification of unique fragments, the gels are analyzed by GeneTools software (Syngene) (**Figure 1**).

4. Contribution of miRNA-based markers on plant genome response to abiotic stress and for genotyping applications

Genomic polymorphism of plants is the basis of their survival and ability to different climatic conditions. The cognition and mapping of plant genome variability using molecular markers is a prerequisite for extending the genetic base of crops to reduce their susceptibility to adverse environmental conditions [26]. An ideal molecular marker should be polymorphic, stable, reproducible, providing sufficient resolution, fast, and with fairly low cost [31]. The miRNA-based marker system is characterized by relatively high polymorphism, reproducibility, transferability across species, and ease of use with putative functionality [23]. The high level of transferability demonstrates the usability of miRNA-based markers for comparative genome mapping and phylogenetic studies [24].

5. MiRNA-based markers in genotyping applications

Recognizing relationships between species or within species help to focus more closely on a wide range of human interests, from basic description and disaggregation, through efficient resource genetic management to the production of quality and safe food, whether plant or animal origin [32].

Within our research, we focused on the use of miRNA-based markers to highlight their broad spectrum of regulatory impact activities in different plant species of nutritional and pharmaceutical uses (**Table 3**): flax (*Linum usitatissimum* L.), medlar (*Messpilus germanica* L.), milk thistle (*Silybum marianum* (L.) Gaertn.), ginkgo (*Ginkgo biloba* L.), common ivy (*Hedera helix* L.), avocado (*Persea americana* Mill), and ribwort plantain (*Plantago lanceolata* L.). A total of 13 miRNA-based markers were applied of which 28 primer combinations were made.

Useful molecular markers produce fragments between 150 and 500 bp in length, as this size of fragments can easily be distinguished using agarose or PAGE gels [23]. In our experiments, this size of fragments varied predominantly from 40 to 300 bp and could be clearly identified on agarose gels (**Figure 2**).

This range is due to variability of stem-loop structure of which the mature miRNA sequences are part of and the length of stem-loop structure ranges from less than 100 to over 900 nt. The primers' design based on miRNAs sequences can be linked to different places of the same stem-loop structure. Another possibility is that primers amplify regions between neighboring miRNAs [23].

Research purpose	Plant species	Reference
Genome profiling with regard to genotype origin	*Linum usitatissimum*	[30]
Spatial and temporal abundance of individual miRNA markers	*Linum usitatissimum*	[33, 34]
Functional markers of commercial type of the crop	*Linum usitatissimum*	[35, 36]
Genotyping	*Mespilus germanica*	[36]
Genomic authentication of varieties	*Silybum marianum*	[37]
Genotyping	*Ginkgo biloba*	[38, 39]
Genotyping	*Hedera helix*	[40]
Genotyping	*Persea americana*	—
Genotyping	*Plantago lanceolata*	—

Table 3.
The list of realized miRNA-based markers experiments.

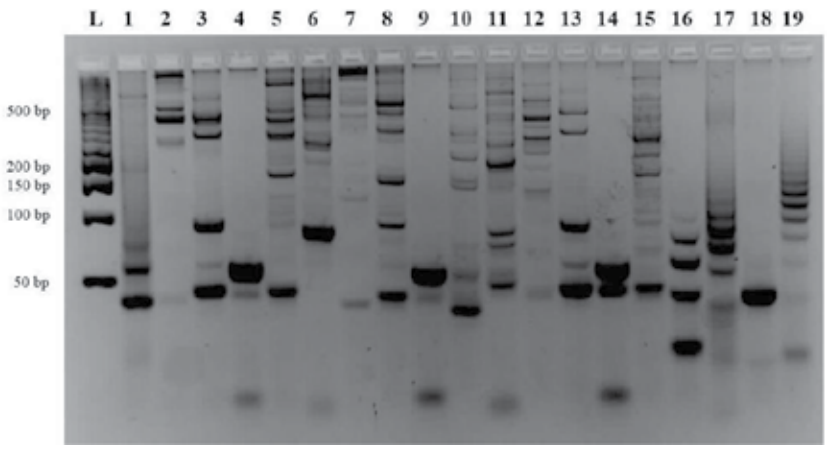

Figure 2.
Representative gel showing amplification profile of miRNA-based markers of Plantago lanceolata (L.). 1–19 combinations of primers. F—forward, R—reverse. 1: lus-miR-R + lus-miR168F, 2: lus-miR-R + gm-miR156bF, 3: lus-miR-R + hyp-miR414F, 4: lus-miR-R + gm-miR171aF, 5: lus-miR-R + lus-miR156aF, 6: gm-miR-R + lus-miR168F, 7: gm-miR-R + gm-miR156bF, 8: gm-miR-R + hyp-miR414F, 9: gm-miR-R + gm-miR171aF, 10: gm-miR-R + lus-miR156aF, 11: miR-R + lus-miR168F, 12: miR-R + gm-miR156bF, 13: miR-R + hyp-miR414F, 14: miR-R + lus-miR171aF, 15: miR-R + lsa-miR156aF, 16: lsa-miR169aF + lsa-miR169aR, 17: lus-miR156aF + lus-miR156aR, 18: hvu-miR827F + hvu-miR827R, 19: lsa-miR396aF + lsa-miR396aR.

The level of polymorphism varied from 70 to 90%. Mature miRNAs are expressed as small 21–24 nt endogenous molecules. As a result of different transcriptional activity among *MIRNA* genes, the miRNAs abundance in the cell varies greatly in dependence of miRNA family [11]. It should be noted that the level of polymorphism depended on the effectiveness of primer combination as well as the level of marker transferability.

The results of the studies have repeatedly confirmed the following:

- MicroRNA-based markers show the cross-genera transferability potential.
- MicroRNA-based markers display sufficient level of polymorphism in analyzed genotypes and are suitable to differentiate within genotypes of one specimen.

- MicroRNA-based markers provide genotype-specific profile of miRNA loci.

- The abundance of selective miRNA-based markers is tissue specific and developmental specific.

5.1 MiRNA-based plant genome response to abiotic stress conditions

Different mechanism of stress response contributes to stress tolerance or resistance at different morphological, biochemical, and molecular level [13]. Many stress-regulated genes are found to be regulated by miRNAs.

In the flax study, we applied nutritional stress factor under *in vitro* conditions [41]. The genome response of the flax genotype CDC Bethune was analyzed under five variants (including control variant) of Murashige-Skoog [42] medium by two miRNA-based markers, lus-miR395 and lus-miR399 [16, 17]. The results show that flax genome responds to the nutritional stress stimulus. Our results have supported the capability of miRNA-based molecules as potential biomarkers of abiotic stress factors.

Another study was conducted in order to test the ultrasound-induced oxidative stress in lettuce (*Lactuca sativa* L.) tissues by miRNA-based markers [43]. We have confirmed that reactive oxygen species (ROS), caused by sonication treatment, induced the polymorphism at the molecular level detected by miRNA-based stress markers. We have observed the statistically significant differences ($p \leq 0.01$) in miRNA markers ability to detect this polymorphism. The response of miR168 marker was statistically more sensitive in comparison with miR156 marker as a result of their specific regulatory nature.

The aim of research into the impact of soil compaction was to identify the barley (*Hordeum vulgare* L.) genome response by stress-responsive miRNA-based markers. A prerequisite for the research was that the plants are exposed to a lack of soil moisture and nutrients due to soil compaction. The effect of soil compaction was analyzed by four different miRNA-based markers (hvu-miR156, hvu-miR399, hvu-miR408, and hvu-miR827), within the leaf, stem, and root tissues of barley plants. We can state that due to soil compaction, the barley plants were exposed to the lack of moisture which subsequently affected the intake and utilization of nutrients from the soil and showed lower plant growth parameters and reduced the yields. Moreover, this genome response was tissue specific. The roots were most affected by dehydration, and the nutrient deficiency was the most pronounced on leaves. The number of amplified miRNA loci was statistically significantly dependent on the stress-sensitive marker applied.

We have conducted experiments in connection with research on the drought resistance of wheat (*Triticum aestivum* L.). Genomes of susceptible and drought-resistant genotypes were screened by stress-sensitive miRNA-based markers (hvu-miR408 and hvu-miR827). Genotypes were tested under *in vitro* conditions on Murashige-Skoog culture medium with different concentrations (0, 5, 10, 15, and 20%) of polyethylene glycol (PEG 6000) to induce dehydration stress. Drought-resistant wheat genotypes responded to dehydration stress, by significantly higher activity of hvu-miR408 biomarker in comparison with susceptible genotypes. This response points to a better genome adaptation ability of the resistant genotypes to abiotic stress. By using the conserved type marker hvu-miR156, which is involved in the regulation of plant growth and development processes, a reduced activity of this type of marker was observed, both in susceptible and resistant genotypes, indicating that the adaptation mechanism of plants to cope with stress conditions is implemented at the expense of growth processes.

6. Conclusions

It can be summarized that the marker system based on microRNA molecules represents (a) flexible marker system based on sequences of regulatory molecules, (b) species-transfer system due to the conserved nature of mature sequences of miRNAs, (c) functionally potential markers, where observed polymorphism points to changes in miRNA loci sequences evoking changes in the target gene regulatory model, (d) tissue-specific and development-specific characters of markers, and (e) screening tool of genome adaptation changes to induced abiotic stress referred to as stress-sensitive biomarkers. It should be noted that in selecting suitable type(s) of miRNA markers for a particular type of study, it is necessary to know the regulatory background, regulation mechanism, and target sequences of particular type of miRNA molecules.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 7

The Role of Noncoding RNAs in Brain Cells during Rat Cerebral Ischemia

Ivan B. Filippenkov, Lyudmila V. Dergunova and Svetlana A. Limborska

Abstract

Ischemic brain stroke is one of the most serious and socially important medical conditions. Transcriptome analysis is a prospective approach to the study of the mechanisms of brain functioning, both under normal conditions and in ischemia. In addition to mRNA encoding proteins, the study of noncoding RNAs in ischemia has exceptional importance for the development of new strategies for neuroprotection. Of greatest interest are microRNAs (miRNAs) and circular RNAs (circRNAs). circRNAs have a closed structure and predominantly brain-specific expression. They can interact with miRNAs, diminish their activity, and thereby inhibit miRNA-mediated repression of mRNA. Recently, it has become clear that the analysis of circRNA-miRNA-mRNA interactions is an important requirement for the detailed study of the mechanisms of damage and regeneration during ischemia. This chapter reviews the most recent data on the role of circRNAs, miRNAs, mRNAs, and their interactions in brain cells under normal conditions and in cerebral ischemia.

Keywords: functional genomics, experimental rat brain ischemia, mRNAs, noncoding RNAs, circular RNAs, microRNAs

1. Introduction

Ischemic stroke is a serious condition and is one of the leading causes of disability and death worldwide. It arises as a consequence of a critical decrease in blood flow in the brain tissues, which leads to the death of neurons and glial cells. Therapy aimed at treating or preventing ischemic stroke is one of the most significant problems of modern medicine. Molecular genetic approaches using experimental models of ischemia based on small laboratory animals are of great importance and provide perspectives for studying the mechanisms underlying the damage to nerve cells and their ability to recover. Events occurring in ischemic stroke in humans caused by the formation of a thrombus are best reflected by the permanent middle cerebral artery occlusion (pMCAO) model. Additionally, the transient middle cerebral artery occlusion (tMCAO) model best reflects the events occurring in ischemic stroke in humans caused by subsequent treatment with thrombolytic drugs. The results of clinical studies suggest that thrombolysis is among the most effective and affordable methods of treating ischemic stroke. At the same time, it is known that reperfusion

after thrombolysis not only contributes to the restoration of penumbra cells but also causes additional damage to brain cells, including disruption of endothelial microvascular cells, the excess oxygen radicals, and activation of apoptosis.

Ischemic brain damage in combination with reperfusion damage is a complex process resulting from changes in the levels of transcripts of genes in response to pathological effects. Currently it has been shown that informational RNA and various types of noncoding RNA (ncRNA), in particular, microRNA (miRNA) and long ncRNA, are actively involved in the response to the pathology. Recently, the idea that long ncRNAs can interact with miRNAs and diminish their activity has been actively developed. Such functions are attributed to circular RNA (circRNA), which is a new and actively studied type of RNA. circRNAs can also participate in the pathogenesis of various neurodegenerative and inflammatory diseases and cancer. These properties of circRNAs can be exploited in medicine to develop technologies to correct pathological processes caused by disruption of gene expression. This chapter will examine the most recent data on the roles of circRNAs, miRNAs, mRNAs, and their interactions in brain cells under normal conditions and in cerebral ischemia.

2. Ischemic stroke

According to the latest data from the World Health Organization, ischemic stroke, which is the result of a permanent or temporary decrease in cerebral blood flow, is in most cases caused by occlusion of cerebral arteries by a thrombus or embolus and is of particular importance among vascular conditions [1–3]. This serious condition is the second most common cause of the general mortality rate of the population in Russia and is the most common cause of impaired brain function [4]. Long-term studies of ischemic stroke have proven the existence of necrosis and penumbra zones in the first hours and days after the development of ischemic stroke. The penumbra is the tissue located around the ischemic nucleus in conditions of limited access of oxygen and glucose, and cells in the penumbra are capable of recovery. The concept of a "therapeutic window" was developed in which this window is a period during which the restoration of penumbra cells is still possible and most effective. The duration of the therapeutic window may vary depending on the organism and model of ischemia, but for most cells, it is limited to 3–6 hours [4–9].

Cerebral ischemia results from biochemical changes in brain tissues after ischemic damage. During ischemia, following the occlusion of the vessel, the glutamate-calcium cascade is activated, contributing to an influx of Ca^{2+} ions, the formation of intracellular mediators (phosphoinositol and diacylglycerol), membrane depolarization, accumulation of glutamate, and further influx of Ca^{2+} leading to damage to the cell macromolecules and ultimately to cell death [4, 10]. Among the factors affecting the development of ischemic stroke, it is important to consider the effects of molecular genetic parameters. High hopes of clinicians are placed on identifying and developing systems of genetic markers, which are an important step toward the development of personalized medicine and individualized prevention. It is extremely important to study the genetic systems that determine the mechanisms underlying the events during the therapeutic window, the death of neurons during ischemic damage, and the restoration of neurological functions.

3. Transcriptomics of ischemic stroke

Recently, as a result of the rapid development of genome-wide analysis and multi-omics technologies, it has become clear that tissue damage and regeneration

during ischemia is a complex process resulting from a change in transcript levels of a significant number of genes in response to pathological effects. Thus, early-response genes such as *c-fos* and *c-jun* [11] and zinc finger genes trigger cell proliferation and differentiation [12, 13], while genes that encode heat-shock proteins are involved in the inflammatory response and cytoskeleton organization [14], and others are predominantly activated after the onset of ischemia. Of great importance and perspective in molecular genetic studies are the models based on small laboratory animals that best reflect certain features of the development of the ischemic process. Study of the molecular mechanisms of cell death using pMCAO and tMCAO models conducted by Ford et al. revealed molecular functions and biological processes unique for each model [15]. Genes unique to tMCAO were predominantly involved in the induction of inflammatory and oxidative stress, while pMCAO resulted in the expression of genes that were more associated with metabolic activity and cellular signaling [15]. A study of the dynamics of changes in gene expression in rat brain a day after pMCAO revealed a substantial number of genes that changed expression significantly and are involved in the development of ischemic damage, including those determining cell survival and death, the immune response, functioning of the vascular system, and also processes associated with hematopoiesis, immune cells, lymphocytes, leucocytes, and other cells [16].

The most frequently used tMCAO model showed a reorganization of the functioning of many genes in various areas of rodent brains, including the infarction center, during the first day after the transient occlusion [15, 17–19]. In particular, activation of the transcription factor Nf-κb was shown. An increase of the mRNA level of *Cox2*, which encodes one of the key enzymes for the synthesis of the pro-inflammatory prostaglandin E2 (PGE2), was accompanied by an increase in the level of the corresponding protein, not only at the source but also in adjacent regions, and accompanied by increased concentration of PGE2 [20–22]. At the same time, as a result of the opening of the blood-brain barrier in brain sections, extensive leucocyte infiltration was observed [21, 23, 24]. An increase of the mRNA level of the gene for INOS, encoding an enzyme for the synthesis of NO, also participating in the development of the inflammatory response in the lesion, was also noted [22, 25]. In the ischemia-reperfusion model, it was also shown that cytokines (IL-1β, IL6), adhesion molecules (ICAM1, E-selectin, MMP-9), MAPK kinase, and c-fos transcription factors were involved in the development of inflammation [17, 20, 23, 26–29]. Wang et al. studied the molecular mechanism of ischemia-reperfusion pathogenesis using genome-wide transcriptome analysis (RNA-Seq) in the hippocampus of rats at 24 h after tMCAO. These investigators detected 182 differentially expressed genes (DEGs), most of which were upregulated [17]. A Gene Ontology analysis showed that these DEGs were mainly associated with inflammation, stress, immune response, glucose metabolism, and apoptosis [17]. Our analysis of gene expression under tMCAO conditions using RNA-Seq confirmed these results. However, in the subcortical structures of the brain that contained the focus of ischemic damage and the penumbra, we identified hundreds of genes that changed expression 24 h after tMCAO using RNA-Seq. Among these, we found activation of genes involved in inflammatory and immune reactions. There were gene encoding chemokines (*Ccl2* and *Ccl3*), heat-shock proteins (*Hspa1* and *Hspb1*), macrophage receptors (*Msr1*), secreted phosphoprotein 1 (*Spp1*), cytokine 3 suppressor (*Socs3*), and other proteins. Mass suppression of genes that ensure the functioning of neurotransmitter systems (*Chrm1*, *Chrm4*, *Cplx2*, *Drd2*, *Gabra5*, and *Gng7*) was also shown [19]. A study of the dynamics of changes of gene expression in rat brain a day after tMCAO conditions revealed a significant activation of the expression of genes involved in biosynthetic cell systems (ribosome, proteasome, DNA replication, and purine metabolism functional categories). The effect obtained indicated

a large-scale reorganization of nucleic acid and protein biosynthesis that was apparently related to the adaptive response of brain cells to the damage caused by ischemia-reperfusion.

4. miRNAs in ischemic conditions

Not only coding mRNA but also various types of ncRNA, which have significant regulatory potential, are involved in the response to ischemia. Much current attention worldwide is paid to the study of the features of the functioning of mRNA, miRNA, and long ncRNA as regulators in the mechanisms of pathogenesis and neuroprotection in ischemic conditions [30–35].

miRNAs are ncRNA molecules with a length of 20–22 nt. They act by direct interaction with target sites on mRNA, which leads to the degradation of mRNA or repression of its translation [36, 37]. miRNAs are critical regulators of central nervous system plasticity and play an important role in ischemia. In particular, miRNA is actively involved in the response to ischemic brain damage [38, 39]. Following ischemic brain damage, miRNAs can play the role of both neuroprotective agents and contribute to pathological manifestations. mRNA of the AMPA receptor subunit GluA2/GluR2 (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor) is the target of miR-181a. Thus, an increase in miR-181a expression may be neuroprotective. Indeed, there are many examples of where miRNAs contribute to the development of the pathological process following ischemic brain damage. Thus, miR-132 increases the expression of the NMDA receptor, which selectively binds N-methyl-d-aspartate, increasing the risk of excitotoxicity [40, 41]. Therefore, the use of miR-132 antagonists may have a neuroprotective effect. Herzog et al. studied the role of steroid hormones 17β-estradiol (E2) and progesterone (P) in the brain as regulatory factors for miR-223-3p, miR-200c-3p, miR-375-3p, miR-199-3p, miR-214-3p, and their target genes in the tMCAO model [42]. The levels of these miRNAs are increased at 12 and 72 h after tMCAO. E2 or P selectively dampened miR-223 and miR-214 but further boosted miR-375 levels. The expression of the genes for NR2B and GRIA2, which are targets for miR-223, was reduced after tMCAO, and E2 and P canceled this effect. Steroid therapy inhibited tMCAO-induced increases in the expression of genes for BCL-2 and RAD1, which are targets for miR-375. Thus, E2 and P have a role as indirect regulators of translation of proapoptotic and pro-inflammatory genes, which leads to the weakening of ischemic damage of tissue [42].

5. Long ncRNAs and circRNAs

Long ncRNAs have lengths greater than 200 nt [30]. Analysis of GENCODE [32], LNCipedia [43], and NONCODE [44] databases indicates the number of annotated long ncRNAs reaches several tens of thousands in humans. Their number is several times greater than the number of human protein-coding genes. Long ncRNAs are classified according to the region of the genome from which they are synthesized [32, 45]. Intergenic long ncRNAs are the most common in humans (59.2%). In second place are sense long ncRNAs that overlap with protein-coding genes (24.4%). Intronic and antisense long ncRNAs account for approximately 10% each [45]. Many long ncRNAs have specific evolutionarily stable expression. In addition, long ncRNAs exhibit tissue-, sex-, developmental stage-, and disease-specific expression [34, 46]. According to Mercer et al., in mice 64% of long

ncRNAs are associated with brain tissue [47]. Cabili et al. found that long ncRNAs may have a more pronounced tissue-specific expression than protein-coding genes [48].

To date, there is evidence that a substantial part of long ncRNA exists in a circular form [49–54]. Circular RNA (circRNA) is a newly discovered and relatively poorly studied class of long ncRNA, found predominantly in mammalian cells. The mammalian circRNAs are distinguished by a variety of structural organization. A common property of all cyclic structures is their resistance to treatment with RNase R, which depletes linear forms of RNA [55, 56]. A specific feature of the structure of exonic circRNAs is the unusual order of exon connection, in which the 3′-end of the downstream exon is linked with the 5′-end of the upstream exon. The mechanism of circRNA formation is called back-splicing. circRNAs may consist of exon or intron sequences [51]. More recently, information has appeared on the existence of circRNAs containing, simultaneously with exons, sequences of un-spliced introns [57] and recursive (RS) exons [58]. We come to the study of circRNAs through the analysis of peculiarities of the structure and expression of the human *SGMS1* gene. This gene encodes the enzyme sphingomyelin synthase 1, which provides the synthesis of sphingomyelin and diacylglycerol from phosphatidylcholine and

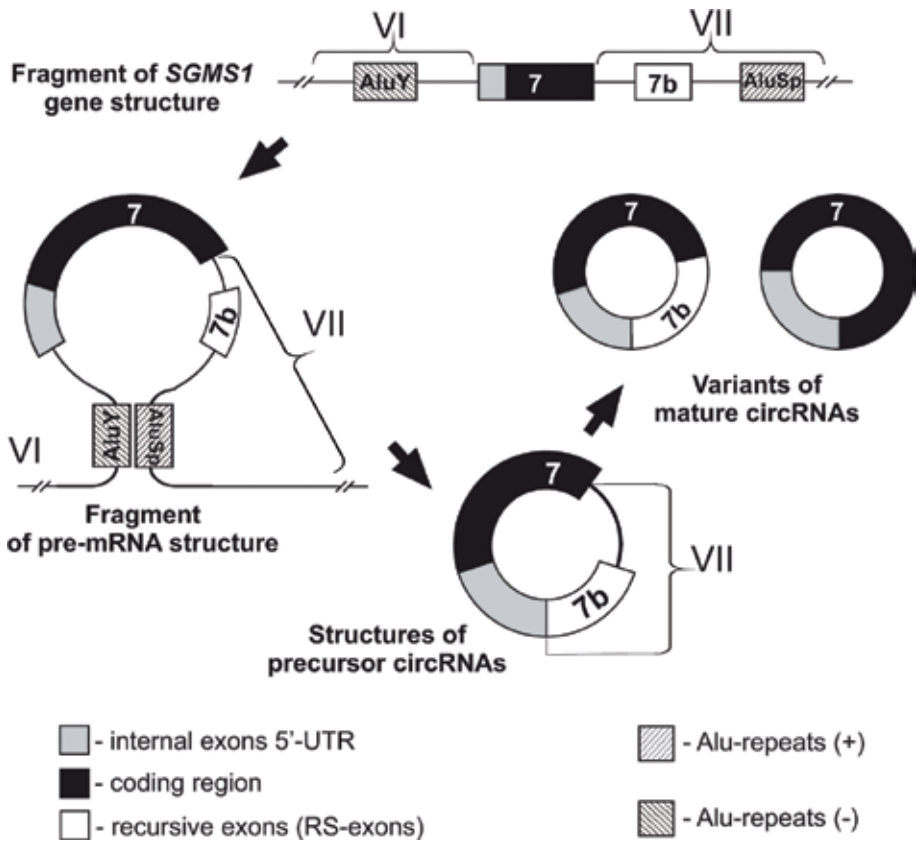

Figure 1.
A model of circRNA formation with the participation of recursive exon (RS-exon). Exons are shown as numbered blocs. Roman numerals indicate introns. Exon 7 is part of the mRNA, and RS-exon 7b is located inside the intron VII. The convergence between the 5′-end of exon 7 and the 3′-end of RS-exon 7b is effected using an interaction of highly homologous repeats of the Alu subtype, which are located near the back-splicing sites. Thus, the structure of the precursor of circRNA is formed according to the back-splicing. Next, part of intron VII is excised up to RS-exon 7b, and a linkage of the main exon with the RS-exon is formed. This leads to the formation of circRNA, which includes RS-exon 7b. Otherwise, the RS-exon 7b is excised along with the rest of intron VII and leads to the circularization of the main exon 7.

ceramide [59–63]. In addition to mRNAs providing protein synthesis, 13 circRNAs that predominantly contained sequences of the multi-exon 5′-untranslated region of the gene (5′-UTR) have been identified [54]. The RS-exons that participate in the multistep splicing of long introns of the gene were found within six circRNAs of the *SGMS1* gene. Based on the human *SGMS1* circRNAs formation from pre-mRNA with the participation of RS exons, the model of recursive back-splicing was proposed (**Figure 1**). Intronic circRNAs often have loop-like (lariat) structures with an abnormal 2′–5′ phosphodiester bond [50, 51]. More than half of circRNAs contain only protein-coding exons, while a smaller proportion contains sequences corresponding to the UTRs [64]. In related species, the circRNAs are often encoded by genes that are orthologous for human genes. So, homologous exons of these genes are detected in circRNA [64]. Most human and rodent circRNAs have predominantly brain-specific expression [54, 65–68]. In particular, it has been shown that circRNAs are predominantly localized in areas of neurons (axons and dendrites). Their level depends on the stage of development of synapses and homeostatic plasticity [69]. It is believed that the accumulation of circRNAs upon neuronal differentiation could result from the combined effect of augmented transcription of circRNA-producing genes and diverse decay rates of circRNAs and their linear counterparts [70]. The specific expression and stability of circRNAs allow them to be considered as potential biomarkers for various diseases [71].

6. Competitive endogenous RNAs

Relatively recently, it was shown that miRNA activity in the human cells can be regulated by the so-called sponge transcripts of competitive endogenous RNA (ceRNA). These transcripts compete with mRNA for binding to miRNA and diminished the effect of miRNA on the transcriptional and posttranscriptional levels of gene expression regulation [72, 73]. Long ncRNAs may act as ceRNAs in mammals. There are examples of pseudogenic and intergenic noncoding transcripts that can perform the functions of ceRNA [74]. One example is regulation of the expression of the tumor suppressor gene *PTEN* using the RNA of its pseudogene PTENP1. The 3′-terminal region of the pseudogenic RNA (PTENP1) is highly homologous to the corresponding 3′-terminal region of the mRNA of *PTEN*. Competitive binding of the 3′-terminal region of the PTENP1 pseudogenic RNA with miRNAs (miR-19b and miR20a) ensures stable transcription of *PTEN* and translation of its mRNA [75]. The expression level of PTENP1 is about 100 times higher than that of mRNA of *PTEN*. This provides a competitive advantage of PTENP1 for binding miRNAs and performing the functions of ceRNA [72]. Among the recent most important and interesting studies of the functioning of ncRNA in ischemia, it is worth mentioning the work of Li et al. [76]. Malat1 ncRNA acts as ceRNA for ULK2 when the endothelial cells of the brain capillaries are damaged. Malat1 acts as an endogenous sponge for miR-26b. This leads to an increase in the expression of ULK2 and contributes to the autophagy of the endothelial cells of the brain capillaries and to the survival of oxygen-glucose in the conditions of deprivation/reoxygenation (OGD/R). Xing et al. showed that miR-155 inhibition may play a protective role in ischemic stroke by S6K phosphorylation on the Rheb/mTOR pathway [77].

Effective ceRNAs should have multiple miRNA binding sites and a high level of expression or increased stability [73, 78]. Of particular interest are circRNAs, which have a covalently closed structure and are often formed from protein-coding genes during back-splicing [52, 58]. circRNAs are not exposed to exonucleases [51, 52], so they can more effectively act as ceRNAs because of their increased stability.

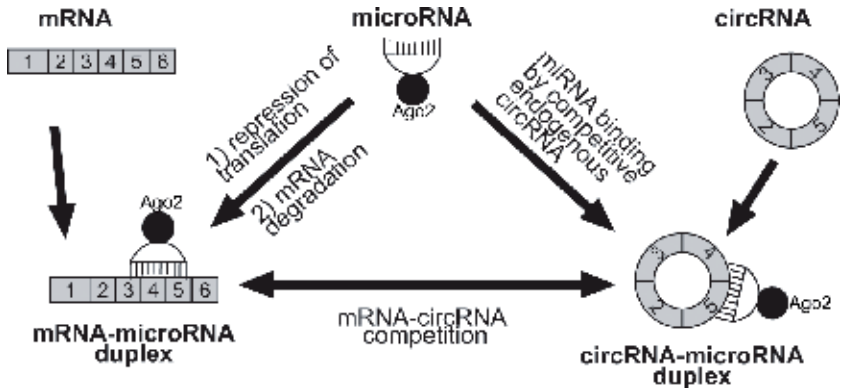

Figure 2.
Scheme of mRNA, miRNA, and circRNA interactions. Exons are shown as numbered blocs.

Currently, great attention is being paid to the function of circRNAs as miRNA sponges. CircRNA acting as ceRNA competes with mRNA for binding to miRNA and diminishes the effect of miRNA on transcriptional and posttranscriptional levels of regulation of gene expression [65, 79] (**Figure 2**). The function of several circRNAs as miRNA sponges has been investigated in various pathologies. In particular, the role of circRNA CIRs-7 in preventing models of neuropsychiatric disorders in mice is associated with its functioning as a ceRNA [79]. In addition, in Alzheimer disease [80] and various types of cancer [81–83], circRNA-miRNA-mRNA competition may be associated with regulation of pathogenesis.

7. The role of circRNA-miRNA-mRNA competition in ischemic conditions

The transcriptional profile and functional properties of circRNAs under conditions simulating brain ischemia have been investigated. Cell culture of HT22 hippocampal cells under conditions of OGD/R simulating damage during cerebral ischemia with reperfusion produced results consistent with the hypothesis that miRNA sponges are assigned to circRNA [84]. In this model, circRNA expression was associated with metabolic pathways related to apoptosis and immunity. In a tMCAO model, biological regulation, metabolism, cellular communication, and protein and nucleic acid binding were the main biological and molecular functions controlled by circRNAs, whose expression was changed during the day after occlusion [85]. Bioinformatics showed that 16 circRNAs contain binding sites for many miRNAs. In a mouse tMCAO model, microarrays detected a change in the expression of over a thousand circRNAs associated with signaling pathways regulating cell survival and death [86]. Moreover, Liu et al. predicted possible pathways of interactions between circRNA and miRNA that could provide information potentially elucidating the mechanisms of brain damage during stroke. We have investigated the expression of genes for glutamate metabotropic mGluR3 and mGluR5 receptors (*Grm3* and *Grm5*) in a tMCAO model [87]. These genes are important participants in the metabolic pathways associated with neuro-signaling. Rat *Grm3* and *Grm5* encode homologues for human and rodent circRNA. In the subcortical structures of rat brains containing a lesion, the level of such circRNAs is more stable than the corresponding mRNAs. Bioinformatics analysis revealed the distribution of miRNA binding sites along the mRNA molecules of human *GRM3* and *GRM5*, which are

homologous to the corresponding genes in rats. A sufficiently large number of binding sites are located inside the exons, which are also part of conservative circRNA. A functional role of circRNAs of the genes under study is implicated by ceRNA in the response of brain cells to ischemia. In an experimental ischemia-reperfusion model, we found numerous circRNAs that were differentially represented in the damage zone 24 h after occlusion. These circRNAs may be key modes for the regulation of the neurotransmission genetic response.

In a recent study, new important information was provided on the functioning of circRNA under ischemia conditions. Bai et al. showed that circRNA of DLGAP4 (circDLGAP4) functions as a miRNA sponge to diminish the activity of miR-143, which inhibits the expression of homologues of E6-AP C-terminal domain E3 ubiquitin ligase 1 [88]. The level of circDLGAP4 was significantly reduced in the plasma of patients with acute ischemic stroke and after tMCAO in mice. Upregulation of circDLGAP4 expression significantly reduced neurological deficit and reduced areas of infarction and damage to the blood-brain barrier in a mouse model of ischemia. Han et al. convincingly showed that circHECTD1 increases expression in the brain of mice after tMCAO, in human glioblastoma A172 cells under conditions of OGD/R, and in the blood of patients with acute ischemic stroke [89]. circHectd1 is involved in the regulation of the regenerative mechanisms of brain cells during ischemia. In particular, suppression of the expression circHectd1 was associated with a reduced infarction size in a mouse model of ischemia [89]. By interacting with MIR142, which negatively affects the mRNA level of the gene for 2,3,7,8-tetrachlorodibenzo-p-dioxin inducible poly [ADP-ribose] polymerase (TIPARP), circHECTD1 diminished the miRNA activity, with consequent circHECTD1-MIR142-TIPARP competition leading to the modulation of astrocyte activity through autophagy during cerebral ischemia.

8. Conclusion

The data presented in this review indicate that in addition to protein-coding mRNA, ncRNAs play an important role in the regulation of intracellular processes, both under normal conditions and in pathologies. An active study of the features of the functioning of ncRNAs in ischemia is of exceptional importance for the development of new strategies for neuroprotection and repair of nerve tissue and for the development of effective new drugs. circRNAs are a new class of RNAs that have enhanced resistance and preferential brain-specific expression. An analysis of circRNA-miRNA-mRNA interactions is an important component of any detailed study of the mechanisms of damage and regeneration in the case of pathological effects and the action of therapeutic agents, especially during the therapeutic window, when treatment is possible and most effective.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 8

lncRNAs in Hallmarks of Cancer and Clinical Applications

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Abstract

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides in length that, in general, do not appear to have protein-coding potential. lncRNAs act in gene regulation involved with several biological processes. Furthermore, lncRNAs have been associated with a significant number of cancers, suggesting a potential role in tumorigenesis and progression. For example, HOTAIR regulates proliferation processes and other lncRNAs like highly upregulated in liver cancer (HULC), H19, PTENP1, HEIH, and antisense noncoding RNA in the INK4 locus (ANRIL). Other lncRNAs as AFAP1-AS1 and lincRNA-p21 can interact with *BCL-2* and *TP53*, acting in apoptosis. Moreover, NORAD plays a vital role in genomic stability. Additionally, due to deregulated expression and high tissue specificity level, lncRNAs exhibit great potential as prognostic markers. In this chapter, we review the most highlighted lncRNAs acting in hallmarks of cancer and clinical application.

Keywords: cancer, hallmarks, lncRNAs, ncRNAs, tumor

1. Introduction

The landscape of human transcriptome is more complicated than was imagined. In the last decade, the technology of RNA sequencing reveals more than 100,000 different RNA molecules produced by mammalian organisms [1, 2], most of them, without protein-coding potential, named as noncoding RNAs (ncRNAs). These molecules called the attention for their multiple roles in cell physiology. NcRNAs are classified, by the size, as small (microRNAs 22~25 bp) or long, with more than 200 nucleotides [3]. Previously, it was considered that lncRNAs were "dark matter" or "transcriptional noise" of the human transcriptome, with no biological functions. Recently, lncRNAs were found in all the branches of the tree of life, and their amount and diversity are more correlated with organismal complexity than protein-coding genes [4].

The majority of lncRNAs is transcribed by RNA polymerase II, capped and polyadenylated with some lncRNAs being also spliced. They are described as noncoding RNAs. New studies have shown functional micropeptides derived from some of the lncRNAs [5]. Until now, 16,000 lncRNAs were identified in the human genome with approximately 30,000 distinct lncRNA transcripts according to the Encyclopedia of DNA Elements (ENCODE) Project Consortium (GENCODE release 30). This number continues to increase, mainly through sensitive RNA sequencing and advanced

bioinformatics pipelines. lncRNAs have a lower expression level than other RNAs, and they show specific expression in tissues [6, 7], cell types, and subcellular compartments [8]. lncRNAs are classified according to their relative position to protein-coding genes in a sense, antisense, bidirectional, intronic, and intergenic [9]. Also, lncRNAs can be regulated by well-established transcription factors and associated with epigenetic signatures that modify chromatin states, making the lncRNA loci more accessible in the cell [10].

The current knowledge about lncRNAs is essential to understand cell biology, especially in cancer cells. Cancer is a complex disease characterized by extreme genetic and epigenetic changes that can fundamentally alter cell homeostasis to promote uncontrolled cell growth. Emerging evidence suggests that lncRNAs are involved with cancer-associated phenotypes like resisting cell death, invasion, proliferation, gene deregulation, and genomic instability and evade growth suppressors [11]. lncRNAs also interact with transcriptional regulation of tumor suppressors or oncogenes [12, 13]. One example is lincRNA-p21 that acts as a repressor in p53-dependent transcriptional responses [12]. Alternatively, HOTAIR can increase metastasis in primary breast tumors and hepatocellular carcinomas [14].

lncRNAs can participate in gene regulation at transcriptional and posttranscriptional levels [15]. For example, related to epigenetic mechanisms, lncRNAs can recruit methyltransferases [16] and polycomb complex [17] to prevent DNA accessibility through histone modification. lncRNAs are also involved in several posttranscriptional processes, such as splicing and nuclear export, mRNAs localization and stability, and in protein translation process [18–22].

lncRNAs can serve as a molecular scaffold, enhancing the interactions between protein-protein, protein-RNA, and protein-DNA, by base complementarity or interaction by secondary structures [23]. Alternatively, lncRNAs can function as a decoy when they titrate transcription molecules and other proteins away from the target [24]. Additionally, lncRNAs can work as binding platforms regulating miRNAs competing with mRNAs for miRNA response elements, known as competitive endogenous [25].

The development of several discoveries about the role of lncRNAs, especially in cancer, highlighted the importance of gene regulation in cellular functions. The evidence that we show here supports the idea that lncRNAs have an essential role in tumorigenesis and are associated with several cellular processes. Here, we review the current knowledge about lncRNAs in hallmarks of cancer and their potential for clinical application.

2. lncRNAs act in hallmarks of cancer

The transformation of a regular cell into cancer involves several processes, including molecular and environmental alterations [26]. The healthy cells must acquire different abilities to change the cell physiology and dictate malignant growth. The hallmarks of cancer comprise the biological capabilities acquired during the multistep development of human tumors. These changes include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion/metastasis [27]. Additionally, genomic instability, inflammation, reprogramming of energy metabolism, and evading immune destruction were also included [28]. There are many lncRNAs well described associated with cancer [29], and, herein, we highlighted some lncRNAs with strong evidence for cancer process association and with molecular details, for exemple (**Figure 1**). The influence of those long noncoding RNAs in hallmarks of cancer is due to the regulation of different pathways.

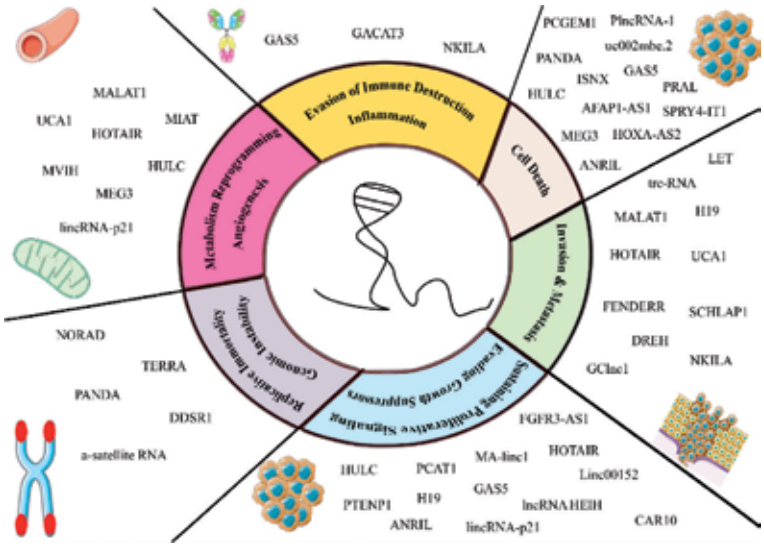

Figure 1.
lncRNAs in hallmarks of cancer.

lncRNAs have virtual association in all the hallmarks of cancer, some of them linked with more than one hallmark. For example, the expression of ANRIL, growth arrest-specific transcript 5 (GAS5), urothelial cancer-associated 1 (UCA1), and HULC regulates the resistance to cell death. Also, ANRIL, HULC, and H19 are associated with the evasion of growth suppressors, GAS5, and H19, sustaining cell proliferation. UCA1 and H19 are also associated with invasion and metastasis. Moreover, UCA1 is associated with metabolism reprogramming and GAS5 with an inflammatory process.

In more detail, ANRIL may inhibit apoptosis by silencing *KFL2* and *p21* genes, involved in cell proliferation. Moreover, regulating MET and MMP3 proteins that facilitate cell migration and invasion [30–34].

The lncRNA GAS5 can sustain proliferation by regulating glucocorticoid receptors (GR), with strong influences in cell growth and repression of anti-apoptosis genes. lncRNA binds to the DNA-binding domain of GR, competing with glucocorticoid response element [35].

lncRNA is also observed in cell cycle regulation, whereas it arrests cells in the G0/G1 phase. In stomach cancer, GAS5 binds to YBX1 to regulate *p21* expression, enhancing G1 arrest. In bladder cancer, GAS5 associates with CDK6 and reduces both *CDK6* mRNA and protein levels, resulting in the inhibition of cell proliferation. Besides, GAS5 acts in the immune response by NF-κB and ERK1/2 pathways. The knockdown of *GAS5* increases expression and secretion of interleukin-10 (*IL-10*) and vascular endothelial growth factor (*VEGF-A*), the essential cytokines involved in inflammation [36].

Due to its oncogenic regulatory potential, the lncRNA UCA1 regulates the proliferation and migration targeting the transcription factor KLF832 in pancreatic cancer. UCA1 also promotes invasion and metastasis through activation of *MMP14*, *FGFR1/ERK*, and *ZEB1/2-FSCN1* [37–40]. In hypoxic conditions, *HIF-1α* effectively activates UCA1 transcription by binding in the *UCA1* promoter, inducing proliferation, migration, invasion, and apoptosis resistance of cancer cells [41].

Several pathways regulate the switch of oxidative phosphorylation to aerobic glycolysis, such as the PI3K, AMPK, p53, and HIF-1 pathways. These pathways

are involved in metabolism deregulation associated with the Warburg effect, which consists in the special metabolism of glucose in the cytosol even in the presence of oxygen [42, 43], and UCA1 acts indirectly in genes involved in this process [44, 45].

HULC contributes to malignant phenotypes by, at least, three mechanisms. The first is regulating the expression of P18, an auxiliary protein of cell cycle that inhibits the tumor suppressors *CDK4* and *CDK6*. Also, hepatitis B virus infection activates the *HULC* promoter and induces cell cycle progression by downregulation of *P18* [46]. The other two mechanisms are related to angiogenesis. In breast and liver cancer, HULC sequesters miR-107 and regulates the transcription factor *E2F1*/*SPHK1* [47, 48]. In glioma, HULC acts through PI3K/Akt/mTOR signaling pathway, inducing *ESM-1*/*VEGF-A* and affecting vascular permeability and cell mobilization [49].

Some lncRNAs have been described more exclusively in only one hallmark, for example, telomeric repeat-containing RNA (TERRA) associated with replicative immortality. We know that cells have a limited survival rate that can be explained by telomere end loss, which generate a waste of genetic conservation, restricting the number of mitosis in a tissue. Thus, neoplastic cells can escape this telomere process with the help of telomerase. This enzyme can increase the size of telomere adding repeats on the edge 3′ in chromosomes [50]. TERRA comprises a heterogeneous class of lncRNAs transcribed from telomeric regions [51]. TERRA transcripts negatively regulate the activity of telomerase, acting as a tumor suppressor. Besides acting as telomere maintenance and genome stability, TERRA is also regulated by genes, such as *TP53* and *RB*, highlighting that TERRA transcripts can be crucially involved in tumorigenesis [52–54].

Another critical feature of neoplastic cells is in genomic instability, which can be related to defects in the DNA repair machinery. Activation of telomerase, following by individual DNA variations, activating proto-oncogenes or deactivating tumor suppressor genes, conferring a selective advantage on subclones of tumor cells, enabling their survival and outgrowth [28].

One lncRNA that is important in this tumor cell feature is NORAD. lncRNA protects the cells against aneuploidy by binding to *PUM1/PUM2* proteins and suppressing their binding to other targets, including those that maintain genomic stability [55, 56]. An alternative mechanism to define the relationship among NORAD and genomic stability is that a nuclear ribonucleoprotein complex, named NORAD-activated ribonucleoprotein complex 1 (NARC1), is joined by NORAD, recruiting proteins known to act as suppressors of genomic instability, such as topoisomerase I (*TOP1*), *ALYREF*, and the *PRPF19-CDC5L* [57].

The implication of lncRNAs in cancer development and progression has been proved in the last decade and indicates that those new class of RNAs has a great potential as biomarkers on cancer and a future perspective to targeted specific therapies.

3. Clinical application for lncRNAs

As we discussed in earlier topics, we have examples of lncRNAs that participate in essential processes in tumor development. Many of them have great potential as diagnostic/prognostic markers and therapeutic targets. A great example is the lncRNA prostate cancer antigen 3 (PCA3), already used as a molecular marker in prostate cancer [58, 59]. PCA3 is a prostate-specific lncRNA overexpressed in 95% of prostate cancer cases. PCA3 may be detected by *in vitro* nucleic acid amplification

in urine specimens, and the US Food and Drug Administration approved the test in 2012 [60].

In cases of suspicion of prostate cancer, the PCA3 test is recommended, based on prostate-specific antigen (PSA) level and post-digital rectal examination with biopsy results. PCA3 has a high expression in prostate cancer without any correlation to prostatic volume and other prostatic diseases. This feature makes a PCA3 an attractive biomarker [61], but some recent studies question the use of this isolated biomarker and propose that the test should be carried out in association with another test, like *TMPRSS2:ERG* quantification [58].

Although there are few lncRNAs used in medical practice, many are being discovered and tested. For example, a treatment protocol for triple-negative high-risk breast cancer predicted by the integrated mRNA-lncRNA signatures is initiated in the clinical trials evaluated, to validate the efficacy of lncRNA signature [62].

Also, in clinical trials, there is an early phase study to evaluate the HOTAIR as a potential lncRNA biomarker in thyroid cancer. Many lncRNAs have a different expression in tumors when compared to healthy tissues and are strongly associated with clinical parameters, making them a candidate for tumor markers or even therapeutic targets [63].

A study found the downregulation of expression of downregulated in liver cancer (DILC) in colorectal cancer tissues compared to their adjacent healthy tissues and the normal colorectal tissues. The downregulation of DILC was associated with aggressive clinical characteristics, including depth of invasion and advanced TNM stage, and the lower expression of DILC was associated with more reduced survival and disease-free survival. With multivariate analyses, the authors confirmed that the expression of DILC was an independent prognostic factor in colorectal cancer [64]. Most of lncRNA papers characterize biomarkers that are specific to one type of cancer, such as those cited above. However, some lncRNAs are found differentially expressed in several types of cancer compared with healthy tissues, like the loc285194 lncRNA [65].

In addition to the association of lncRNAs with stage and prognosis, their association with drug resistance is also possible [66]. Several works have tried to reallocate the lncRNAs in the mechanism of resistance to the primary drugs used in the treatment of cancer. For example, Campos-Parra et al. [67] have identified several lncRNAs that participate in resistance mechanisms to several drugs utilized in the therapy of breast cancer. Most studies with lncRNAs measure their expression in tissues, but it is possible to detect and quantify their presence in other types of samples, like whole blood, plasma, urine, gastric acid, and saliva [68].

Within the use of lncRNAs as biomarkers in cancer, these molecules have applications as therapeutic targets in the development of new treatments and drugs. New therapeutic strategies are already focusing on noncoding RNAs, such as silencing via small interfering RNA (siRNA), an antisense oligonucleotide (ASO)-based strategies and other molecular inhibitors further modulating lncRNA expression by gene editing [69, 70]. An experimental model that aims to modulate lncRNAs in cancer cells is the use of siRNAs, which can decrease the amount of a target lncRNA, since they are complementary molecules to the sequence of lncRNA, promoting lncRNA binding and subsequent degradation. Although this methodology may be functional in many studies, some lncRNAs are not efficiently reduced by siRNA [70]. This methodology is efficient for cytoplasmic lncRNAs since the siRNA mechanism is located predominantly in the cytoplasm. In this case, siRNA does not silence nuclear lncRNAs [11, 71].

Another mechanism used to block lncRNAs activity relies on the ability of lncRNAs to bend and create secondary structures and on the ability of protein

interactions in lncRNAs. RNAi molecule can compete with the protein for the binding site; or when it binds to the target lncRNA, it changes the structure of the RNA, disrupting the binding site of the protein [72, 73].

For nuclear lncRNAs, an alternative strategy is the use of antisense oligonucleotides, which function predominantly in the nucleus [71]. ASOs modulate gene expression by inducing ribonuclease H cleavage of the duplex DNA-RNA. A limitation of the ASO and siRNA strategies is the possibility of non-specific targets, as well as the inconvenience of incomplete knockdown and transient modulation [74]. One method that has shed light on ncRNA-based cancer therapy and solves the problem of target specificity is genome editing by clustered regularly interspaced short palindromic repeats-associated endonuclease 9 (CRISPR/Cas9). The Cas9 nuclease can act guided to generate site-specific DNA cleavage in the genome, by an optimized equivalent single-guide RNA (sgRNA) [75]. That is, it can delete lncRNA genes or introduce RNA-destabilizing elements into their locus.

A limitation to apply CRISPR/Cas9 system to noncoding genes is that tiny indels may not necessarily generate a functional loss of a specific noncoding gene and the most protocols can perform small point mutations; plus not all lncRNAs CRISPR can be applied. Another limitation is that, although it is more specific than other systems, this technique may still have off-target effects [76, 77].

Currently, many studies performed lncRNA modulation technologies, both *in vitro* and *in vivo*. Moreover, while these models may resemble reality, the clinical use of modulation technology has a barrier that still needs to be broken: an efficient delivery to the target. There are some techniques for delivering lncRNA modulation systems to live cells, based on viral and non-viral methods, but both ways have limitations and problems to be solved before the utilization in clinical practice.

The main advantage of viral vectors is their innate ability to efficiently transfer the genetic material into the cell and the possibility of infecting specific cells. However, this technique also has a significant disadvantage, which is relatively high immunogenicity and toxicity. The possibility of generating an immune response is the main challenge for the use of this tool [78, 79].

Non-viral vectors are becoming recognized as an alternative to the immunogenicity of viral vectors, although their transfection capabilities usually do not reach such levels. Besides its main strengths are the low capacity to generate an immune response and the relatively easy and inexpensive synthesis with large-scale production and safety, which make them very attractive delivery systems for *in vivo* application. However, the target specification still needs to be better developed [79].

In order to choose the best delivery system, it is necessary to consider the type of cell/tissue, since some tissues are more accessible than others. When the target of therapy is a difficult-to-access tissue, some strategies may improve delivery efficiencies, such as binding to specific targeting elements like antibodies, carbohydrates, and synthetic peptides. These molecules also have their advantages and limitations; for example, although antibodies have a high recognition specificity and interaction with different receptors on target cells, they may be immunogenic and chemically unstable. Peptides and carbohydrates, on the other hand, demonstrate low immunogenicity, but the binding affinity to the target is lower [79, 80].

All the techniques cited in this topic have their limitations. Although *in vitro* studies present satisfactory results that have the potential to use in clinical aspects. However, these techniques require better improvements, especially in the delivery of drugs in specific targets. Despite this, our knowledge of lncRNAs linked with clinical applications creates hope for the development of better biomarkers and therapeutic targets. In **Table 1**, we list the main lncRNAs that have excellent potential for biomarker and therapeutic target.

	Cancer															
lncRNAs	**Head/ neck**	**Gastric**	**Lung**	**Breast**	**Pancreas**	**Liver**	**Colon**	**Uterine**	**Ovarian**	**Osteosarcoma**	**Prostate**	**Bladder**	**Renal**	**SNC**	**Leukemia**	**Refs.**
AFAP1-AS1	x	x	x	x	x	x	x		x			x	x			[81]
ANRIL	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	[82, 83]
CCAT1/ CCAT2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	[84]
CRNDE		x	x	x	x	x	x	x	x			x	x	x	x	[85]
DANCR		x	x	x		x	x			x	x		x			[85]
GAS5		x	x	x	x	x	x	x	x	x	x	x	x	x	x	[86]
H19	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	[87]
HOTAIR	x	x	x	x	x	x	x	x	x	x	x	x	x	x	X	[88]
HULC	x	x		x	x	x	x	x	x	x	x	x		x	x	[89]
LINC00152	x	x	x	x	x	x	x		x			x	x	x		[90]
LincRNA-p21		x	x			x	x				x				x	[91]
Linc-RoR	x		x	x	x	x	x		x					x		[92]
MALAT1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	[93]
MALAT2		x														[94]
MEG3	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	[95]
MIAT		x	x	x	x		x		x		x		x		x	[96]
NEAT1	x	x	x	x	x	x	x	x	x	x	x		x	x	x	[97]
PANDA		x	x	x	x	x	x			x			x			[98]

	Cancer															
lncRNAs	**Head/ neck**	**Gastric**	**Lung**	**Breast**	**Pancreas**	**Liver**	**Colon**	**Uterine**	**Ovarian**	**Osteosarcoma**	**Prostate**	**Bladder**	**Renal**	**SNC**	**Leukemia**	**Refs.**
PCA3									x		x					[99, 100]
PCAT-1	x	x	x	x		x	x	x		x	x	x		x		[101, 102]
TUG1	x	x	x	x		x	x	x	x	x	x	x	x	x	x	[103, 104]
XIST	x	x	x	x		x	x	x	x	x	x	x		x	x	[105]

Table 1.
lncRNAs with potential to be used as biomarkers, in several types of cancer.

4. Conclusion

The vast number of studies describing lncRNAs associated with several tumor types and regulating several processes of cancer cells is shown here. The great advance in RNA sequencing technology allows us to identify new molecules and characterized better lncRNAs. From the discovery of these molecules, in the beginning, they appeared to have no important functions; however, today many researches in this area propose that more information about these molecules may help us understand numerous characters of tumor cells that are still unknown. Some lncRNAs are associated with several hallmarks of cancer demonstrating the importance of these molecules in the mechanism of disease, like MALAT and HOTAIR. Other are already utilized as biomarker in prostate cancer like PCA3. Considering the challenges for *in vivo* experimental designs, lncRNAs continue to be promising as biomarkers and potential therapeutic targets.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

The Function of lncRNAs as Epigenetic Regulators

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Abstract

Recently, the non-coding RNAs (ncRNAs) have been classified in different categories, and its importance in regulating different cellular processes has been unravelled. The long non-coding RNAs (lncRNAs) can interact with DNA, other RNAs and proteins, including epigenetic modifiers. Some lncRNAs are related to genomic imprinting and are associated with chromatin-modifying complexes that can regulate gene transcription. It is well established that cancer cells have different epigenetic alterations and some of these modifications are associated with lncRNAs. Studies of cancer-associated lncRNAs have defined its function in the process of tumorigenesis, its impact on cell proliferation, cellular signalling, angiogenesis and metastasis. Therefore, having a better knowledge of their role might contribute to a better understanding of the diseases. In this chapter, we will discuss about lncRNA classification and functions, epigenetic marks and how they can guide transcription. Nevertheless, we will discuss how these mechanisms can interact and guide gene expression, as well as recently findings of dysregulation of lncRNAs in cancer.

Keywords: epigenetics, lncRNAs, DNA methylation, histone modifications, cancer

1. An overview

The patterns of gene expression of a cell are altered throughout its lifetime, and these changes occur as a response to different stimuli. For example, during the differentiation stage of an embryonic cell, a group of active genes dictates the cell fate, while after differentiation those genes are silenced since they are no longer needed for that task. In this manner, shifts in gene expression may occur within different mechanisms. However, the most important alterations occur in the epigenome level. The epigenome is dynamic, being constantly altered by different chemical modifications such as DNA methylation, histone modifications, nucleosome positioning and chromatin remodelling. Those changes make the DNA sequences more or less accessible to the transcriptional machinery, altering gene expression in a cell. The regulation of these mechanisms is complex and involves enzymes, proteins and RNA molecules. In the last years, it has been shown that long non-coding RNAs are also responsible for regulating transcription and they can do it in three different levels: pre-transcriptional, transcriptional and post-transcriptional. Besides these regulatory functions, they can also alter gene expression by altering the epigenome. Epigenomic alterations may alter gene expression and are related to the onset of many diseases and have been reported

to be crucial to cancer development. In cancer cells, tumour suppressor genes are silenced, and oncogenes are overexpressed, and these alterations can be driven by epigenetic modifications regulated by lncRNAs. In this chapter, we will discuss about lncRNAs and epigenetic marks. Nevertheless, we will approach how they can interact with each other to regulate gene expression and their role in cancer.

2. Long non-coding RNA

The discovery of ribonucleic acid (RNA) molecules that do not code for proteins has drastically altered our understanding of molecular biology. Until recent years, the central dogma of biology described the DNA as the source of information from which an encoded gene was transcribed into a RNA strand and after it would be translated into a protein. However, in the human genome, approximately 93% of the DNA can be transcribed into RNA, but only around 2% of that would be protein-coding messenger RNA (mRNA). The remaining transcripts were therefore classified as transcriptional noise. With the rapid advance in molecular biology techniques, including large-scale sequencing, it is now known that many thousands of non-coding transcripts are encoded by the genome. These transcripts represent more than 70% of the genome, and they are transcribed into non-coding RNA (ncRNA) molecules. This knowledge opens up a completely new universe, and currently more than 40 types of non-coding RNAs have already been described. Among the most well-known ncRNAs are the transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs) and, more recently, the long non-coding RNAs (lncRNAs), which are the focus of this chapter.

2.1 Characteristics of lncRNAs

The lncRNAs, as the name suggests, are long RNA transcripts, with more than 200 nucleotides which are not translated into protein. The first long non-coding RNA was described in 1971, in a viroid plant pathogen; however, the first time a long non-coding RNA had its regulatory role described was only in the early 1990s, when the scientific community discovered transcripts involved in epigenetic mechanisms. One of the first identified lncRNAs was *H19* (imprinted maternally expressed transcript), firstly described in mouse [1]. Shortly after, X-inactive-specific transcript (*XIST*) was suggested to be a functional lncRNA, with a structural role in the cell nucleus. lncRNAs present relatively low levels of evolutionary conservation and originated from genes that are usually shorter than protein coding genes, with fewer exons [2]. However, they present similar features with protein-coding transcripts, as they are typically transcribed by RNA polymerase II and can be capped, polyadenylated and spliced [3].

lncRNAs can be transcribed from both mitochondrial and nuclear genomes, in sense and antisense directions. Also, strong evidence suggest that the post-transcriptional cleavage of the lncRNAs might be the substrate to smaller RNAs, as they can act as precursors to smaller molecules such as miRNAs, piRNAs, siRNAs and others.

One of the main characteristics of lncRNAs is their ability to fold themselves into secondary or higher thermodynamically stable structures, which are highly conserved [4]. The longer the lncRNA, the higher is the probability of it to form those structures. Because lncRNAs have the capacity to bind through bonds, they are able to fold themselves into structures such as double-helix, hairpins, loops, pseudonodes and more. Due to these complex structures, they are able to bind to

more than one molecule at a time, regulating gene expression at different levels through RNA-protein, RNA-DNA and RNA-RNA complexes.

lncRNAs can be expressed in different cell compartments, and their function is directly related to their location. A substantial proportion of lncRNAs are exclusively expressed in the nucleus. Nuclear lncRNAs often play a role in modulating gene expression by recruiting transcription factors, by remodelling or by modifying the chromatin or by RNA-DNA triplex formation [5]. Other lncRNAs must be transported to the cytoplasm, where they may interfere in post-translational modification, participating in protein localization processes, mRNA translation and stability [6]. Not only that, lncRNAs may also be transported to distant regions through extracellular vesicles, such as exosomes and microvesicles; however, the mechanisms which regulate the expression of these circulating lncRNAs are still not well understood [7, 8].

2.2 The lncRNA classification

Because lncRNAs are a very diverse class of molecules, there is still a debate on which would be the best way to classify them into categories, as the classification can infer information regarding their localization, regulatory function, biological function and so on. The simplest method of lncRNA classification is related to their size [9, 10]: small lncRNA (200–950 nt), medium lncRNA (950–4800 nt) and large lncRNA (>4800 nt). According to this classification, most human lncRNAs fall into the small-lncRNA group (58%).

Another classification by the catalogue of human lncRNAs, made in 2012, defines five biotypes of lncRNAs according to the GENCODE (**Figure 1**):

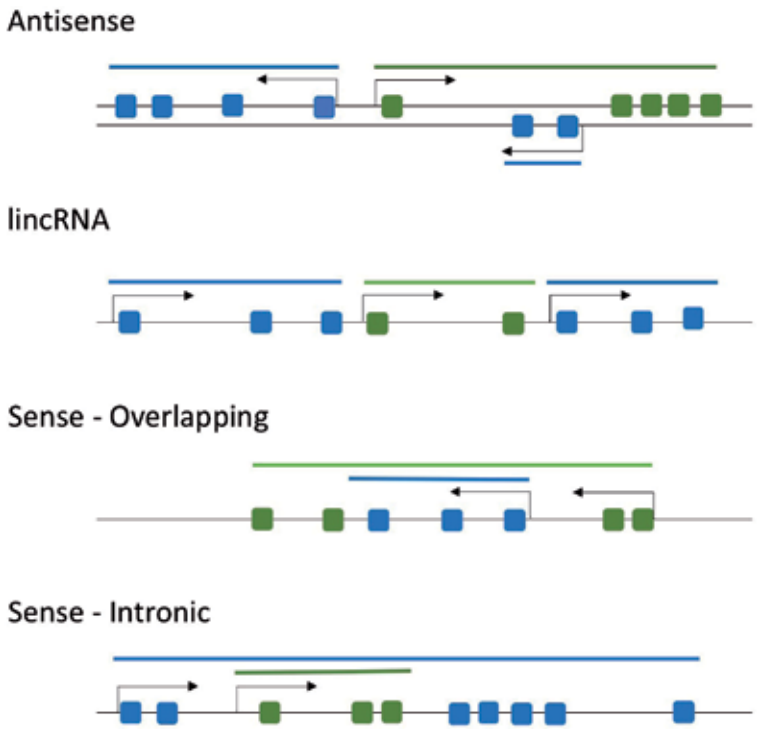

Figure 1.
lncRNA biotype classification. The image shows the lncRNA biotypes: antisense, lincRNA, sense overlapping *and* sense intronic. *Blue squares represent gene coding exons, and green squares represent lncRNA exons; directions of transcription are indicated by arrows.*

1. *Antisense*: located on the opposite strand from protein-coding genes, containing an intersection with some exons or introns or published evidence of antisense gene regulation

2. Long intergenic non-coding RNA (*lincRNA*): transcripts originated from intergenic loci; that is, located between two protein-coding genes

3. *Sense overlapping*: transcripts containing 'protein-coding gene sequences in their introns', located in the same strand as them and that do not overlap with any exon

4. *Sense intronic*: located within introns of a protein-coding gene and with no intersection with exons

5. *Processed transcripts*: locus where all transcripts have no open reading frame (ORF) and do not fit in any of the above biotypes, due to their complex structure

It is important to note that even though this classification is widely used, additional biotypes of lncRNAs are also described in GENCODE, such as *macro lncRNAs*, *pseudogenes*, 3 *prime overlapping ncRNA* and *bidirectional promoter lncRNA*, among others. Alternatively, lncRNAs can be categorized according to the molecular mechanisms that may be involved in their functions into five archetypes:

1. *Signal archetype*: acts as a molecular signal or indicator of transcriptional activity

2. *Decoy archetype*: binds and captures other molecules, such as proteins and other regulatory RNAs, inhibiting its function

3. *Guide archetype*: binds and recruits ribonucleoprotein complexes to specific targets

4. *Scaffold archetype*: plays a structural role as a platform upon which other molecules can bind simultaneously, assembling a complex

5. *Enhancer archetype*: controls higher-order chromosomal looping

Nevertheless, they can also be classified based on the region of the DNA sequence impacted by the lncRNA. lncRNAs can influence a neighbouring gene on the same allele from which it is transcribed (cis) or in further genomic region and other chromosomes (trans):

1. *Cis-lncRNAs*: lncRNAs regulating the expression of genes in close genomic proximity. They may be transcribed from promoter regions and may interfere in the transcription activity of neighbouring genes. They may act by recruiting transcription factors, inducing chromatin remodelling or forming DNA-RNA triplex structure.

One of the most well-known examples of cis-acting lncRNA is XIST. In mammals, the females have two copies of the X chromosome (XX), while the males have only one (XY). This unbalance could result in a variety of problems associated with the expression of genes from chromosome X. However, the lncRNA X-inactive-specific transcript (XIST) is expressed from the X-inactivation centre (XIC) locus

and acts in *cis* along the whole chromosome from which it is transcribed, resulting in this chromosome silencing (**Figure 2A**).

2. *Trans-lncRNAs*: lncRNAs may also function in trans-mode by influencing distant gene loci. In such case, they may also act as chromatin modification complexes, as well as affect transcription by binding to transcription elongation factors or to RNA polymerases.

Another well-studied lncRNA, HOX transcript antisense RNA (HOTAIR), also recruits the polycomb repressive complex 2 (PRC2) to inactivate gene expression. However, in this case, HOTAIR is transcribed from the HoxC locus on chromosome 12 and represses the HoxD locus on chromosome 2, therefore acting in trans (**Figure 2B**).

2.3 Gene expression regulation mediated by lncRNAs

Long non-coding RNAs are functionally very diverse and are involved in numerous biological roles, such as imprinting, epigenetic regulation, apoptosis and cell cycle control, transcriptional and translational regulation, splicing, cell development and differentiation and ageing. They have been described in almost every stage of gene regulation: pre-transcriptionally, guiding proteins to specific areas of the genome; as decoys, keeping proteins away from chromatin; by epigenetic alterations, by histone modifications or DNA methylation [3]; transcriptionally, modulating the transcriptional process; and post-transcriptionally, by RNA-RNA interactions.

2.3.1 Pre-transcriptional regulation

It is well understood that, in eukaryotic cells, the DNA is packaged in the chromatin and the availability of those structures to the transcriptional machinery has a strong influence in the gene expression, as the transcriptional factors must have access to the chromatin in order to transcribe the encoded gene. The lncRNAs can regulate this expression in the nucleus by associating and recruiting chromatin-remodelling factors. The examples of *XIST* and *HOTAIR* mentioned above illustrate this pre-transcription regulation, as in both examples, *XIST* and *HOTAIR* recruit the PRC2 to interact and repress the expression of genes through the K27 trimethylation in H3 histones [11].

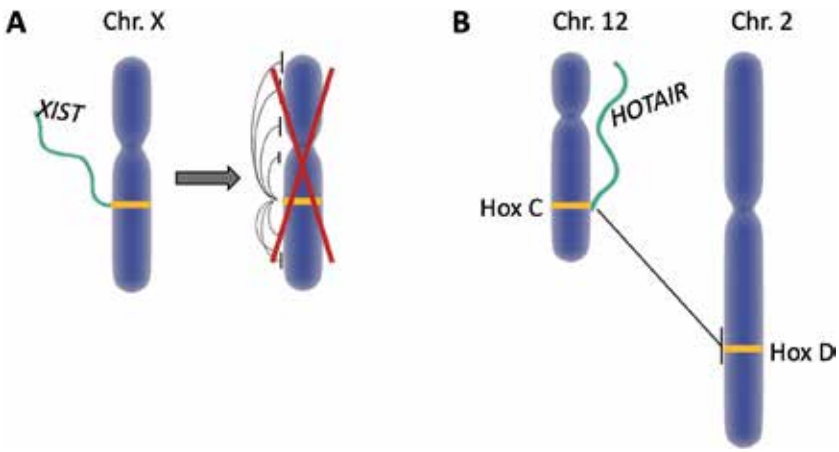

Figure 2.
Cis and trans regulation of gene expression by lncRNAs. (A) Inactivation of the X chromosome by the lncRNA XIST, cis*-acting in the same chromosome; (B) lncRNA* HOTAIR *is transcribed in chromosome 12 and acts in chromosome 2 blocking gene expression.*

2.3.2 Transcriptional regulation

The lncRNAs located in the cell nucleus can participate in the transcription regulation and are divided into two different categories, according to its function: promoter-associated lncRNAs (plncRNAs) and enhancer-like lncRNAs (elncRNAs).

The plncRNAs may act as inhibitor or promoter of gene expression. For example, the dihydrofolate reductase (DHFR) gene contains two promoters, with the downstream major promoter being responsible for 99% of the RNA transcription. However, the transcription from the upstream minor promoter generates a lncRNA transcript that interacts both with the major promoter and the transcription factor IIB (TFIIB), forming a triplex structure between DNA and RNA, which inhibits the binding of TFIIB with the major promoter causing the inhibition of DHFR gene expression [12].

Another example is the lncRNA *Evf-2*, which acts on cis as a distal-less homeobox 2 (DLX2) protein coactivator, creating a stable complex that activates the transcription of the adjacent locus distal-less homeobox 5/6 (DLx5/6) [13].

2.3.3 Post-transcription regulation

lncRNAs may act upon the post-transcription regulation in different ways. In the nucleus or in the cytoplasm, they can alter the mRNA stability, splicing or even cellular compartmental distribution. The zinc finger E-box binding homeobox 2 (*ZEB2*) gene is transcribed in the DNA sense strand of chromosome 2. On its opposite strand, the lncRNA ZEB2 natural antisense transcript (*ZEB2NAT*) can mask the splicing site of an intron in the 5'UTR region of the ZEB2 mRNA by complementary binding. This interaction avoids the spliceosome attachment, allowing the expression of the ZEB2 protein [14].

In another case, in the cytoplasm, lncRNAs can also act as miRNA 'sponges', when the mRNA and the lncRNA have similar miRNA binding sites. Therefore, when the lncRNA binds to the miRNA, the miRNA is no longer available for mRNA attachment, increasing the concentration of the mRNA in the cytoplasm. The phosphatase and tensin homolog (*PTEN*) gene and the lncRNA phosphatase and tensin homolog pseudogene-1 (*PTEN1*) illustrate this action mode. They both share similar nucleotide sequences. The miRNA families miR-17, miR-21, miR-214, miR-19 and miR-26 contain in their 3'UTR region a perfect match binding sequence to *PTEN1*. Therefore, the lncRNA acts as bait and sequesters the miRNAs that would otherwise bind to PTEN mRNA [15].

3. Epigenetic regulatory functions of lncRNAs

As mentioned above, lncRNAs can interact with epigenetic mechanisms altering gene expression. But what are those mechanisms? Epigenetics refers to chemical modifications of the chromatin, without alterations in the nucleotide sequence, which are transmitted throughout mitosis and play a key role in gene expression regulation and genomic stability. Among epigenetic mechanisms are DNA methylation, post-translational histone modifications, nucleosome positioning and chromatin accessibility. All these epigenetic marks interact with each other in a dynamic way altering patterns of gene expression along embryogenesis, throughout lifetime by environmental stimulus and in the transition health-disease stage.

3.1 Chromatin structure and epigenetic marks

Different states of chromatin organization allow different transcriptional factors to bind to DNA and regulate gene expression. This interaction between transcriptional factors and DNA is only possible due to a chromatin open state, known as euchromatin. The inactive form of chromatin is called heterochromatin and is characterized by epigenetic marks that make this structure highly condensed. The negatively charged phosphate backbone of DNA is wrapped around an octamer of histone proteins, forming the nucleosome. The histone octamer is made out of a pair of each histone protein H2A, H2B, H3 and H4, and these structures are linked by the histone H1 protein. The epigenetic marks are written by enzymes that can add methyl groups to cytosine in the genomic DNA (DNA methyltransferases; DNMTs) and acetyl and methyl groups to amino acid residues of histone proteins (histones acetylases and methylases; HATs and HMTs, respectively). These marks can be interpreted by proteins that bind to methylated DNA, such as methyl CpG-binding domain (MBDs) proteins, and to modified histones, as proteins containing chromo- and bromodomains. Epigenetic marks can also be further erased by enzymes, such as histone deacetylases and demethylases (HDACs and HDMs), respectively, as well as the family of ten-eleven translocation (TETs), which oxidases the 5-methylcytosine.

3.1.1 DNA methylation

DNA methylation is the process in which a methyl group is added to the fifth carbon of a cytosine resulting in 5-methylcytosine (5mC). The methyl group is donated from S-adenosyl methionine (SAM), and this reaction is catalyzed by DNMT enzymes. In mammals, the methylation process usually occurs in the CpG dinucleotide context, but is not limited to this condition. There are five types of DNMTs in mammals: DNMT1, DNMT3A, DNMT3B, DNMT2 and DNMT3L. During the replication process of a cell, DNMT1 recognizes 5mC in the hemimethylated DNA and is responsible for the reestablishment of the methylation patterns in the daughter strand, which makes the epigenetic marks heritable during cell division. For this reason, DNMT1 can be called as a maintenance DNA methyltransferase. DNMT3A and DNMT3B do not prefer hemimethylated DNA, being able to establish de novo (new) methylation patterns especially during cell differentiation in embryogenesis. DNMT3L does not have catalytic activity but can act as a cofactor of DNMT3A to improve its affinity to DNA and further improve the methylation process. Despite the fact that DNMT2 has no strong catalytic activity to DNA, it was recently showed that this enzyme is capable of adding methyl groups to tRNA.

The methylation pattern of the human genome is said to be bimodal; in other words, that means that some regions have a low methylation level, as transcription start sites (TSS) with high content of CpGs and imprinting control regions (ICR), although the other CpG sites in the genome are kept methylated. The CpG islands (CGI) are known as CpG-rich regions within the DNA sequence, and they can be related with gene expression. For example, it is well established that TSS with high methylation of CGI are related with a long-term silencing. Methylation of promoter regions is associated with transcriptional repression, while the non-methylated promoters are associated with active transcription. Methylated DNA acts as a physical barrier for transcription factors and, additionally, recruits MBDs that also act as repression complexes. However, methylation at the gene body is related with active transcribed genes; for example, when the methyl-CpG binding protein 2 (MeCP2) identifies methylated exons, it may regulate alternative splicing. Furthermore, transposable elements can also be silenced by DNA methylation, contributing to genome stability.

Demethylation of the genome is an important process during the pre-implantation phase and germ cell development. The enzymes responsible for this multistep process are the TET proteins capable of oxidizing 5mC, leading to the loss of DNA methylation. DNMTs and TETs can dynamically regulate transcription repression and activation across the genome. DNA methylation is a stable mark for gene silencing, but is not the only one.

Interactions between DNA methylation marks and lncRNAs may happen in order to control gene expression. As an example, one of TET proteins, TET2, has been shown to negatively regulate the expression of the lncRNA antisense ncRNA in the *INK4* locus (*ANRIL*) through its binding affinity with the lncRNA promoter, regulating not only its expression but the expression of its downstream genes as well. In addition, another negative correlation has been observed between TET2 and the methylation pattern of the lncRNA maternally expressed gene 3 (*MEG3*) promoter. This is particularly interesting considering that *MEG*3 promoter methylation has been associated with poor survival in myeloid malignancies and the same trend has been observed in breast, cervical, colon, liver, lung and prostate cancer cell lines [16, 17]. As mentioned previously, lncRNA *XIST* is responsible for X-chromosome inactivation. However, the active X chromosome expresses the lncRNA *TSIX*, antisense to *XIST* through its 5′ end, that interacts with PRC2 and enhances DNA hypermethylation through DNMT3A, resulting in *XIST* silencing. Conversely, neither PRC2 nor DNMT3A is essential for *XIST* expression, which implies that more than one pathway acts in the *TSIX/XIST* regulation complex. Another lncRNA that interferes in DNA methylation is *H19* that was discovered to interact with methyl-CpG-binding domain protein 1 (MBD1), which recruits different enzymes that are related to gene silencing. Besides that, it was also identified to bind with S-adenosyl homocysteine hydrolase (SAHH) and hydrolysis S-adenosyl homocysteine (SAH) and block DNA methylation by DNMT3B [18, 19]. It is also known that *H19* is part of an imprinted gene network that is only expressed from maternal allele. Interestingly, the silencing of the paternal allele is due to CpG methylation of the *H19* promoter. Genomic imprinting is an epigenetic mechanism that restricts the expression of a gene to only one allele, either maternal or paternal. The lncRNA *Nespas* belongs to the *Gnas* imprinted cluster. *Nespas* transcription through the paternal allele is inversely correlated with *Nesp*. *Nesp* promoter in this context is encountered methylated due to *Nespas* ability to recruit KDM1B (histone demethylase 1B) and therefore demethylate lysine 4 of histone 3 [20].

3.1.2 Histone modifications and chromatin remodellers

Other epigenetic marks are the post-translational modifications of histone proteins. Histone modifications are more plastic and can lead to repression and activation of transcription, depending on the modification and the residue to be modified. The main targets of modifications are the amino acids located in the N-terminal portion of histone tails that shape the nucleosome. Lysine is the amino acid that can accommodate more combinations of modifications; however, other histone residues can also be modified, such as arginine, serine, threonine and tyrosine. Some of the most studied modifications are methylation and acetylation, but modifications as phosphorylation, ubiquitination and ADP-ribosylation also occur. Histone acetylation is a mark related to active gene expression. The acetyl group neutralizes the positive charge of histones, and due to this new charge, since the DNA has a negative backbone, the DNA-histone interactions are lowered, and this repulsion makes this loosen structure more accessible to the transcription machinery. An example of acetylation controlling gene expression is the acetylation of histone H3: high-acetylated genome regions are related to highly expressed

genes, while low acetylation is present in silenced genomic regions. While histone acetylation profiles can indicate active or repressive regions of the genome, the effect of histone methylation depends on the residue where it occurs and their degree of methylation, whether once, twice or three times methylated (mono, di or tri, respectively). Just like DNA methylation, the methyl group added in histone residues is donated from SAM; however, this reaction is catalyzed by HMTs. The most commonly methylated histones are H3 and H4, and some marks are already related with different states of chromatin accessibility. For example, trimethylation of lysine 9 in histone H3 (H3K9me3) and trimethylation of lysine 20 in histone H4 (H4K20me3) are associated with inactive regions of the genome, while mono-methylation of lysine 9 in histone H3 (H3K9me1) and mono-methylation of lysine 20 in histone H4 (H4K20me1) are related with active genome regions. The mechanism that erases those marks are guided by different enzymes, including HDACs and HDMs, responsible for removing, respectively, acetyl and methyl groups from the histones. The process of histone modification and its cooperation with DNA methylation makes the epigenome very dynamic and plastic during different stages of cell development.

The nucleosome occupancy in the chromatin and the changes that may occur in this structure are important to guide gene expression. Chromatin remodellers use the energy of adenosine triphosphate (ATP) hydrolysis to alter the nucleosome position. These remodellers are multi-protein complexes that can modulate nucleosome occupancy with the help of transcriptional cofactors, pioneer factors and non-coding RNAs. The coordinated mechanism of nucleosome modifications and DNA accessibility can occur in at least four ways: (1) sliding of the nucleosome: supported by imitation switch (ISWI), chromodomain-helicase-DNA (CHD) binding and chromatin remodeller families; it is characterized by the transfer of the nucleosome to a new position but without changing its chromatin region; (2) nucleosome ejection: mediated by imitation switch/sucrose non-fermentable (ISWI/SNF) remodeller family; the nucleosome is taken out of its position, and the DNA in that area becomes accessible; (3) nucleosome-selective dimer removal: mediated by SWI/SNF family, destabilizes the nucleosome since it leaves only two tetrameters within the nucleosome; (4) nucleosome histone replacement: histone variants such as H2A is replaced by H2A.Z, mediated by the inositol (INO)-requiring family. Therefore, all those modifications are important to maintain the dynamic regulation of gene expression. The histone-modifying complexes (PRC1/PRC2) and the trithorax group/mixed-lineage leukaemia (MLL) protein complexes (TrxG/MLL) are important players on the control of chromatin structure and, therefore, are important regulators of gene activity. PRC2 promotes the methylation of histone H3 at lysine 27 (H3K27me3), inhibiting gene activity, while on the other hand, TrxG/MLL stimulates the methylation of histone H3 at lysine 4 (H3K4me3), triggering gene expression.

In the literature, many lncRNAs have been reported to alter gene expression through histone modifications and chromatin remodelling. lncRNAs can interact with chromatin remodellers in order to promote or repress gene expression according to its genomic regions. For instance, they can interact with multiple regulatory complexes at the same time and bind with different enzymes that can change chromatin marks, such as DNA methylation, histone modifications and nucleosome modifications. lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) can act as a molecular scaffold, and it is related to both gene silencing and gene activation. The lncRNA can bind to PRC2 complex, resulting in the methylation of histone H3 in lysine 27 (H3K27), which is a repression mark of gene expression. In other context, it can interact with nuclear speckles, structures that are thought to be associated with splicing and processing of pre-mRNA, coordinating gene

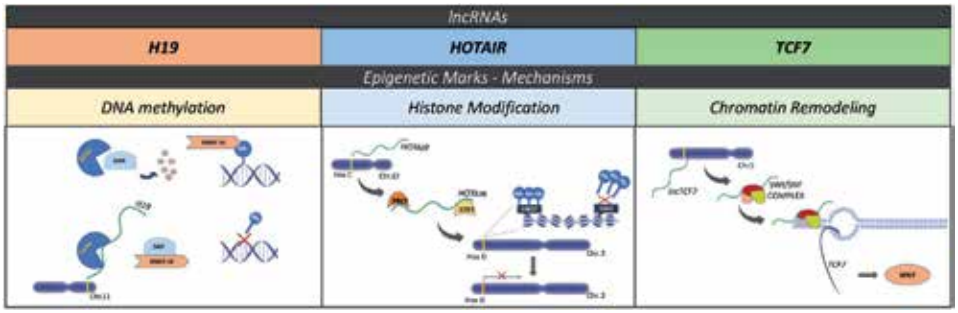

Figure 3.
lncRNAs as epigenetic regulators. The image shows the lncRNA H19 *that binds with SAHH and inhibits SAH hydrolysis blocking DNA methylation by DNMT3B; lncRNA* HOTAIR *silencing expression of target by binding with PRC2 and LSD1 promoting H3K27me3 and demethylation of H3K4me2; and* TCF7 *binding with the SWI/SNF complex, promoting the activation of gene expression by chromatin remodelling.*

transcription and regulating splicing of mRNA [21]. The elncRNA *HOTTIP* (HOXA distal transcript antisense RNA) is transcribed from the 5′ tip of the homeobox A (HOXA) locus. *HOTTIP* binds with the WD repeat-containing protein 5 (WDR5) and recruits the methyltransferase MLL complex driving the histone H3 lysine 4 trimethylation (H3K4me3) and coordinating the transcription of several genes from HOX cluster [22]. In addition, the lncRNA Foxf1 adjacent non-coding developmental regulatory RNA (*FENDRR*) also recruits silencing complexes such as PRC2, to guide then to regions that will be silenced. However, *FENDRR* also interacts with the TrxG/MLL complex at a specific set of promoters, suggesting that there is a fine balance between FENDRR/PRC2 and FENDRR/MLL gene regulation [23, 24]. lncRNAs urothelial cancer associated 1 (*UCA1*), highly upregulated in liver cancer (*HULC*) and *PVT1* lncRNAs can interact with the histone methyltransferase complex PRC2 promoting trimethylation of lysine 27 on histone H3, silencing gene expression in gallbladder cancer, colorectal carcinoma and gastric cancer, respectively. Some lncRNAs can also interact with the chromatin remodellers of the ISWI/SNF family in order to recruit this complex to genome regions to activate transcription; an example is the lncRNA transcription factor 7 (*TCF7*) in hepatocarcinoma cells. Other lncRNAs, such as lncRNA nuclear enriched transcript 1 (*NEAT1*) may also interact with the SWI/SNF complex; however, the specific mechanisms and function are still unclear. Together, these recent findings of how lncRNAs regulate gene expression through epigenetic marks still need to be more elucidated. Accumulating evidence over the last years indicates that lncRNAs play a major role in regulating gene expression through epigenetic marks. However, the comprehension of these mechanisms still needs to be more elucidated. Nevertheless, based on the topics discussed, the main interactions between lncRNAs and the epigenetic machinery are illustrated with one example of each in **Figure 3**.

3.2 Epigenetic regulation, lncRNAs and cancer

Human cancers are complex diseases involving multiple genetic and epigenetic alterations, while only part of the DNA mutations corresponds to the malignant phenotype; recent research has demonstrated the importance of the epigenetic alterations in the development of tumours. The switch from silenced genes to actively transcribed genes and vice versa is regulated by complex mechanisms and alterations within the cell machinery. Cancer cells present variations within DNA methylation patterns, such as global hypomethylation profile and CpG island hypermethylation in promoter regions. Due to these modifications, the cancer cell

genome presents chromosomal instability, loss of genomic imprinting and changes in gene expression, both for protein coding and regulatory non-coding RNAs.

With the development of high-throughput sequencing, a number of studies have provided an ever-expanding survey on genetic aberrations in cancer. However, these abnormalities also affect lncRNAs, disrupting their functions and consequently leading to deregulation of their targets. Some of the recurring molecular mechanisms that govern how lncRNAs regulate cellular processes were highlighted earlier in this chapter. Most well-characterized lncRNAs to date show a functional role in gene expression regulation, typically transcriptional rather than post-transcriptional regulation. With advances in cancer transcriptome profiling and accumulated evidence supporting lncRNA functions, a number of differentially expressed lncRNAs have been associated with several types of cancers, which simultaneously acquire one or more dynamic modifications within their structures. Here, a few lncRNAs that have already been reported to participate in cancer progression will be mentioned, as detailed below.

XIST is one of the best-studied lncRNAs, and as such, it has been searched for and found in many different human neoplasias. Its expression can be either upregulated or downregulated, acting as an oncogene or as a tumour suppressor in multiple types of cancer. Overexpression of *XIST* is associated with advanced tumour stage, lymph node or distant metastasis and overall poor prognosis in human cancers. In breast cancer, *XIST* acts as a tumour suppressor by positively regulating the expression of non-X-chromosome gene PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1), which in turn catalyses dephosphorylation of protein kinase B (AKT) [25]. In non-small-cell lung cancer (NSCLC), nasopharyngeal and hepatocellular carcinoma, osteosarcoma and gastric, colorectal, pancreatic and bladder cancer, its expression is upregulated, acting as an oncogene and promoting cell proliferation and migration. *XIST* was also described acting as a sponge for miR-186-5p, and its knockdown suppresses multiplication and invasion in NSCLC.

MALAT1 is another well-studied lncRNA. Its high expression has been associated with gastric cancer, melanoma, breast cancer tumour and metastatic progression. *MALAT1* also functions in key spots of cancer development process, since it regulates transcription of oncogenic targets and regulates itself interacting with transcription factors [26]. This lncRNA can mediate transcription factors binding to target gene promoters or can act as a sponge to sequester miRNAs, controlling miRNA suppressor effects on oncogenic targets. On the other hand, epigenetic modifications occurring at histone level, for instance, demethylation of histone H3 in lysine 9 position (H3K9) by a demethylase that binds to the *MALAT1* promoter, may result in *MALAT1* lncRNA overexpression [27, 28].

HOTAIR is a lncRNA involved in gene silencing by interaction with two chromatin-modifying complexes and plays numerous roles in cancer development. Altered expression of *HOTAIR* is found in many types of cancers [29], promoting metastasis and tumour invasiveness through epigenetic gene silencing. Cancer stem cells from breast, oral and colon carcinomas express high levels of *HOTAIR* associated with increased stemness and metastatic potential [30]. High levels of *HOTAIR* correlating with metastasis and poor prognosis have been found in lung cancer [31], hepatocellular carcinoma [32], breast cancer [29], gastric cancer [33], colorectal cancer [34], cervical cancer [35], ovarian cancer, head and neck carcinoma and oesophageal squamous cell carcinoma. Just recently, elevated *HOTAIR* expression was also identified in adrenocortical carcinoma, and it was demonstrated to induce cell proliferation. In addition, another recent study showed the potential of *HOTAIR* to promote osteosarcoma development. Evidence supporting *HOTAIR*'s role in mediating drug resistance has emerged from an investigation with different

types of cancer. *HOTAIR* overexpression was found in samples from drug-resistant patients with NSCLC. Similar results demonstrated *HOTAIR*'s potential to promote resistance to cisplatin or other types of chemotherapy drugs. Those studies have been conducted with hepatocellular carcinoma, breast cancer, gastric cancer, colorectal cancer, cervical cancer and ovarian cancer [36–40].

The lncRNA *H19* has been well-studied in cancer. Aberrant expression of *H19* is observed in numerous solid tumours, including hepatocellular and bladder cancer. Functional data on *H19* points in several directions, and it has been linked to both oncogenic and tumour suppressive qualities. For example, there is evidence for its direct activation by cMYC as well as its downregulation by p53 during prolonged cell proliferation. The siRNA knockdown of *H19* impairs cell growth and clonogenicity in lung cancer cell lines in vitro and decreased xenograft tumour growth of Hep3B hepatocellular carcinoma cells in vivo [41, 42].

DNMT1-associated lncRNA (*DACOR1*) was described to be repressed in colon cancer. The lncRNA was directly associated with demethylation of CpG sites by guiding DNMT1 methylation patterns across the genome at thousands of different CpG sites [43].

The long non-coding RNA *PTENP1* is a well-known tumour suppressor gene. Studies have shown that *PTENP1* increased PTEN protein levels by competing for a set of PTEN-targeting miRNAs, which downregulate PTEN independent of its protein-coding function. In colon cancer, the loss of focal copy number at the

lncRNA	Associated epigenetic marks	Described in	Ref.
XIST	Histone modification, chromatin remodelling	Gastric, oesophageal	[21, 51, 52]
MALAT1/a/NEA	Histone modification	Breast, lung	[53–56]
HOTAIR	Chromatin remodelling/histone modification	Breast, pancreas	[36–40]
H19	DNA hypermethylation	Gastric	[41, 42, 57]
HULC	Chromatin remodelling	Hepato-, colorectal carcinoma	[58, 59]
GCLnc1	Histone modification	Gastric cancer	[60]
DACOR1	DNA methylation	Colon cancer	[38]
FENDRR	Histone modification	Gastric, lung cancers	[61]
UCA1	Chromatin remodelling	Colorectal cancer	[62, 63]
TCF7	Chromatin remodelling	Liver cancer	[64]
TP53TG1	CGI hypermethylation	Colorectal, gastric cancers	[65]
ANRIL	Chromatin remodelling	Prostate cancer, leukaemia	[66]
MEG3	Promoter/imprinting control hypermethylation	Brain tumour	[67]
GAS5	Histone modification	Breast cancer	[68]
NEAT1	Chromatin remodelling	Cervical renal, lung	[69–73]
PVT1	Histone modification	Renal, gastric	[70–75]

The table shows some examples of lncRNAs, with their respective epigenetic mark and in what cancer they were described.

Table 1.
lncRNAs altered in cancer and associated epigenetic marks.

PTENP1 locus was associated with the downregulation of PTEN expression in colon cancer patients. A similar relationship was shown between the oncogene KRAS and its pseudogene KRAS1P in colon cancer [15, 44]. *PTENP1* has been downregulated or suppressed in several cancers, such as GC, hepatocellular carcinoma (HCC), renal cell carcinoma, head and neck squamous cell carcinoma (HNSCC), melanoma, endometrial cancer and oral squamous cell carcinoma (OSCC) [45–50].

Besides the alterations mentioned above, some lncRNAs were also related to specific epigenetic marks in cancer. However, not all of these marks were well elucidated in all types of cancer. **Table 1** shows some lncRNAs that were described in different cancer types and what epigenetic mark they can regulate.

4. Conclusions

Gene expression can be regulated by different mechanisms; however, epigenetic alterations have a major effect in this process. We discussed the different ways by which lncRNAs may interact with epigenetic marks to guide gene expression. Furthermore, the complete panorama of how these interactions work remains unclear, as we are only beginning to understand the connections between lncRNAs and the epigenome. Since accumulating evidence shows that lncRNAs can regulate gene expression in cancer cells, understanding the mechanisms by which these molecules work on it is essential for comprehending cancer development and progression, in order to develop better diagnostic tools and treatments. There is still a long way to go on this road, until we can finally elucidate the rules that guide these interactions as well as the functional implications of these associations.

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Conflict of interest

No potential conflicts of interest were disclosed.

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Noncoding RNAs (ncRNAs) are an attractive research field in the RNA world. ncRNAs play important roles in regulating gene expression in animals, plants, and various human diseases. It is clear that current and further research on ncRNAs will change our knowledge of the nature of genome composition by editing previous dogmas. This book provides an overview of current knowledge on ncRNA research by dealing with miRNA and ncRNA-related human diseases, plant miRNA markers, and the roles of lncRNAs in cancer and epigenetics.

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