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Long Non-Coding RNAs
Function, Mechanisms, and Applications

Edited by Yusuf Tutar and Lütfi Tutar

Long Non-Coding RNAs - Function, Mechanisms, and Applications

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Genetics

Volume 6

Aims and Scope of the Series

"Genetics," which has been proud of its tradition since Mendel presented his research results in 1865, initially progressed quite slowly due to simple observational approaches of individuals and groups. However, the discovery of double-stranded DNA by Watson and Crick about 70 years ago triggered rapid progress in life sciences, including genetics, which was primarily conducted using Escherichia coli and bacteriophages infecting E. coli. Subsequently, genetics has achieved remarkable developments, such as understanding genetic disorders, including cancers, through research on the biogenesis and differentiation of plants and animals.

The two topics of this book series - Human Genetics, and Genomics - will address important areas of advancement in genetics.

Human Genetics: After fundamental genetics, initially studied with the main goal of revealing the functions of individual genes and proteins, genetics expanded from understanding the genetic system itself to understanding many infectious diseases caused by bacteria and viruses. Consequently, human beings are now overcoming infectious diseases by developing medicinal chemicals, including antibiotics and vaccines. However, genetic disorders remain challenging to cure up to now. Nevertheless, even the cure for them, including various cancers, is coming closer to reality due to the rapid progress of human genetics. In this way, the welfare of human life continues to improve, and even longevity, which was once a dream, has been achieved to some extent in recent years.

Genomics: On the other hand, the understanding of the comprehensive interrelationship of whole genes or whole proteins functioning in one organism has become possible now, as research has entered the era of genomics, owing to the rapid progress of base sequence analysis and bioinformatics. The development of genomics has further made it possible to understand the evolutionary processes of organisms through comparative studies among the genomes of many organisms.

This book series will discuss the findings obtained during the advancement of human genetics and genomics. It is also expected that this series will trigger the formation of a better world composed of human beings and all other organisms on Earth through discussions of research results obtained under the development of general genetics.

Meet the Series Editor

Kenji Ikehara graduated from the Department of Industrial Chemistry, Faculty of Engineering, Kyoto University in 1968. He received his B. Eng. (1968) and subsequently earned M. Eng. (1970) and D. Eng. (1976) degrees from Kyoto University. He began his career as a research associate in the Faculty of Science at the University of Tokyo before moving on to become an associate professor in the Faculty of Science at Nara Women's University. He was later promoted to professor and subsequently served as the dean of the Faculty of Science at Nara Women's University. Additionally, he held the position of director at the Nara Study Center of the Open University of Japan. For approximately 15 years, he focused his research on sporulation initiation of Bacillus subtilis. Later, he shifted his focus to the origins and evolutionary processes of microbial genes, the genetic code, proteins, and life. He has proposed several hypotheses, including the GC-NSF(a) hypothesis on the origin of genes, the GNC-SNS hypothesis on the genetic code, the protein 0th-order structure hypothesis on the origin of proteins, and the [GADV]-protein world hypothesis (GADV hypothesis) on the origin of life. Furthermore, he served as the local chair of the International Conference, Origin 2014, held in Nara in 2014.

Meet the Volume Editors

Dr. Yusuf Tutar currently conducts his research at the Recep Tayyip Erdoğan University, Faculty of Medicine, Division of Biochemistry. Dr. Tutar is the Dean of the Health Sciences Faculty. He obtained his M. Sc. and Ph.D. at Oregon State University and Texas Tech University, respectively. He pursued his postdoctoral studies at Rutgers University Medical School and the National Institutes of Health (NIH/NIDDK), USA. His research focuses on Biochemistry, Biophysics, Genetics, Molecular Biology, and Molecular Medicine with specialization in the fields of drug design, protein structure-function, protein folding, prion, microRNA, pseudogenes, molecular cancer, epigenetics, metabolites, proteomics, genomics, protein expression and characterization by spectroscopic and calorimetric methods. Having received 22 awards from national and international institutions, Dr. Tutar is the editor of several leading scientific journals and books.

Dr. Lütfi Tutar is currently an Assistant Professor at the Department of Molecular Biology and Genetics, Faculty of Art and Sciences, Kırşehir Ahi Evran University, Turkey. His interdisciplinary research focuses on bioinformatics analysis of high-throughput data, microRNAs, small RNAs, and Heat Shock Proteins (HSPs) in human diseases and other multicellular organisms.

Contents

Preface

Long non-coding RNAs (lncRNAs) play a multifaceted and critical role in cancer progression through diverse mechanisms affecting gene expression and cellular behavior. These RNA molecules, which do not encode proteins, regulate gene expression at multiple levels, including chromatin remodeling, transcriptional control, and post-transcriptional modulation. In cancer, lncRNAs can interact with chromatin-modifying complexes, serving as scaffolds that facilitate epigenetic changes to activate oncogenes or silence tumor suppressor genes. This contributes to tumorigenesis by altering the transcriptional landscape of cancer cells and promoting uncontrolled proliferation.

LncRNAs also function as competing endogenous RNAs (ceRNAs), sequestering tumor-suppressive microRNAs (miRNAs), thereby preventing miRNAs from binding to their mRNA targets. This modulation enhances the expression of oncogenic mRNAs, further supporting cancer cell growth, survival, and metastasis. Furthermore, lncRNAs regulate epithelial-mesenchymal transition (EMT), a vital process in cancer metastasis where epithelial cells acquire mesenchymal traits facilitating migration and invasion. By orchestrating the gene networks involved in EMT, lncRNAs contribute to tumor dissemination and resistance to anticancer therapies.

Beyond EMT, lncRNAs influence key cellular processes such as apoptosis, metabolism, and drug resistance, impacting cancer progression and therapeutic outcomes. Their aberrant expression patterns are often tumor type-specific, making them promising biomarkers for cancer diagnosis, prognosis, and stratification of patient subgroups. The complex regulation by lncRNAs extends to various signaling pathways critical for maintaining cellular homeostasis and responding to microenvironmental cues, highlighting their role as central regulators in cancer biology.

Given their diverse functions and disease associations, lncRNAs are emerging as potential targets for novel therapeutic interventions and as components of liquid biopsy platforms for non-invasive cancer detection. Ongoing research explores their utility in modulating gene expression networks to counteract tumor progression and improve treatment responses. Overall, lncRNAs represent a dynamic and versatile class of molecules that substantially influence cancer development and metastasis, underscoring their importance in both basic research and clinical applications.

This book has four sections for lncRNAs: 1. Biochemistry of Long Non-Coding RNAs, 2. Long Non-Coding RNA's in Diseases, 3. Long Non-Coding RNA's in Biotechnology, and 4. Long Non-Coding RNA's in Plants. The biochemistry section covers four chapters that provide a comprehensive review and overview of the relationships of pseudogenes

with lncRNAs, X-Chromosome Dosage Compensation, and skeletal pathophysiology. The disease section covers lncRNAs as therapeutic targets for β-Thalassemia and cardiovascular disorder. The book covers CRISPR technology and stem cells over lncRNA in the Biotechnology section. The final section focuses on lncRNAs' role in stress tolerance and crop resilience. The content provides recent advances in the field to the book's audience.

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Section 1

Biochemistry of Long Non-Coding RNAs

Chapter 1

Perspective Chapter: Decoding Cancer's Silent Players – A Comprehensive Guide to LncRNAs

Abhijit Mandal and Sarbani Giri

Abstract

Long non-coding RNAs (LncRNAs) are RNAs that do not code for proteins and were thus earlier known as Junk RNAs. Recently, LncRNAs have emerged as critical regulators in the expression of coding genes and various important biological signaling pathways, thus controlling crucial biological and developmental processes. Reports of LncRNAs association with several diseases including cancer have also been implicated. LncRNAs play a crucial diverse role in regulating cancer pathways, thus influencing tumorigenesis, progression, and metastasis. They can function both as oncogenes or tumor suppressors, modulating key signaling pathways and cellular processes. Mutation or epigenetic-induced aberrant expression of LncRNAs dysregulates different essential biological pathways, leading to malignant phenotype and cancer hallmarks in different types of cancer. Tumor cells secrete specific endogenous LncRNAs into biological fluids depending on the cancer type, giving rise to stable circulating LncRNAs, thus proving to be of great potential as non-invasive or minimally invasive diagnostic biomarkers. In this chapter, we explore the multifaceted roles of LncRNAs in various cancer types, highlighting their potential as diagnostic/prognostic biomarkers and therapeutic targets. Additionally, we discuss innovative strategies for targeting LncRNAs in cancer treatment, including RNA interference and CRISPR technology. This chapter will provide a comprehensive overview of LncRNAs' implications in cancer research and personalized medicine.

Keywords: long non-coding RNAs, cancer, mechanisms, biomarkers, prognosis, therapeutic target, personalized medicine

1. Introduction

With the advent and development of advanced high-throughput sequencing technologies such as microarray and RNAseq, besides coding genes, many non-coding RNAs that do not code for proteins were identified. The most distinguished non-coding RNAs are microRNAs (miRNAs) and long non-coding RNAs (LncRNAs). LncRNAs are transcripts that are greater than 200 nucleotides in length and have no long open reading frames (>100 amino acids), regulating gene expression of target

genes at transcriptional, post-transcriptional, translational, and epigenetic levels by acting as guides, scaffolds, decoys, or signals [1–5]. LncRNAs play vital roles in important biological and developmental processes, such as genomic imprinting, X-chromosome inactivation, chromatin modification, and alternative splicing, as well as affecting various cellular activities, such as proliferation, differentiation, and survival [1, 6]. Due to their multifaceted and diverse functions, LncRNAs have been implicated in a large number of human diseases including coronary disorders, type 2 diabetes, neurological disorders, and various types of cancer [6]. Other than cardiovascular disorders, cancer is thought to be the leading cause of mortality worldwide [7]. Increasing evidence of LncRNAs association with cancer has been found in the literature [1, 8]. LncRNAs play a crucial role in regulating cancer pathways, thus influencing tumorigenesis, progression, and metastasis [1]. Literature suggests that altered expression of LncRNAs in tumors is closely linked to the manifestation of cancer hallmarks such as unregulated proliferative signaling, avoiding growth suppressors, preventing cell death (apoptosis), uncontrolled replication, triggering angiogenesis, initiating invasion and metastasis, aberrant metabolic pathways, immune evasion, genomic instability, and inflammation [5]. Although the mechanisms are poorly understood, LncRNAs play a pivotal role in tumorigenesis and progression by regulating gene expression and fine-tuning signaling pathways [1, 9]. Several LncRNAs, such as MALAT1, PCA3, HOTAIR, ANRIL, and MEG3, have been reported to be upregulated or downregulated in a variety of human cancers [8, 10–12]. Research indicates that LncRNAs, known for their high specificity and precision, hold potential as cancer biomarkers [12, 13]. Their unique expression and diverse functions across cancer types make them promising for diagnosis, prognosis, and treatment. Additionally, LncRNAs can be collected noninvasively from body fluids, tissues, and cells, serving either as independent or supplementary biomarkers to enhance diagnostic and prognostic accuracy [13]. This chapter provides an overview of the roles of different LncRNAs in the development and progression of various cancer types, emphasizing their promise as diagnostic, prognostic markers, and therapeutic targets (**Figure 1**). It also covers novel approaches to targeting LncRNAs, such as RNA interference (RNAi) and CRISPR, offering a thorough overview of their impact on cancer research and personalized medicine.

2. LncRNAs in tumorigenesis and cancer progression

LncRNAs can function both as oncogenes or tumor suppressors, regulating various aspects of cancer initiation and progression [14]. Abnormal expression of oncogenic and tumor-suppressive LncRNAs disrupts tumor suppressor gene activity by reducing their transcription and translation, while promoting the expression and translation of oncogenes. This imbalance drives processes such as cellular proliferation, differentiation obstruction, migration, invasion, metastasis, genomic instability, transformation into malignant type, tumor initiation and progression, and resistance to chemotherapy and radiotherapy [10].

2.1 LncRNAs in tumorigenesis

Several LncRNAs have been reported to be overexpressed in a variety of human cancers, for example, MALAT1 [10, 15], HOTAIR [16, 17], ANRIL [18], etc., whereas several others are downregulated, for example, MEG3 [19], PANDA [20], PCAT29

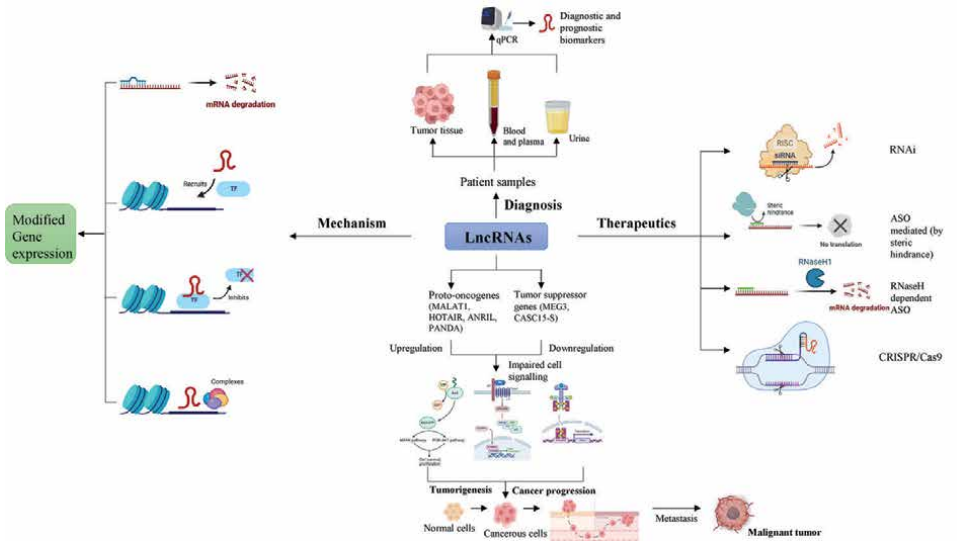

Figure 1.
Schematic representation of the influence of LncRNAs in cancer research, highlighting their role in tumorigenesis, cancer progression, diagnosis/prognosis and therapeutics.

[21], and CASC15-S [22]. Also, it has to be noted that the same LncRNA can either act as an oncogene or a tumor suppressor depending on the type of cancer; for example, MALAT1 was initially reported to be upregulated in human mantle cell lymphoma, renal cell carcinoma tissues, bladder cancer, pancreatic ductal adenocarcinoma tissues, human melanoma, ovarian cancer, lung cancer, and liver cancer [10]. On the other hand, two recent in-depth studies show that MALAT1 has tumor-suppressive effects in colorectal and breast cancers [10, 23]. Thus, demonstrating the cancer type-specific role of LncRNAs in cancer development.

MALAT1 (metastasis-associated in lung adenocarcinoma transcript 1) is one of the most researched LncRNAs in cancer because it is readily detectable and functionally investigated, and it is substantially expressed in cancer tissues [10]. Cancer tissues are thought to release MALAT1, an intergenic transcript found on human chromosome 11q13.1, into the bloodstream through exosomes since it is highly expressed in both serum and cancer tissues. As a result, its expression in blood is similar to that of the primary tumors [11]. MALAT1 was initially found to be significantly linked to non-small cell lung cancer (NSCLC) metastases [5]. Further research showed that, in contrast to normal cervical squamous cell samples, LncRNA-MALAT1 expression was elevated in cervical cancer cell lines [24]. Also, the laryngeal cancer cells' expression levels of LncRNA-MALAT1, miRNA-503-5p, and FOXK1 were investigated. The levels of miRNA-503-5p were low, while those of LncRNA-MALAT1 and FOXK1 were high. This implies that LncRNA-MALAT1 and miRNA-503-5p have a negative correlation and that miRNA-503-5p is one of its target regulatory genes [11]. Despite the fact that MALAT1 has been found to be overexpressed in the majority of human cancer tissues, its oncogenic or tumor-suppressive function in breast cancer remains up for debate [10]. MALAT1 has been found to be abnormally upregulated in human breast cancer tissues, and high expression levels of MALAT1 are associated with a poor prognosis for patients. By binding miRNA-1, miRNA-124, and miRNA-448, MALAT1 functions as a competitive endogenous RNA (ceRNA) to decrease CDC42 and increase CDK4

expression, which promotes invasion, migration, and cell cycle progression in breast cancer [10, 25]. However, recent comprehensive studies show that MALAT1 has tumor-suppressive effects in breast cancers. By binding and sequestering miRNA-17, miRNA-20a, and miRNA-106b, PTEN increases the expression of MALAT1, which in turn inhibits the migration and invasion of breast cancer cells by lowering integrin β4 (ITGB4) and the pro-metastatic Epithelial Cell Adhesion Molecule (EpCAM) [23].

LncRNA HOTAIR (HOX Transcript Antisense Intergenic RNA) dysregulation is also frequently found in several types of human cancer. Because it is overexpressed in many malignancies, HOTAIR can function as a pro-oncogene and be linked to a number of cancer hallmarks, including apoptosis inhibition, cellular proliferation, and genomic instability [5]. Overexpression of HOTAIR, an oncogene that is transcribed from the HOXC locus during normal development, can accelerate the development of gastric cancer [9]. Progesterone receptor (PGR), protocadherin 10 (PCDH10), protocadherin β5 (PCDHB5), and junctional adhesion molecule 2 (JAM2) are tumor suppressors that can be inhibited by overexpression of HOTAIR, hence increasing tumor growth [9, 26, 27]. The aberrant expression of LncRNA HOTAIR promotes the proliferation, cell cycle, and migration of gastric cancer cells, and it can suppress the expression of miRNA-217 and increase the resistance of gastric cancer cells to doxorubicin and paclitaxel [9, 28]. It has also been reported that prostate cancer (PCa) tissues and cells have high levels of HOTAIR expression which is linked to anti-apoptosis, migration, invasion, proliferation, and tumor formation [5]. LncRNA HOTAIR has also been reported to be upregulated in breast carcinoma, colorectal cancer (CRC), hepatocellular carcinoma (HCC), laryngeal squamous cell carcinoma (LSCC) tissues [5]. Peng et al. [29] in their study have shown that HOTAIR downregulates miRNA-34a to promote the development of colon cancer.

MEG3 (Maternally Expressed 3), a tumor suppressor on chromosome 14q32.2, is typically downregulated in cancer cells. In bladder cancer, MEG3 overexpression induces autophagy and promotes P53 accumulation [9]. In NSCLC, elevated Heterogeneous nuclear ribonucleoprotein A2B1 (HNRNPA2B1) expression correlates with distant metastasis, poor survival, and serves as an independent prognostic marker. HNRNPA2B1 knockdown *in vitro* and *in vivo* reduces cell proliferation and metastasis, while its overexpression has the opposite effect. Mechanistically, HNRNPA2B1 mediates m^6A modification of MEG3, and its inhibition leads to decreased MEG3 m^6A while increasing the mRNA levels. MEG3 functions as a miRNA-21-5p sponge to upregulate PTEN, inhibiting PI3K/AKT signaling and suppressing proliferation and invasion. Low MEG3 or high miRNA-21-5p expression predicts poor NSCLC survival, identifying the HNRNPA2B1/MEG3/miRNA-21-5p/PTEN axis as a potential therapeutic target [19].

LncRNA-TSLNC8 (Tumor Suppressor Long Non-Coding RNA on Chromosome 8p12) plays a critical role in various cancers, including glioma, liver, lung, breast, melanoma, and gastric cancers [30]. Acting as a tumor suppressor, TSLNC8 inhibits tumor progression and reduces chemoresistance when overexpressed. It functions as a ceRNA, sequestering specific microRNAs, and is involved in multiple signaling pathways implicated in cancer development and progression [30].

The oncofetal LncRNA H19, typically repressed postnatally, is re-expressed in various cancers, including HCC and rectal cancer [31]. Although not a classical oncogene, H19 was the first LncRNA identified as overexpressed in HCC. It influences cancer progression through pathways such as acting as a precursor to miRNA-675, whose increase promotes tumor growth, or functioning as a ceRNA that sponges miRNAs like let-7, reducing tumor suppressor activity and encouraging proliferation [9, 31]. Cancer susceptibility candidate

9 (CASC9) is a LncRNA overexpressed in oral SCC and HCC. It binds to hnRNP L to form a complex that regulates HCC cell viability by modulating the PI3K/AKT signaling pathway [32]. Small nucleolar RNA host gene 1 (SNHG1), located on chromosome 11q12.3, hosts eight small nucleolar RNAs (snoRNAs), which is another LncRNA overexpressed in various cancers [33]. In HCC, LncRNA SNHG1 levels are elevated in tissues and cell lines compared to normal counterparts. It promotes HCC progression by sponging miRNA-195, increasing AEG-1 protein expression [33, 34]. SNHG1 is also significantly upregulated in breast cancer, esophageal squamous cell carcinoma, PCa, NSCLC, colon cancer, and bladder cancer [33].

Several other LncRNAs have been reported to be involved in cancer development and progression, which has been mentioned in **Table 1.**

LncRNA	Cancer type	Oncogenic or Tumor suppressor	Expression	Mechanism	Reference
MALAT1	Colorectal	Tumor suppressor	Down-regulated	Non-canonical PTEN miRNA- MALAT1 axis	[23]
	Breast	Tumor suppressor	Down-regulated	Non-canonical PTEN miRNA- MALAT1 axis	[23]
	Bladder	Oncogenic	Upregulated	TGF-β induced MALAT 1 upregulation resulting in EMT	[15]
	Gallbladder	Oncogenic	Upregulated	Functions as a ceRNA to regulate miRNA-206	[35]
	Malignant Melanoma (Skin)	Oncogenic	Upregulated	Acting as a sponge for miRNA-22	[36]
PVT1	Lung	Oncogenic	Upregulated	Interacts with miRNAs and proteins	[37]
CASC9	HCC	Oncogenic	Upregulated	PI3K/AKT-signaling cascade	[32, 38]
PCAT29	Prostate	Tumor suppressor	Down-regulated	Directly regulated by the androgen receptor (AR), which binds to the promoter of PCAT29	[21]
loc285194	Colon	Tumor suppressor	Down-regulated	Reciprocal repression of loc285194 and miRNA-211 possibly via pathway involving RISC complex	[13]
DRAIC	Prostate	Tumor suppressor	Down-regulated	AR binds to the *DRAIC* locus and represses *DRAIC* expression	[39]
TUG1	Colorectal	Oncogenic	Up-regulated	SP1, an upstream transcription factor interacts with TUG1 and regulates the downstream miRNA-421/KDM2A/ERK axis	[40]

LncRNA	Cancer type	Oncogenic or Tumor suppressor	Expression	Mechanism	Reference
CCAT1	hepatocellular carcinoma	Oncogenic	Up-regulated	Acting as a let-7 sponge	[41]
	gallbladder cancer	Oncogenic	Up-regulated	Acting as a miRNA-218 sponge	[42]
GMDS-AS1	Colorectal	Oncogenic	Up-regulated	GMDS-AS1 targets HuR directly activating STAT3/Wnt signaling	[43]
MEG3	NSCLC	Tumor suppressor	Down-regulated	HNRNPA2B1-mediated m^6A modification of the LncRNA MEG3 facilitates tumor growth and metastasis by modulating the miRNA-21–5p/PTEN pathway.	[19]
PTTG3P	NSCLC	Oncogenic	Up-regulated	PTTG3P/ILF3/E2F1 axis	[44]
G077640	ESCC	Oncogenic	Up-regulated	Enhancing HIF1α stability and downstream glycolytic signaling pathway	[45]
GAS5	Cervical	Tumor suppressor	Down-regulated	siRNA-mediated knockdown of GAS5	[46]
HOTAIR	Pancreatic	Oncogenic	Up-regulated	Polycomb Repressive Complex 2 (PRC2) -dependent and -independent	[16]
	Gastric	Oncogenic	Up-regulated	Possibly via interaction with PRC2	[17]
ANRIL	NSCLC	Oncogenic	Up-regulated	Possibly by regulating (silencing) KLF2 and P21 transcription	[18]
TP73-AS1	Ovarian	Oncogenic	Up-regulated	Modulation of MMP2 and MMP9	[47]

Table 1.
List of some LncRNAs reported to be involved in cancer development and progression.

2.2 LncRNAs and metastasis

Metastasis, the spread of cancer to distant sites, is a leading cause of cancer-related deaths. It involves steps like epithelial–mesenchymal transition (EMT), migration, anoikis resistance, and angiogenesis [48, 49]. LncRNAs play a key role in promoting metastasis by driving EMT [5], angiogenesis, invasion, migration [32, 50, 51], organ-specific colonization, apoptosis evasion [37], and shaping the metastatic tumor microenvironment [49]. They directly regulate metastasis *in vitro* and *in vivo* across various cancers [48].

LncRNA MALAT1 was initially linked to NSCLC metastasis. Its overexpression promotes brain metastasis in NSCLC by driving EMT and enhances tumor proliferation and metastasis in osteosarcoma through the PI3K/Akt pathway [5]. MALAT1 also regulates EMT *via* this pathway in osteosarcoma progression and influences HCC through the mTOR pathway [5, 52, 53]. In ESCC, MALAT1 upregulation promotes proliferation and metastasis by dephosphorylation of the ATM-CHK2 pathway, while in gastric cancer, it accelerates tumor growth and spread [54, 55]. Clinical studies identify MALAT1 overexpression as a poor prognostic marker in pancreatic cancer [5]. In breast cancer, MALAT1 enhances angiogenesis, potentially *via* miRNA-145 regulation [56]. MALAT1's role in cancer metastasis is primarily mediated through EMT [48].

Hou et al. [57] found that LncRNA-ROR is upregulated in breast tumor tissues, promoting EMT by acting as a ceRNA for miRNA-205 in mammary epithelial cells. In triple-negative breast cancer (TNBC), ROR serves as a ceRNA sponge for miRNA-145, affecting its target ARF6, a key regulator of tumor cell invasion and metastasis. ARF6 influences E-cadherin localization and cell–cell adhesion. These findings highlight the LncRNA-ROR/miRNA-145/ARF6 pathway in regulating TNBC metastasis [48, 58]. LncRNA-TTN-AS1, an oncogene overexpressed in ESCC tissues and cell lines, promotes proliferation and metastasis. It enhances Snail1 expression by sponging miRNA-133b, triggering the EMT cascade. Additionally, LncRNA-TTN-AS1 upregulates FSCN1 expression through miRNA-133b sponging and HuR upregulation, driving ESCC invasion. miRNA-133b directly regulates FSCN1, a key protein linked to ESCC metastasis [59]. Another LncRNA SNHG1, highly upregulated in PCa and sublocalized in the nucleus, promotes PCa metastasis by activating the EMT pathway [33]. Tan et al. [60] found that SNHG1 competitively interacts with hnRNPL, disrupting CDH1 translation through the hnRNPL-CDH1 axis, thereby triggering EMT and tumor spread. LncRNA LOXL1-AS1 upregulation is linked to increased proliferation, migration, metastasis, and EMT while suppressing apoptosis in various cancers, including ovarian, cervical, endometrial, gastric, CRC, esophageal, lung, laryngeal, liver, breast, and PCa [61]. Low GAS5 expression is associated with increased apoptosis in TNBC and ER-positive breast cancer, correlating with lymph node metastasis, advanced clinical stage, and poor survival [5, 62]. In CRC, reduced GAS5 levels are linked to tumor size, advanced TNM stage, lymph node metastasis, low histological grade, poor survival, distant metastasis, and higher recurrence rates [5]. However, overexpression of GAS5 has been linked to early-stage CRC liver metastases [63]. PCAT-1 upregulation is strongly linked to TNM stage and metastasis in HCC and osteosarcoma, as well as tumor invasion and lymph node metastasis in gastric and esophageal cancers [5]. Zhang et al. [64] revealed that PCAT-1 acts as a ceRNA against miRNA-122, and PCAT-1 silencing reduced Wnt/b-catenin signaling *via* miRNA-122 suppression and WNT1 expression, which in turn prevented the advancement of ESCC. The overexpression of HOTAIR was found to be associated with tumor growth, metastasis, migration, cell proliferation, invasion, TNM stage, decreased survival, and a poor prognosis for patients with CRC as compared to healthy controls [5].

2.3 LncRNAs' interaction with signaling pathways

Abnormal signal transduction is a key driver of tumorigenesis and cancer progression, with LncRNAs acting as critical regulators by modulating various signaling pathways [1]. Several key signaling pathways regulated by LncRNA to promote cancer development and progression have been discussed in brief below.

Wnt/β-catenin pathway: The Wnt/β-catenin pathway is often dysregulated in tumorigenesis, influencing proliferation, invasion, metastasis, and apoptosis. LncRNAs regulate this pathway, acting as tumor suppressors or promoters [1]. For example, HOTAIR promotes breast cancer invasion and metastasis by activating the Wnt/β-catenin pathway [65].

STAT3 signaling pathway: The STAT3 signaling pathway, particularly JAK/STAT, is a key oncogenic driver in various cancers. For example, some miRNAs and LncRNAs act as STAT3 negative regulators, binding to its mRNA or modulating its expression to inhibit CRC progression. Conversely, certain LncRNAs, like HOTAIR, promote CRC development by enhancing STAT3 activity, functioning as oncogenes [66].

MAPK pathway: The MAPK pathway is involved in regulating cell proliferation, differentiation, transformation, and apoptosis, contributing to inflammation and tumor development by phosphorylating key cellular components [67]. Many LncRNAs modulate this pathway. For instance, LncRNA XIST targets miRNA-194-5p, reducing its expression and regulating MAPK1, while its silencing inhibits hepatoma cell proliferation and invasion [67]. Similarly, MALAT1 knockdown suppresses MEK/ERK/MAPK/JNK phosphorylation leading to inhibition of ERK/MAPK pathway, reducing gallbladder carcinoma metastasis and invasiveness [68].

PI3K/AKT signaling pathway: The PI3K/AKT signaling pathway is associated with the regulation of cell proliferation, survival, and migration and is abnormally activated in cancers like breast, colorectal, ovarian, pancreatic, and endometrial cancer [50]. Interaction of phosphatidylinositol-3,4,5-trisphosphate (PIP3) and pleckstrin homology (PH) domain drives AKT phosphorylation at Ser473 and Thr308 by PDK1 and mTOR resulting in AKT activation, which then regulates tumor growth and metastasis [50, 69, 70]. LINK-A enhances interactions between PtdIns(3,4,5)P3 and the AKT PH domain, driving AKT hyperphosphorylation, resistance to AKT inhibitors, and promoting tumorigenesis and metastasis [71].

Notch signaling pathway: The Notch signaling pathway regulates cell differentiation, proliferation, and apoptosis, playing diverse roles in tumorigenesis and progression by influencing genes like MYC, cyclin-D1, and p21 [1, 72]. LncRNA LINC01152 acts as an oncogene in glioblastoma multiforme by activating Notch signaling through miRNA-466 interaction and upregulating MAML2, a transcriptional co-activator 2, thereby promoting tumor progression [73].

TGF-β signaling pathway: The TGF-β signaling pathway maintains homeostasis in normal tissues, regulating cell proliferation, motility, and differentiation. However, in tumors, it promotes malignancy. LncRNAs significantly influence TGF-β pathway activity, impacting cancer progression [1]. For instance, downregulation of LncRNA-ANCR promotes breast cancer metastasis *via* association with TGF-β signaling pathway [74].

Unsurprisingly, multiple signaling pathways may be regulated by a single LncRNA. MALAT1 acts as an oncogene by activating the Wnt/β-catenin pathway *via* β-catenin upregulation and GSK3β downregulation. It also activates the Notch signaling pathway by increasing JAG1 (Jagged1) expression through miRNA-124 inhibition [1, 75]. Additionally, LncRNAs can link multiple pathways; for example, BCAR4 in breast cancer connects the Hippo and HH pathways. Hippo effector YAP promotes BCAR4 expression, activating HH signaling to drive glycolysis by upregulating HK2 and PFKFB3, reprogramming glucose metabolism [1, 76].

3. Diagnostic and prognostic potential

Cancer's high mortality rate is partly attributed to inadequate early detection methods and unreliable diagnostic tools like certain protein biomarkers. Most current biomarkers are protein-based, such as glycoproteins, detected through tissue biopsy and immunohistochemistry (IHC) to identify cancer subtypes. However, protein markers often produce false-positive or false-negative results. Conventional serological markers like CA153, CA125, CA27.29, and CEA are also criticized for low specificity and sensitivity. These limitations arise from antibody-based detection, where antibodies may lack specificity or cross-react with other tissues. Furthermore, traditional histology relies on invasive biopsies, discouraging some patients from undergoing diagnostics [77]. Therefore, non-invasive, non-protein biomarkers are urgently needed. Liquid biopsy enables early cancer detection through minimally invasive, serial testing of body fluids, allowing real-time tumor progression monitoring [78]. LncRNAs, detectable in blood, plasma, serum, and urine *via* real-time PCR, are stable in circulation and resistant to nuclease degradation. Their abundance and accessibility make circulating LncRNAs highly promising diagnostic biomarkers [78, 79]. Several LncRNAs, including PCA3, HOTAIR, HULC, MALAT1, and H19, are promising minimally invasive diagnostic and prognostic cancer biomarkers found in body fluids (**Table 2**). Some of these LncRNAs have already been proven effective for diagnostic and prognostic use in clinical settings [12, 79]. PCA3 has been approved by the US FDA as a urine biomarker for PCa, offering better sensitivity and specificity than the Prostate-Specific Antigen (PSA) blood test due to its higher expression in PCa patients [12]. It is more reliable than other circulating nucleic acids due to its high stability in the bloodstream and resistance to nuclease degradation [77]. Genome-wide studies have identified thousands of LncRNAs with differential expression between normal tissues and tumors, highlighting their potential as cancer-specific biomarkers. LncRNA expression profiles can also help identify cancer subtypes, providing insights into tumor behavior and prognosis [8]. MALAT1 is a promising diagnostic biomarker for lung cancer, detectable in blood, and is also elevated in the

LncRNA	Cancer type	Bioavailability	Prognosis	Reference
PCA3	Prostate	Urine	Poor prognosis	[80]
HOTAIR	OSCC	Saliva	Risk of metastasis	[81]
MALAT1	Lung	Blood	Increased risk of metastasis	[82]
H19	Gastric	Plasma, gastric juice	Poor prognosis	[83]
HULC	Pancreatic	Serum	Associated with tumor size, T staging, M staging, vascular invasion and overall survival	[84]
MEG3	Cervical	Tissue	Associated with tumor size, lymph node metastasis and overall survival	[85]
LncRNA-ATB	Breast	Serum	Associated with advanced TNM stage, large tumor size, high M stage and positive lymph node metastasis	[86]

Table 2.
List of some LncRNAs with potential diagnostic and prognostic value.

plasma and urine of PCa patients [12]. LncRNA HOTAIR, which is found to be highly expressed in the saliva samples of oral squamous cell carcinoma (OSCC) patients, is a strong candidate for diagnosing metastatic oral cancer, as its levels are higher in metastatic cases [12, 81].

Some LncRNAs are better suited as complementary biomarkers rather than stand-alone cancer diagnostic tools [12]. For instance, a five-LncRNA signature (AK001094, AK024171, AK093735, BC003519, and NR_003573) shows strong diagnostic and prognostic potential for gastric cancer (GC), with a combined AUC of 0.95 ± 0.025 in ROC curve analysis [87]. Similarly, three LncRNAs (lnc-MB21D1-3:5, lnc-PSCA-4:2, and lnc-ABCC5-2:1) were significantly dysregulated in GC and showed promising diagnostic performance with an AUC of 0.902 when combined [79]. For CRC, a panel of four LncRNAs (ZFAS1, SNHG11, LINC00909, and LINC00654) showed high diagnostic performance, particularly for early-stage disease, with an AUC of 0.937. Notably, SNHG11 showed the greatest potential for detecting precancerous lesions and early-stage tumors, indicating it could be a promising biomarker for CRC detection and a potential therapeutic target [88].

4. Therapeutic targeting of LncRNAs

LncRNAs have become promising pharmacological targets for treating complex malignancies due to their versatility in gene regulation and tissue-specific expression [89]. They offer advantages such as higher specificity and sensitivity, making them valuable for predicting therapeutic responses [90]. Unlike conventional treatments like chemotherapy, radiotherapy, and surgery, which often cause significant physical and mental stress and may lead to relapse, LncRNA-based approaches provide a more targeted and potentially effective alternative [91]. Furthermore, there is a growing need for personalized cancer therapy which relies on understanding genetic and molecular variations among patients that influence treatment responses [92]. A detailed analysis of LncRNA interactions with proteins, chromatin, and other RNAs can enable their use as precise biomarkers for diagnosis, prognosis, and targeted therapies in personalized medicine [93].

Abnormal levels of LncRNA in bodily fluids and tissues are consistent indicators of cancer and may be the focus of a potential treatment. Indeed, it has been demonstrated that cellular abnormalities linked to cancer can be successfully normalized by interfering with dysregulated LncRNA levels both *in vitro* and *in vivo* [91]. LncRNAs can be targeted through various approaches: (i) degrading pathogenic RNAs *via* siRNAs or chemically modified ASOs using dicer-, AGO-, or RNase H-dependent pathways; (ii) modulating LncRNA genes through promoter blockade or genome editing; and (iii) inhibiting RNA-protein interactions or formation of secondary structure by using ASOs or RNA-binding small molecules [94].

RNA interference (RNAi) is a well-established post-transcriptional gene silencing mechanism that is initiated by double-stranded RNA matching the target gene's sequence. It is a highly effective method for suppressing genes involved in specific biological or pathological processes by employing siRNAs that degrade mRNA during translation [95]. Traditional siRNA approaches have successfully targeted several LncRNAs, such as MALAT1, in human PCa cells, where they inhibited growth, invasion, and migration while inducing cell-cycle arrest [94, 96]. Similarly, siRNA-mediated knockdown of HOTAIR reduced matrix invasion in human breast cancer cells [27].

Another promising therapeutic tool for targeting LncRNAs is antisense oligonucleotides (ASOs), which interact with RNA through Watson–Crick base pairing. By binding to their target RNA, ASOs can modulate gene expression through mechanisms such as steric hindrance, splicing modification, or triggering RNA degradation [94]. In a luminal B breast cancer mouse model (MMTV–PyMT), subcutaneous delivery of MALAT 1-specific ASOs led to primary tumor differentiation and reduced metastasis by nearly 80% compared to nonspecific ASO controls. Additionally, ASOs targeting MALAT 1 decreased branching morphogenesis in 3D organoid models derived from MMTV–PyMT tumors and HER2-amplified mouse mammary tumors [97]. Beyond breast cancer, MALAT1-targeting ASOs have demonstrated strong antimetastatic effects in lung cancer xenograft models [94, 98].

Advances in genome editing technologies, such as CRISPR/Cas9, have enabled the transcriptional silencing of LncRNA-expressing genes through a technique known as CRISPR interference (CRISPRi). This method involves fusing a catalytically inactive Cas9 (dead-Cas9) with transcriptional repressors, which are guided to specific gene promoters by guide RNAs to inhibit transcription [94]. CRISPRi has been effectively utilized to selectively deactivate LncRNA genes across seven human cell lines, including six cancer types and one line of induced pluripotent stem cells (iPSCs) [99].

Nonetheless, there are several challenges associated with studying LncRNAs in the context of cancer. Many LncRNAs are expressed at low levels, raising concerns about their functional relevance in clinical oncology [100]. Differential expression patterns, alternative splicing, and tumor heterogeneity further complicate accurate analysis [101]. Techniques like RNAseq, single-cell RNAseq, and fluorescent RNA *in situ* hybridization (FISH) may address these issues but are time-consuming and resource-intensive [101, 102]. LncRNA-targeted therapies face delivery challenges, including poor membrane permeability of ASOs and siRNAs and difficulty targeting sub-nuclear LncRNAs [100, 101]. Additionally, toxicity and off-target effects remain significant hurdles, although bioinformatics and RNA-capture sequencing can help mitigate these problems. Functional validation of therapeutics *in vivo* is complicated by poor LncRNA conservation across species. Engineered models using larger human genomic segments or proteins may offer solutions. High-throughput methods and CRISPR-Cas9 technology could improve functional screening [101]. Profiling LncRNAs in body fluids or single cells is challenging due to low RNA abundance, fragmentation, and technical limitations in RNA sequencing. Advances like total RNA sequencing, RNA-capture sequencing, and specialized protocols such as SMART-seq are helping overcome these barriers [103]. Furthermore, conventional methods like RT-qPCR and microarray hybridization are time-consuming, while polyA-selection in RNAseq often misses non-polyadenylated LncRNAs [102, 103]. Emerging techniques, including total RNA sequencing, isothermal amplification and nanotechnology-based delivery systems, may improve clinical application feasibility [102, 103]. Addressing these challenges with innovative methods and technologies could unlock the full potential of LncRNAs in cancer diagnostics and therapeutics.

5. Conclusion and future perspectives

LncRNAs have been found to play a critical role in regulating cancer pathways and influencing tumorigenesis, progression, and metastasis. Aberrant expression of LncRNAs dysregulates essential biological pathways, leading to cancer hallmarks and malignant phenotype. Cancer-specific endogenous LncRNAs are secreted from

tumor cells into biological fluids, giving rise to stable circulating LncRNAs, thus proving to be of great potential as minimally invasive diagnostic and prognostic biomarkers. Also, LncRNAs have emerged as promising pharmacological targets for treating different types of cancer due to their higher specificity and sensitivity, diverse functional role in gene regulation, and tissue-specific expression. However, there are various challenges that are encountered while studying LncRNAs in the context of cancer such as low and differential expression patterns, off-target effects, profiling in single cells, delivery challenges, and technical limitations. Emerging techniques such as total RNA sequencing, isothermal amplification, and nanotechnology-based delivery systems, may help in addressing these challenges so that the full potential of LncRNAs in cancer diagnostics and therapeutics can be unlocked. Nanotechnology-based delivery systems, such as lipid or polymer nanoparticles carrying ASOs or siRNAs, represent a promising avenue for targeting oncogenic LncRNAs in cancers like TNBC [89, 100]. Furthermore, LncRNAs show significant promise for personalized medicine, particularly when combined with coding genes and SNPs. This approach could soon bring precision treatments closer to clinical application, especially for complex diseases like cancer, where there are genomic, epigenomic, and transcriptomic variations [93, 104].

The advancement of LncRNA applications in the future depends on technology that can recognize and validate their functions, structures, and mechanisms. Attempts to annotate LncRNAs like the formation of GENCODE and databases such as LncRNAtor, LNCipedia, and NONCODE are rapidly expanding knowledge of LncRNA loci [93]. Cutting-edge methodologies, including genome-wide DNA binding analysis such as ChiRP, CHART, RAP, and RNA-protein interaction mapping such as CLIP-seq and PAR-CLIP-seq [92], are paving the way for functional insights. Altogether, it can be concluded that there exists a multifaceted role of LncRNAs in various cancer types, highlighting their potential as diagnostic, prognostic biomarkers and therapeutic targets. Additionally, innovative strategies like RNAi and CRISPR technology may help in improving LncRNA-based cancer therapies as well as refine targeted approaches in personalized medicine.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

Uncovering the Crosstalk between Pseudogenes and Non-Coding RNAs in Tumorigenesis

Duygu Sari-Ak, Lütfi Tutar and Yusuf Tutar

Abstract

The expression of genes gets influenced by pseudogenes through their interactions with non-coding RNAs (ncRNAs) that include microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) and PIWI-interacting RNAs (piRNAs). This chapter seeks to describe pseudogene-ncRNA interactions, to highlight their new roles in cancer biology, and to discuss their potential in clinics. Pseudogenes act as competing endogenous RNAs (ceRNAs) to bind miRNAs and control the expression of oncogenes and tumor suppressors, thus affecting oncogenic and tumor-suppressive pathways. Furthermore, pseudogene-derived lncRNAs and piRNAs regulate transcription and genome stability, which are crucial for cancer progression. Recent studies have shown that pseudogene-ncRNA networks can be used as biomarkers for early cancer detection, prognosis, and therapeutic intervention. However, their roles are quite different from one cancer type to another. This chapter reviews the current understanding of pseudogene-ncRNA interactions, with a focus on their significance and potential to improve cancer diagnosis and treatment.

Keywords: pseudogenes, non-coding RNAs, tumorigenesis, ceRNAs, cancer biomarkers

1. Introduction

Pseudogenes are sequences of genomic DNA that are similar to protein-coding genes but have mutations, frameshift insertions, or premature stop codons and therefore cannot produce functional proteins. Unlike other non-coding RNAs (ncRNAs), pseudogenes mainly result from retrotransposition or gene duplication events [1]. Once considered as "junk" DNA, pseudogenes are now classified as active regulators of gene expression and have been shown to influence various biological processes including cancer through interactions with microRNAs and long non-coding RNAs (lncRNAs) [2].

In cancer biology, pseudogenes regulate gene expression, thus playing a crucial role in tumorigenesis through the interaction with ncRNAs. Pseudogene-miRNA interactions are accurately predicted by computational tools, including ensemble learning-based approaches such as ELPMA, and this is significant for targeted cancer

therapy [2]. For instance, in esophageal carcinoma (ESCA), pseudogenes influence cancer progression through the sequestration of miRNAs, thereby influencing transcription factors such as SOX2 and TFAP2A and cell differentiation and reprogramming pathways [3]. In breast cancer, the GBP1P1 pseudogene functions as a miRNA sponge to affect survival, self-renewal, and migration of cancer cells through the GBP1/hsa-miR-30d-5p axis [4]. These specific examples explain the molecular pathways through which pseudogenes act.

Pseudogenes also operate through RNA-independent mechanisms, including lncRNAs-induced chromatin remodeling that significantly affects oncogenic processes [5]. Furthermore, during embryonic development, pseudogenes such as PI4KAP1 and TMED10P1 interact with proteins and miRNAs, indicating their wider physiological functions beyond cancer [6]. Because pseudogene-ncRNA interactions are important in biology, it is essential to investigate them in detail. This chapter provides a review of the existing evidence on the regulatory function of pseudogenes in tumorigenesis, with an emphasis on the specifics of the molecular mechanisms of their interactions with ncRNAs. An analysis of these complex regulatory networks will help to elucidate their clinical significance and provide new ideas in cancer research, diagnosis, and treatment.

2. Pseudogenes and functional regulators

2.1 Evolutionary perspective

Pseudogenes affect embryogenesis and diseases through their evolutionarily conserved regulatory functions. Dynamic pseudogene expression, including genes such as PI4KAP1, TMED10P1, and FBXW4P1, plays important role in the maintenance of embryonic stem cell (ESC) self-renewal through modulation of transcriptional and post-transcriptional processes by protein and miRNA interactions [6, 7]. The correlated expression patterns between pseudogenes and their parent genes indicate that there are common regulatory elements that are necessary for proper embryonic development [6].

Furthermore, the conserved expression of pseudogenes across species highlights their basic function in organ identity. Pseudogenes are also related to congenital diseases, which further prove their importance in developmental biology and disease [7]. Studies on regulatory DNA mutations and promoter motifs have demonstrated that pseudogenes have evolving regulatory capabilities that are consistent with their ability to adapt to different developmental stages and environmental conditions [8]. However, to understand the function of pseudogenes in embryogenesis and their role in various diseases, more extensive studies are required.

2.2 Classification and general features of non-coding RNAs

Non-coding RNAs are functional RNA molecules that regulate gene expression without encoding proteins. Classified based on their size, structure, and biological roles, the main types of ncRNAs include long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and PIWI-interacting RNAs (piRNAs) (**Figure 1**).

LncRNAs, generally longer than 200 nucleotides, regulate gene expression at chromatin, transcriptional, and post-transcriptional levels through diverse mechanisms [9, 10]. Aberrant expression of lncRNAs is associated with various diseases

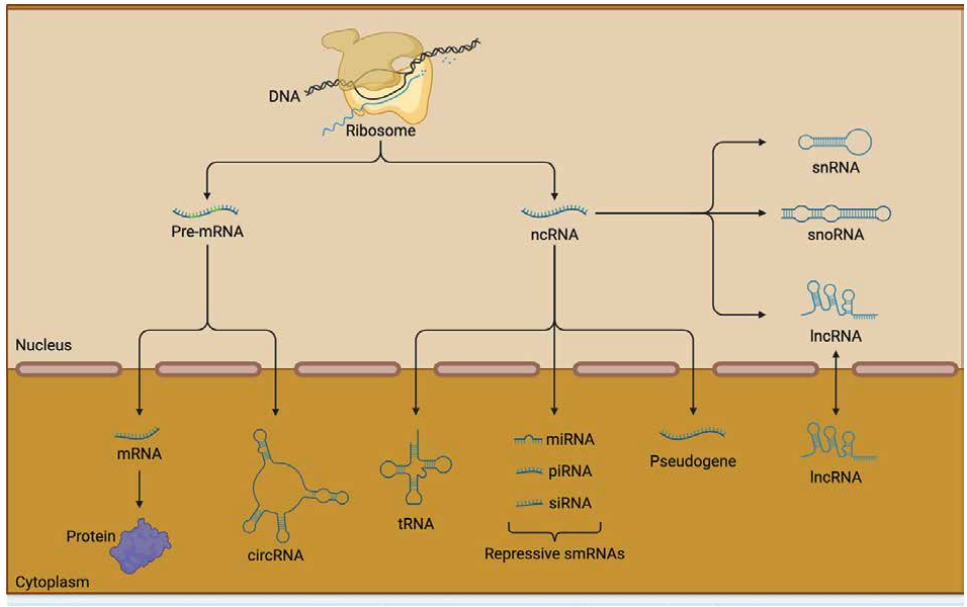

Figure 1.
The classification system for coding versus non-coding RNAs based on their structural attributes and functional relevance (created in BioRender. Tutar Y. (2025) https://BioRender.com/l65g280).

including cancer and cardiovascular disorders, making them valuable biomarkers and therapeutic targets [9, 11].

MiRNAs are short RNA molecules (~22 nucleotides) that primarily mediate post-transcriptional gene silencing by binding to complementary sequences on target messenger RNAs (mRNAs), leading to their degradation or translational inhibition. These small regulatory RNA molecules control essential cellular functions including proliferation and differentiation and apoptosis, while their altered expression causes cancer and multiple pathological states [11].

PiRNAs with lengths ranging from 24 to 31 nucleotides serve primarily to protect germline cell genomes through transposable element silencing that prevents genomic instability. The therapeutic field of piRNAs has emerged through recent studies to show promise in clinical practice especially for cancer diagnostics and therapeutics [12].

2.3 The role of pseudogene-ncRNA interactions in cancer biology

The interactions between pseudogenes and ncRNAs play a crucial role in controlling gene expression and different cellular functions in cancer biology. Primarily, pseudogenes act as competing endogenous RNAs (ceRNAs) to bind miRNAs. The ceRNA function of pseudogenes enables them to control miRNA availability that in turn regulates the expression of key oncogenes and tumor suppressors for maintaining cellular homeostasis. The disturbance of this complex equilibrium can have major implications for cancer development and its progression.

Studies in recent years have described certain ways through which these interactions occur. ESCA pseudogenes function to manage miRNAs, which later help in cancer development through their effects on cell differentiation and reprogramming processes [3]. Or, in breast cancer, GBP1 expression is to enhance with miRNA, which leads to increased cancer cell survival, clonogenic potential, and metastatic capabiliti [4].

The pseudogene-derived lncRNA SNRPFP1 sequesters the tumor-suppressive miR-126-5p in hepatocellular carcinoma, which leads to cancer cell proliferation and migration and thus drives tumor progression [13].

In hormone-dependent cancers such as prostate and breast cancer, pseudogene-ncRNA networks comprising both miRNAs and lncRNAs greatly affect cancer progression, metastasis, and patient prognosis [14].

Although extensive research proves the importance of these interactions in cancer biology, it is essential to completely understand their multifaceted regulatory functions to achieve therapeutic applications from these molecular findings.

3. Pseudogenes' functional roles and mechanisms

3.1 Pseudogenes as competing endogenous RNAs

Some pseudogenes have a regulatory function through their action as ceRNAs, which regulate protein-coding gene expression. Their main function to achieve this is by sequestering miRNAs, which otherwise would target mRNAs. The tumor suppressor PTEN is an example where PTENP1 acts as a ceRNA to bind miR-19b and miR-21 thus leading to the enhancement of PTEN expression that halts oncogenic processes [2].

The ceRNA network establishes functional crosstalk between pseudogenes, miRNAs, and mRNAs [15]. In breast cancer, for instance, GBP1P1 functions within the GBP1/hsa-miR-30d-5p axis, indirectly regulating GBP1 expression and contributing to cancer progression [4].

In addition to cancer, pseudogenes regulate miRNA activity in inflammatory diseases as well as other pathological conditions [16]. Understanding pseudogene ceRNA mechanisms in detail will generate new insights about disease progression and develop new diagnostic and therapeutic approaches [15].

3.2 Transcriptional and post-transcriptional regulation beyond ceRNA activity

Pseudogenes function beyond acting as miRNA sponges to regulate gene expression by creating endogenous siRNAs and through transcriptional interference. The described pathways emphasize the complex nature of pseudogenes in maintaining cellular balance and disease progression.

The transcription of nearby genes may be disrupted by pseudogenes. The mechanism of disruption occurs through the competition or obstruction of transcriptional machinery that binds to a pseudogene with machinery that binds to adjacent genes thus changing expression patterns [6]. In cancer, such interference can contribute to oncogenic pathways by affecting genes involved in differentiation and reprogramming [3].

Some pseudogenes produce endogenous siRNAs that serve to destroy mRNA transcripts or block their translation that adds an additional layer of post-transcriptional gene expression regulation [6]. These siRNAs have been found to regulate oncogenes and tumor suppressors, which make them important for tumorigenesis [17].

The detailed molecular mechanisms behind the regulatory functions of pseudogenes continue to be the subject of current research despite their growing recognition. The lack of complete annotation of pseudogenes together with species variations creates obstacles for thorough functional characterization [18, 19].

3.3 Dual roles of pseudogenes in cancer: Tumor suppressors and oncogenes

The biological processes of cancer are affected by pseudogenes which act as either tumor suppressors or oncogenes through their regulatory relationships with non-coding RNAs and gene expression networks.

The tumor suppressor CSPG4P12 along with other pseudogenes functions to block epithelial-mesenchymal transition (EMT) in colorectal cancer (CRC). The regulatory network leads to E-cadherin elevation and vimentin and N-cadherin and MMP9 repression, which results in reduced cancer cell proliferation and invasion as well as motility [19]. The transcriptional repressors ZEB1 and SNAIL are downregulated by CSPG4P12 that enables EMT suppression through epithelial identity restoration. The suppression of EMT serves to reduce metastatic potential and therefore functions as a tumor suppressor in CRC. PTENP1 functions as a decoy for miRNAs that target the PTEN tumor suppressor thus maintaining PTEN expression and inhibiting tumor progression [17].

In contrast to certain pseudogenes, some have oncogenic activity. OCT4-pg5 for example acts as an oncogene in bladder cancer by sponging miR-145-5p, leading to an increase in OCT4B expression and activation of the Wnt/β-catenin signaling pathway. This process increases the EMT and leads to the cancer cell dissemination and invasion [20]. In breast cancer, GBP1P1 enhances oncogenic activity through the regulation of the GBP1/hsa-miR-30d-5p axis, which leads to the upregulation of GBP1 and enhances cell viability, migration, and colony formation [4]. GBP1P1 binds to miR-30d-5p, which normally suppresses GBP1 expression. Through acting as a sponge, GBP1P1 re-established GBP1 protein levels and enhanced oncogenic signaling. This mechanism enhances the ability of cancer cells to invade and metastasize, thus highlighting its potential as a target for therapy.

Furthermore, pseudogenes can affect cancer stemness. For instance, CTB-63M22.1 has been implicated in the regulation of stemness-related pathways in hepatocellular carcinoma and may influence the prognosis and treatment of the disease [21].

These examples reveal that pseudogenes play both oncogenic and tumor suppressor roles in tumorigenesis and that they may be useful as therapeutic and diagnostic tools for different types of cancers.

4. Functional interplay between pseudogenes and long non-coding RNAs

LncRNAs are important regulators of gene expression and they do so through several mechanisms including chromatin remodeling, transcriptional regulation, and post-transcriptional regulation. They can function as scaffolds, guides, or decoys in gene expression control [9]. LncRNAs bind to chromatin modifying complexes and enzymes and determine chromatin states and transcriptional output. They also bind to mRNAs and affect their splicing, stability, and translation and thus determine the phenotype [22–24].

The aberrant expression of lncRNAs has been found to be associated with the diseases such as cardiovascular diseases and cancer. Their rapid evolutionary diversification and cell-specific functions make their classification difficult but highlight their biological significance [9, 25]. Elucidation of their structural characteristics and interaction with pseudogenes may help to elucidate their functions in disease mechanisms [22].

Pseudogenes interact with lncRNAs through various mechanisms. Some pseudogenes can function as ceRNAs, sequestrating miRNAs and thereby affecting lncRNAs

levels, for instance, GBP1P1 in breast cancer which targets the GBP1/hsa-miR-30d-5p axis [4]. Others share promoter elements or exhibit antisense transcription that can hinder lncRNA transcription [17].

A notable example is the regulatory relationship between PTENP1 and the lncRNA HOTAIR, which both affects PTEN expression through the PI3K/AKT pathway. This axis is crucial for cell proliferation and metastasis, especially in cancer [26].

Furthermore, pseudogene-derived lncRNAs and associated microRNAs have been identified as potential cancer biomarkers due to their specificity and expression profiles [27]. The network of their functions affects cancer progression, and therefore, their potential in designing diagnostic and therapeutic approaches must be further investigated.

5. Small non-coding RNAs with their critical roles

5.1 Pseudogene-miRNA interactions in gene regulation

While the ceRNA function of pseudogenes is mentioned earlier, this section provides further insight into the broader category of small ncRNAs and their roles and uses. MiRNAs regulate gene expression by binding to the 3′-untranslated regions (3′-UTRs) of target mRNAs, leading to translational repression or degradation through Argonaute-containing silencing complexes. They are key regulators of proliferation, differentiation, and apoptosis. Altered miRNA expression is involved in numerous diseases, especially cancer where they can act as oncogenes or tumor suppressors [11, 28, 29]. Their specific expression patterns also render them useful as biomarkers and therapeutic targets.

Pseudogenes affect miRNA function through the mechanism of ceRNAs. As decoys, pseudogenes sequester miRNAs and thus prevent them from targeting their target mRNAs, thereby indirectly affecting gene expression. For example, PTENP1 acts as a sponge for miR-19b and miR-21 that otherwise degrade PTEN mRNA leading to PTEN expression preservation and tumor suppression [26, 30, 31]. This interaction is depicted in **Figure 2**, where PTENP1 protects PTEN mRNA from being degraded by miRNAs through competition for shared miRNAs, thereby increasing PTEN protein expression and reducing cancer progression.

In the same manner, KRASP1 binds to miR-141 and miR-185 and increases KRAS expression in colorectal and lung cancers that leads to tumor progression [32, 33].

The pseudogene network with miRNAs and mRNAs depends on the transcriptional environment together with cell tissue origin and disease condition. ELPMA and ceRNAR are analytical tools that enable researchers to study pseudogene-miRNA interactions and their regulatory functions [2].

Pseudogene networks play essential roles in both cancer biology and normal physiological developmental processes including embryonic development. Pseudogenes serve as dual regulators of developmental processes while affecting disease states, which make them useful markers for disease diagnosis and prognosis and therapeutic targets [6].

5.2 PIWI-interacting RNAs in cancer

PiRNAs bind to PIWI proteins to defend genome stability by suppressing transposable elements. PiRNAs get processed independently of Dicer and function mainly in germline cells, whereas miRNAs do not. Research now demonstrates that

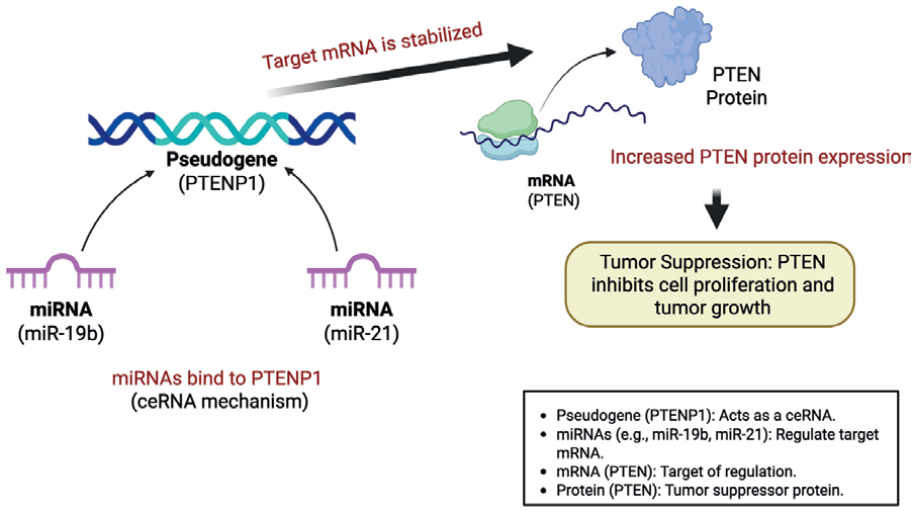

Figure 2.
Pseudogene-ncRNA interactions in gene regulation. The pseudogene, PTENP1 operates as a ceRNA by engaging with microRNAs, including miR-19b and miR-21, thus preserving the target messenger RNA, PTEN, from degradation. Consequently, this interaction facilitates an increase in PTEN protein expression, which is crucial for inhibiting tumor progression (created in BioRender. Tutar Y. (2025) https://BioRender.com/c19g442).

piRNAs play a role in cancer development through epigenetic silencing of gene expression and oncogenesis [34–36].

Abnormal piRNA expression leads to cancer onset through the inhibition of cancer-controlling genes and activation of cancer-promoting genes. The piR-823 gene accelerates multiple myeloma progression but the piR-651 gene enhances gastric cancer proliferation, while the piR-932 gene functions as an oncogene suppressor in colorectal cancer and leads to worse outcomes due to decreased expression levels [37–39].

Research has revealed that pseudogenes serve as possible sources of piRNA expression. Pseudogene-derived piRNAs control PIWI protein activity in ovarian cancer, which affect tumor progression [37]. The findings indicate that pseudogenes interact through a new regulatory mechanism with the piRNA pathway to emphasize their importance in cancer research.

Scientists investigate piR-823 among other piRNAs because of their stable expression in cancer tissues and their ability to act as cancer-specific biomarkers for early detection. Therapeutic interventions focused on oncogenic piRNA inhibition or tumor-suppressive piRNA reactivation represent possible treatment approaches [10, 39].

Current knowledge about piRNAs and their pseudogene origins does not fully explain their biological functions. Additional studies need to occur to establish the functions of these molecules within somatic tissues while evaluating their clinical value. The intricate nature of piRNA biogenesis alongside their pseudogene interactions demonstrates why extensive research must be conducted to understand their complete effects on cancer development and treatment [10, 40].

6. Cancer types and pseudogene-ncRNA networks

The development of oncogenesis through pseudogenes and ncRNAs occurs through intricate mechanisms that differ between various cancer types and their associated regulatory pathways.

6.1 Breast cancer

The interactions between pseudogenes and ncRNAs serve essential functions in the development of breast cancer and its metastatic progression. The pseudogene PTENP1 binds to miR-19b and miR-21, which results in maintaining PTEN tumor suppressor expression and reducing tumor progression [4]. According to Mohebifar et al., the pseudogene GBP1P1 functions through the GBP1/hsa-miR-30d-5p axis to drive cancer cell proliferation and migration while also enhancing colony formation [4]. HOTAIR functions as a long non-coding RNA to establish chromatin conformation changes and modify gene expression when paired with pseudogenes, which leads to metastasis development [41].

6.2 Colorectal cancer

The pseudogenes KRASP1 and BRAFP1 function as competing endogenous RNAs to bind miR-185 and miR-141 which result in KRAS oncogene activation through downstream MAPK signaling pathways [42]. MALAT1 pseudogene-lncRNA networks help CRC cells undergo EMT while also making them resistant to chemotherapy and radiotherapy. The interactions affect essential cellular processes that include proliferation, apoptosis, and differentiation. The detailed study of these complicated ceRNA interactions creates new opportunities to identify biomarkers and therapeutic approaches [11, 12, 43, 44].

6.3 Lung cancer

The process of oncogenic transformation in lung cancer relies on pseudogenes to regulate miRNAs and piRNAs. DUXAP8 acts as an miR-29a-5p sponge to enhance cell proliferation, while HSPA7 binds to miR-34a which disrupts cell cycle checkpoints [42, 45, 46]. PiRNA derived from pseudogenes acts to silence tumor suppressor genes during cancer development. The piRNAs piR-651 among others contribute to elevated cell proliferation in lung cancer cells as well as gastric cancer cells [46–48].

6.4 Prostate and ovarian cancers

PTENP1 in prostate cancer binds to oncogenic miRNAs like miR-17, which helps preserve PTEN expression to create a tumor suppressive outcome [30, 31]. PCA3 stands as a prostate-specific long non-coding RNA, which shows increased levels in prostate cancer tissues and functions as an accurate biomarker [49]. PIWI protein activity suppression by pseudogene-derived piRNAs in ovarian cancer leads to accelerated tumor progression [14].

7. Clinical implications and future directions

The detailed molecular connections involving pseudogenes and ncRNAs hold substantial importance in clinical situations, especially regarding cancer diagnosis, prognostic evaluations, and treatment plans. These regulatory frameworks have created opportunities to develop innovative personalized medicine approaches by investigating pseudogenes and ncRNAs for disease progression prediction and targeted treatment strategies.

7.1 Pseudogenes and ncRNAs as biomarkers in cancer diagnosis

Pseudogenes and ncRNAs are increasingly acknowledged as significant biomarkers for cancer diagnosis due to their consistent expression profiles and specificity. PTENP1 pseudogenes along with piRNAs and lncRNAs have proven valuable across different cancer types, which make them suitable for non-invasive diagnostic procedures. The metastatic potential and prognostic value of HOTAIR lncRNA in cancer patients has been established. These biomarkers improve diagnostic precision and allow for the early identification of tumors. **Table 1** presents a summary of pseudogenes and ncRNAs together with diagnostic tools that demonstrate effectiveness in cancer detection and prognosis. Each category presents particular examples alongside their clinical importance and the methods used to apply these biomarkers for diagnosis.

Although pseudogenes and nc-RNAs hold great promise for cancer diagnostics, several significant problems still exist regarding the standardization of these biomarkers across different populations and cancer types. To validate these biomarkers in clinical settings and incorporate them into current diagnostic protocols, additional empirical research is necessary. Moreover, the integration of multi-omics panels could enhance the accuracy of cancer risk assessment and improve the efficacy of early detection practices [54].

7.2 Therapeutic targeting of pseudogene-ncRNA networks

Pseudogene-ncRNA network modulation is new oncology strategies that disrupts oncogenic pathways. Some approaches include synthetic analogs and suppressors, antisense oligonucleotides (ASOs), RNA interference (RNAi), and CRISPR/Cas9 to reconstitute pseudogene-ceRNA networks. These techniques are currently under rigorous investigation to face the challenges of cancer therapy, particularly in the case of targeting oncogenic pseudogenes and ncRNAs.

Synthetic miRNA mimics/inhibitors modulate ceRNA networks, such as inhibiting KRASP1 to downregulate KRAS in colorectal cancer [33]. The pseudogene transcripts are targeted by ASOs, and the oncogenic functions of these transcripts are disrupted. ASOs, such as DUXAP8, suppress tumor growth in lung cancer models. Recent progress in ASO delivery systems may help to increase their usability [55]. Through RNAi, the pseudogene OCT4-pg4 is targeted, and tumor growth is reduced, and tumor suppressor activity is restored [33, 56]. CRISPR/Cas9 edits pseudogene loci or

Category	Examples	Clinical significance
Pseudogenes as biomarkers	PTENP1	Interacts with miRNAs, influencing prognosis in prostate and breast cancers. Reduced PTENP1 expression correlates with poor outcomes, making it a prognostic biomarker [50]
ncRNAs in cancer detection	piRNAs (e.g., piR-823); miRNAs; lncRNAs (e.g., HOTAIR)	piR-823 is elevated in multiple myeloma, aiding non-invasive detection; a 4-miRNA model achieves over 90% sensitivity for multiple cancers; HOTAIR is linked to metastasis in breast and CRC [50–52]
Diagnostic tools and techniques	Machine learning models, liquid biopsy	Machine learning using piRNAs provides high accuracy in CRC diagnosis; liquid biopsy techniques improve biomarker detection using reverse transcription enzymes [53]

Table 1.
Biomarkers and diagnostic tools in cancer detection.

ncRNA-binding sites to disrupt disease-causing interactions and restore normal gene expression [57]. **Figure 3** shows how pseudogenes and ncRNAs interact with each other during the progression and treatment of breast cancer.

Despite the challenges that exist, these methodologies hold promise. The use of computational techniques and artificial intelligence for the prediction and mitigation of off-target effects is critical for the advancement of small RNA therapeutics [33]. Standardized detection protocols and studies on pseudogene-ncRNA mechanisms are crucial for the therapeutic efficacy.

7.3 Challenges and opportunities in translating findings to clinical applications

The conversion of findings from pseudogene-ncRNA networks into practical clinical applications is a task that is fraught with a number of challenges as well as possibilities. The complexity of these regulatory networks, potential off-target effects, delivery system limitations, and ethical considerations are major challenges. However, progress in multi-omics integration, personalized medicine, and delivery technologies present some possible solutions. The challenges and opportunities are given in **Table 2**.

7.4 Future directions in research

Further research on the complete potential of pseudogene-ncRNA networks in oncology should be directed toward the following topics in the future. Concerning functional characterization, the roles of uncharacterized pseudogenes and non-coding RNAs in cancer pathogenesis should be determined. The therapeutic efficacy of

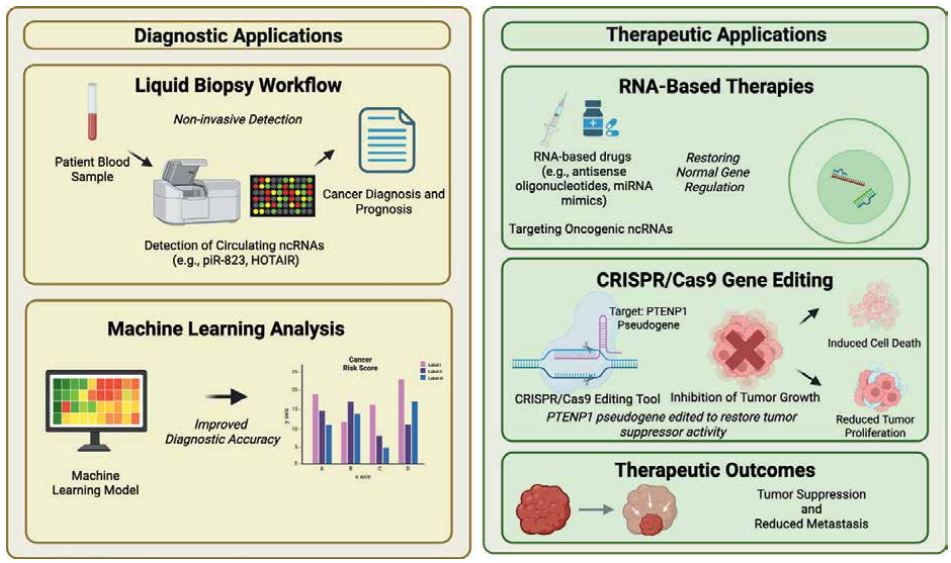

Figure 3.
Diagnostic and therapeutic applications of pseudogenes and ncRNAs in cancer. This chart shows diagnostic uses such as liquid biopsies for detecting circulating ncRNAs (e.g., piR-823, HOTAIR) and machine learning algorithms to analyze expression profiles to enhance cancer diagnosis and risk evaluation. Therapeutic applications including antisense oligonucleotides, miRNA mimetics, and CRISPR/Cas9 targeting pseudogenes (e.g., PTENP1) to restore gene regulation, reduce tumor growth, and mitigate metastasis (created in BioRender. Tutar Y. (2025) https://BioRender.com/p70j490).

Challenges	Opportunities
The ceRNA activity of pseudogenes is highly context-dependent, with variations in expression levels across tissues complicating universal therapeutic strategies [32, 58]	Combining transcriptomics, proteomics, and epigenomics helps unravel pseudogene-ncRNA networks, facilitating biomarker discovery and therapeutic targets [32]
Therapeutic techniques, such as RNAi and CRISPR/Cas9, may interfere with non-target transcripts, disrupting normal cellular functions [59]	Single-cell sequencing reveals pseudogene and ncRNA expression, while spatial transcriptomics maps pseudogene-ncRNA interactions, aiding biomarker and therapeutic target discovery [52, 60–62]
Tissue-specific targeting remains challenging despite the potential shown by nanoparticle-based delivery systems [59]	Advances in lipid nanoparticles and exosome-based systems offer promising solutions for efficient and targeted RNA therapy delivery [59]
Many pseudogenes and ncRNAs are poorly characterized, hindering their development as therapeutic targets. Comprehensive studies are needed to understand their roles in oncogenesis [1]	The specificity of pseudogene-ncRNA interactions supports tailored therapeutic approaches aligned with the personalized medicine goals [58]
Genetic material alterations present ethical and regulatory dilemmas, requiring attention to ensure patient safety and societal acceptance [59]	Panels of pseudogenes and associated ncRNAs as diagnostic tools can revolutionize cancer screening, enabling early detection and improving outcomes [63]

Table 2.
The key challenges and opportunities in pseudogene-ncRNA research and clinical applications.

the alteration of interactions between pseudogenes and ncRNAs should be validated by *in vivo* studies using preclinical models. Clinical trials should be conducted with potential candidates. To understand the relation between pseudogenes and ncRNAs and their impact on biological pathways and diseases, computational approaches and experimental practices should be integrated.

8. Conclusion

Pseudogenes, once considered evolutionary relics, are now recognized as dynamic regulatory elements that are integrated into the gene expression networks through their interactions with non-coding RNAs. Pseudogenes regulate both tumor suppressive and oncogenic pathways through mechanisms that include miRNA sponging, transcriptional interference, and piRNA generation. Their roles span across various levels of cellular regulation and are highly context-dependent, showing tissue-specific and cancer type-specific functions. The increasing knowledge of pseudogene-ncRNA interactions holds great promise for the diagnosis and treatment of cancer. These molecular interactions not only provide useful biomarkers for early detection and prognosis, but also provide potential therapeutic targets, particularly in the context of precision oncology. However, the complexity of their regulatory functions and the heterogeneity across different tumors underscore the need for further mechanistic insights and translational research. With the integration of multi-omics technologies and computational tools, pseudogenes and their ncRNA partners are ready to transform the future of cancer biology and personalized medicine.

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Chapter 3

Perspective Chapter: The Role of Long Non-Coding RNAs in X Chromosome Dosage Compensation

Sylvia Garza-Manero, Hober Nelson Núñez-Martínez, Gustavo Tapia-Urzúa and Félix Recillas-Targa

Abstract

Genetic sex determination required the specialization of an ancestral autosome pair into sexual chromosomes. This evolutionary trajectory involves recombination suppression in the sexual pair and degeneration of one chromosome. In species with a male XY heterogametic system, the Y chromosome is highly degenerated, creating a difference in the dosage of X-linked genes between females and males. To overcome this difference, one X chromosome is transcriptionally silenced in female mammals. Conversely, genes on the single-male X chromosome of lizards and the fruit fly are overexpressed, reaching the transcriptional output produced by two X chromosomes in females. Despite promoting opposite effects on transcription, these dosage compensation mechanisms share relevant features, including the modification of the chromatin landscape and three-dimensional conformation orchestrated, at least in part, by long non-coding RNAs transcribed from the target X. In this chapter, we describe the mechanisms of X chromosome dosage compensation in eutherians, marsupials, Drosophila, and a lizard. We focus on the leading role of the respective long non-coding RNA, XIST in eutherians, RSX in marsupials, roX1 and roX2 in the fruit fly, and the recently described MAYEX in lizards, in the ambitious task of equalizing the transcriptional output of X-linked genes between females and males.

Keywords: long non-coding RNAs, X chromosome, dosage compensation, X inactivation, X overexpression, sex chromosomes, genetic-sex determination

1. Introduction

In animals with sexual dimorphism, specific differentiation programs direct the development of sexual characters, including structures such as ovaries and testes. These programs can be triggered by either environmental or genetic cues (**Figure 1**). Observations along animal evolution support that genetic sex

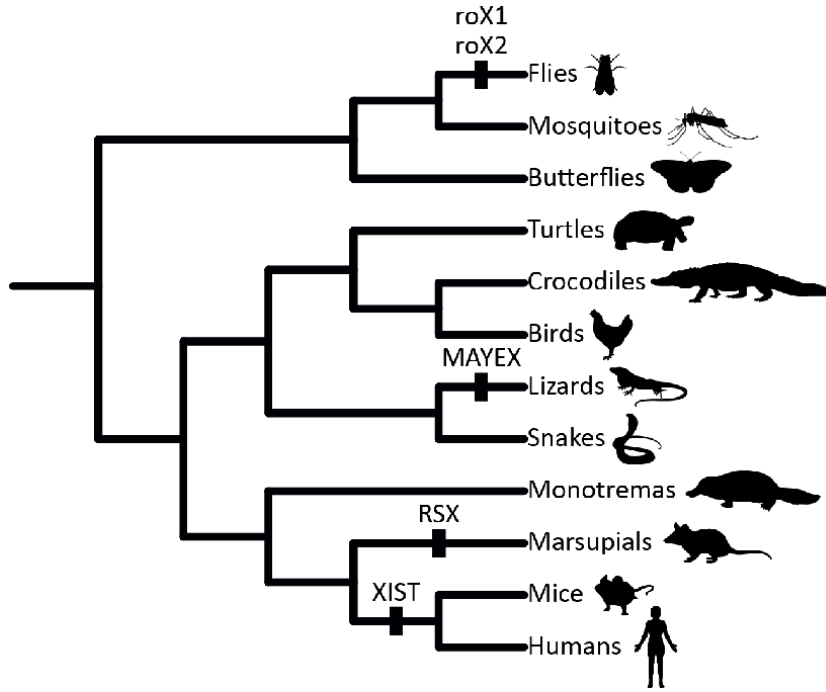

Figure 1.
LncRNAs target global dosage compensation in various XY species. *Phylogenetic associations of representative animal species with either temperature-guided: turtles and crocodiles; or genetic sex determination systems, including both the male heterogametic (XY) and the female heterogametic (ZW) sex determination systems. Groups with XY sex chromosomes include fruit flies, mosquitos, lizards, monotremes, marsupials, and eutherian mammals; while butterflies, birds, and snakes harbor ZW sex chromosomes. Global dosage compensation between sexes has been reported for eutherians, marsupials, fruit flies, and more recently, lizards, mosquitos, and butterflies [1–6]. roX, MAYEX, RSX, and XIST are lncRNAs with a leading role in global X chromosome dosage compensation between sexes, which arose in four of these different groups [7–11]. Branches indicate divergence events and do not represent a time scale. Silhouettes correspond to representative species of each group.*

determination arose after environmental sex determination, perhaps reflecting the selection pressure in response to changing environmental conditions [1]. The most recent ancestor of reptiles and mammals had a temperature-dependent sex determination that was retained in turtles and crocodiles. However, related species that transitioned further away from water, such as mammals, birds, snakes, and lizards, developed genetic sex determination systems in a similar period of time [1]. These genetic sex determination systems rely on sexual chromosomes. Although their origin is evolutionary independent, mammals and lizards developed a male heterogametic system, in which females carry two X chromosomes (XX) and males, one X and one Y chromosome (XY). In contrast, a female heterogametic system operates in birds and snakes, with males carrying two Z chromosomes and females one Z and one W chromosome. The evolution trajectory of sexual chromosomes has been recently reviewed in Ref. [12]. In this work, the authors discuss different models that intersect the main events of sexual chromosome development: the initial acquisition of the genetic cue by an ancient autosome, and the suppression of meiotic recombination between the autosomal pair, the massive loss of homologous genes in the Y or W chromosomes, and the complete degeneration of this chromosome and/or the sexual chromosome turnover [12].

The fact that the sexual chromosome pair is formed by a highly degraded Y or W and a gene-rich X or Z leads to an unbalance in the gene dosage, compared to autosomal pairs and between sexes. Counteracting this unbalance does not seem to be a rule in all studied species with heterogametic sex determination systems [13]. However, distinct mechanisms for dosage compensation have been identified and described for XY model organisms, representing eutherians [2], marsupials [3], and the fruit fly [4]; and have started to be explored in other animal species such as lizards, mosquitos, and butterflies [1, 5, 6]. Thus far, two main strategies equalize the transcriptional output of the X chromosome between females and males. The first consists in the silencing of a whole X chromosome in females, which is referred to as X chromosome inactivation (XCI) and observed in eutherians and marsupials [3, 14]. The second was first described in fruit flies [4] and, more recently, in a lizard [1], where a global twofold upregulation of the male X chromosome or an X chromosome overexpression (XCO) is observed. Importantly, this strategy also equalizes the sexual and autosomal gene expression [1]. Despite having independently evolved in the four animal groups, targeting different sexes and producing opposite transcriptional outputs, XCI and XCO share relevant mechanistic features. These include changes in chromatin organization and compaction of a complete X chromosome, mediated by pre-existent epigenetic machinery that is targeted to the X chromosome with a leading role of long non-coding RNAs (lncRNAs) (**Figure 1**).

LncRNAs are currently recognized as key regulators of gene expression and genome topology [15]. Three of the first examples describing an RNA role in this matter were the mammalian XIST (standing for X-inactive specific transcript) [7] and the Drosophila roX1 and roX2 (roX standing for RNA on the X) [8, 9], the three of them involved in the X chromosome dosage compensation. Since then, these lncRNAs have been widely studied and have become a reference point for proposing the molecular mechanisms by which lncRNAs exert their regulatory functions and for the development of RNA-based techniques [16]. In this chapter, we focus on the role of lncRNAs in X chromosome dosage compensation. First, we describe the extensively studied mechanism by which XIST as well as other lncRNAs achieves XCI in eutherian mammals. We complement the repressive role of lncRNAs on the female X chromosome by describing RSX (standing for RNA on the silent X) from marsupials, and we then contrast it with the role of roX RNAs supporting the global overexpression of the *Drosophila* male X chromosome. The last dosage compensation system has also been reported in reptiles [1], where the recently described MAYEX represents the lncRNA component [10]. Finally, we discuss the advantages of depositing relevant molecular mechanisms for the X chromosome dosage compensation on lncRNAs.

2. X chromosome inactivation in eutherian mammals

In female mammals, one of the two X chromosomes is inactivated to equalize gene dosage with males by a process-denominated X-chromosome inactivation (XCI). XCI occurs randomly in one of two X chromosomes, leading to an active (Xa) and an inactive (Xi) X chromosome [17–19]. This process occurs early in the female development (specifically during the preimplantation stage in mice and humans) to ensure the transcriptional silencing of almost the entire X chromosome across the organism's lifespan [20, 21]. XCI implies coordinated-complex layers of chromosome-wide silencing including RNA polymerase II (Pol II) eviction; deposition of repressive histone marks; and DNA methylation [14].

XCI begins within a highly conserved complex locus (around 2.3 Mb), denominated X inactivation center (XIC), harboring the necessary genetic elements to lead XCI [22–24]. These elements encode lncRNAs, including XIST, TSIX, Repa, Xite, Jpx, Ftx, and Tsx [25–28]. These elements are essential in the initiation and triggering of XCI, as demonstrated by integrating the XIC-containing transgenes into autosomal sites and recapitulating the silencing effect [29]. Efforts in characterizing the roles of these XIC-containing lncRNAs have shed light on their role in driving XCI. For instance, TSIX, Xite, and Ftx prevent XIST activation in the Xa [25, 30, 31]; whereas Jpx and RepA facilitate XIST activation [32, 33]. Thus, XIC represents an intriguing aspect of XCI due to its essential role in the choice of Xi in the same nucleus. In this section, we focus mainly on the mechanisms driving XCI by XIST on mice and humans.

2.1 XIST spreading, coating, and triggering the XCI

The major orchestrator of XCI is the X inactive-specific transcript (XIST), a monoallelic-transcribed lncRNA from the XIC of the future Xi chromosome [6, 15]. XCI initiates with the activation of XIST transcription resulting in a coordinated cascade of molecular waves, including the recruitment of chromatin regulators to induce transcriptional silencing of most of the genes located in the target X [34, 35]. Upon XIST is transcribed, it accumulates at its locus and then spreads to several nucleation sites located at gene-rich regions associated in physical proximity to the XIST locus (**Figure 2A** and **B**). Later, XIST coats the rest of the Xi, including gene-poor regions, to establish chromosome-wide silencing [36, 37]. In addition, XIST recruits numerous proteins through direct interaction with its several repeat domains (A-F) required for the multiple functions of XIST-mediated transcriptional silencing, including initiation of gene silencing (repeat-A), recruitment of Polycomb Repressive Complexes (PRC1 and PRC2) (repeat B-F), and coating across X (repeat C and E) (**Figure 2C**) [38–43]. Thus, XIST diffuses across the future Xi, exploiting its repeat domains and the chromatin structure to trigger XCI.

Histone deacetylation and H2A ubiquitination are some of the earliest events of XCI occurring across the future Xi previous to transcriptional silencing [44]. XIST, through repeat-A, directly interacts with SPEN (also known as SHARP and Msx2-interacting protein) [42]. SPEN interacts with SMRT, a co-repressor complex, to activate the histone deacetylase HDAC3 and promote Pol II eviction from chromatin [45, 46]. Later, XIST leads to PRC1 and PRC2 recruitment to induce H2AK119ub1 and H3K27me3 deposition, respectively [36, 38, 47–49]. XIST binds to heterogeneous nuclear ribonucleoprotein K (HNRNPK), through the repeats B-C, to promote PRC1 recruitment [50, 51]. JARID2, a PRC2 cofactor, interacts with the deposited H2AK119ub1 facilitating PCR2 deposition. Repeats B and F of XIST are critical for JARID2 recruitment and subsequent H3K27me3 accumulation mediated by PRC2 [52, 53]. More recently, multiple RNA G-quadruplexes (rG4s) in XIST modulate the PRC2 histone methyl transferase activity and H3K27me3 enrichment across the Xi [54]. Thus, the histone deacetylation mediated by SPEN-recruited HDACs enables early transcriptional silencing, whereas H3K27me3 deposition by PRC2 facilitates a long-term silencing effect stably maintained in subsequent cell generations. Although the interplay between XIST and PRC2 has been extensively characterized, it remains unclear how Polycomb is recruited to Xi and induces chromosome-wide silencing.

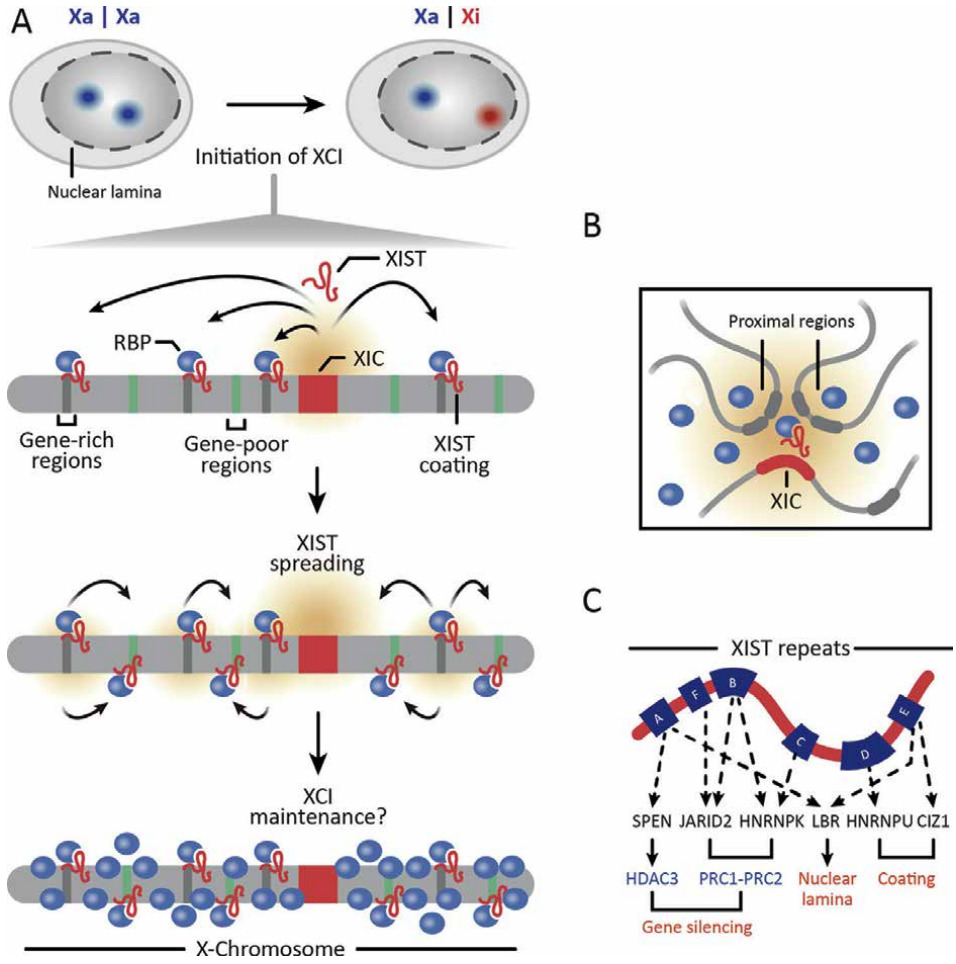

Figure 2.
Role of XIST in X-chromosome inactivation. *(A) During the early stages of development and differentiation of ESCs, one of the two active X chromosomes (Xa) becomes inactive (Xi) due to the transcriptional activation of XIST from the X-inactivation center (XIC). XIST modulates XCI through two main stages: coating and spreading. XIST coats at gene-rich genes located close to its locus (B). Subsequently, XIST spreads to gene-poor and intergenic regions to induce chromosome-wide silencing. The role of XIST at the late stages of XCI is unclear; however, its coating and spreading are essential at the early stages (initiation). (C) Repeat sequences embedded into XIST directly interact with proteins involved in gene silencing, coating, and localization of Xi to the nuclear lamina.*

Additional waves of chromatin remodeling occur later during XCI, including the deposition of H3K9me2, H3K9me3, the macroH2A histone variant, and the DNA methylation at CpG islands of X-linked genes [55–61]. These epigenetic changes may function as additional layers to maintain gene silencing and ensure XCI. Furthermore, XIST depletion analysis in somatic cells displayed limited gene reactivation, leading to the hypothesis that the central role of XIST is the initiation rather than the maintenance of XCI [62, 63]. Thus, XIST exerts a pivotal role in the initiation of XCI due to driving coordinated waves of proteins and chromatin remodelers at the future Xi, so that, the silencing effect will be maintained in subsequent cell divisions.

2.2 XIST shapes Xi topology

Xi displays a distinct nuclear structure from its counterpart Xa in the same cells. This phenomenon was first observed in adult cat neurons, where a difference in the nuclear structure was visible in females but not in males, later it was denominated "Bar body" [64]. This structure corresponds to Xi, which appears as a nuclear foci localized at the nuclear periphery [65, 66]. XIST interacts with the Lamin B receptor, leading to Xi recruitment to the nuclear lamina and inducing changes in the chromatin structure, which is essential for XIST spreading and transcriptional silencing of the future Xi [67]. However, the Xi-lamina location alone displays ineffective XCI, indicating that the localization of Xi at the nuclear lamina has a limited role in the initiation of XCI, and the additional nuclear components are required for XCI regulation [68].

In contrast to Xa, Xi appears as a compacted structure (around 1.2-fold) in somatic cells [69]. At the global level, these differences in compaction appeared insignificant but large-scale chromatin structures look more compacted in Xi, including gene-rich regions [70]. It has been proposed that these differences in the X1 and Xa result from the reorganization of higher-order structures instead of a direct chromatin compaction [71].

The development of high-throughput chromosome conformation capture technologies, such as Hi-C, has revealed the Xi topology [72]. Xa is structured into topologically associated domains (TADs), defined as units of chromatin structures characterized by high internal interaction frequency and low external interaction frequency with adjacent loci, whereas Xi is folded into two megadomains [73, 74]. This asymmetric topology between Xa and Xi could be explained by the differences in the binding patterns of architectural proteins across the chromosomes, including CTCF and cohesins. Cohesins (SMC1A and RAD21) bind the X chromosomes in an allele-specific manner, accounting for ~600 sites in Xa and ~ 20 sites in Xi. Intriguingly, the depletion of XIST in Xi results in the restoration of the cohesin-binding pattern resembling the Xa. The profiling of the XIST protein interactome revealed that XIST binds to numerous chromatin-associated proteins, including the cohesins and CTCF. The authors suggested a model in which XIST antagonizes the cohesin and CTCF binding to prevent the establishment of an Xa-like binding pattern. In the same study, the depletion of XIST and the restoration of cohesin-binding patterns in Xi lead to a transition toward an Xa-like chromatin structure, in which TAD structures appeared in Xi [41]. Thus, XIST functions as a modular platform to recruit numerous proteins and simultaneously prevents the binding of others to ensure the establishment of a Xi conformation.

2.3 TSIX, a negative regulator of XIST

In contrast to XIST, the XIST antisense non-coding transcription unit TSIX is exclusively expressed in the Xa [27]. Previous studies have pointed out that the act of TSIX transcription overlapping the *XIST* promoter is sufficient to induce chromatin remodeling, leading to the prevention of XIST upregulation in Xa [75–78]. However, recent evidence suggests that TSIX RNA by itself recruits the histone methyltransferase G9A to induce H3K9me2 and subsequent XIST silencing on the future Xa [55]. Deletion of TSIX resulted in an increased number of cells expressing XIST in both chromosomes [30]. These findings underpin the central role of TSIX in coordinating the monoallelic choice of XIST activation from both X chromosomes.

3. A parallel system of XCI in marsupials

Marsupials, as monotremes and eutherians (placentals), belong to the group of mammals, all of which evolved from a common ancestor. The sex determination system of this mammalian ancestor remains elusive; however, after its divergence into monotremes and the common ancestor of eutherians and marsupials, a genetic sex-determination system arose independently in the two phylogenetic branches, approximately 160 million of years ago (mya) [3]. Monotremes carry five pairs of sexual chromosomes, where the male is heterogametic ($X_{1–5}Y_{1–5}$) and the female homogametic ($X_{1–5}X_{1–5}$) [79]. There is no evidence of a global dosage compensation system in monotremes; rather, they equilibrate the transcriptional levels of X-linked genes by silencing female X chromosome loci in a gene-by-gene mode [79]. In contrast, eutherians and marsupials share the XY male heterogametic sex determination system and both display XCI. However, marsupial and eutherian XCI differ significantly, suggesting that at least some of the molecular actors and mechanisms evolved after the divergence of these two groups [3].

In marsupials, an apparent global upregulation of X-linked genes in both sexes accompanies the female XCI, restoring gene expression levels to those of autosomes and equalizing them between sexes [80]. Marsupial XCI is less stable and incomplete, despite the accumulation of the repressive histone modification H3K27me3 on the inactive X, which may be reminiscent of its low levels of DNA methylation in comparison with the eutherian inactive X [3]. DNA methylation seems to work differently in marsupials, since sexual and autosomal chromosomes are hypomethylated [81]. However, a flat profile of DNA methylation in the paternal germ line is associated with the predisposition of the paternal X chromosome for XCI [81]. Indeed, another great difference with eutherians is that the marsupial XCI is paternally imprinted, rather than random, meaning that the paternal X chromosome is inactive in all female cells. Finally, there is no XIST homolog in marsupials, and therefore, the leading role in XCI is played by RSX.

3.1 RNA on the silent X of female marsupials

RSX was identified by analysis of RNA-seq data of a South American marsupial, focusing on a differential male-to-female ratio in the reads mapped to the X chromosome [11]. RSX is a large (~27 Kb) lncRNA that is transcribed from and coats the inactive X chromosome in females [11]. It is silenced in the female germ line, where both X chromosomes are active, but expressed from the paternally imprinted X during embryonic development to promote XCI [11, 80]. Interestingly, integration of an *RSX* transgene in mESCs triggers gene silencing in *cis*, suggesting that RSX is a functional analog of XIST [11].

RSX and XIST share no sequence homology; however, both harbor tandem repeats form stem-loop structures critical for recruiting transcriptional repressors to the inactive X chromosome (**Table 1**) [82]. Indeed, the RSX interactome overlaps with that of XIST both in specific proteins, such as the polycomb repressive complex subunits, and in functional categories that include epigenetic transcriptional silencing [83]. Members of the last category that interact with RSX comprise the polycomb catalytic unit EZH2 that deposits H3K27me3, the histone deacetylase HDAC2, and DNA-methyl binding proteins MBD4 and MeCP2, all of which promote transcriptional silencing and chromatin compaction [83]. These findings suggest that the mechanism by which RSX facilitates a repressive chromatin landscape for gene expression is similar to that documented for XIST.

Besides the relevance of the XIST molecule, the particular three-dimensional arrangement of chromatin in the inactive X entails the *XIST* genomic locus [37].

lnRNA	Species	SSE	TOP	Size (Kb)	Repeats	Protein interactions	DNA interactions
XIST	Eutherians	F	XCI	15-17	Repeat A Repeat B Repeat C (murine) Repeat D (human) Repeat E Repeat F	hnRNPK hnRNPU RBM15 SPEN EZH2 SUZ12 CHD4	No DNA binding motif. Association between XIST spreading and LINE1 repeats.
RSX	Marsupials	F	XCI	27-30	Repeat 1 Repeat 2 Repeat 3 Repeat 4	hnRNPK SFPQ EZH2 HDAC2 MBD4 MeCP2	No DNA binding motif.
roX1 roX2	Fruit flies	M	XCO	3.8 0.6	roX-box	MSL2 MLE Polycomb?	Binding motif: MRE $(GA)_4$. Associations between satellite repeats and HAS.
MAYEX	Lizards	M	XCO	3.3		Histone 4 G1KD42 JMJD2C DNAJC2 SPT6 ZFC3H1 RANBP2 HSPA8 SASH1	Long-range interactions with $(TTA)_5$ sequences. A featured repeat sequence downstream MAYEX.

SSE: sex-specific expression. F: female. M: male. TOP: transcriptional output. XCI: X chromosome inactivation. XCO: X chromosome overexpression. Kb: Kilobases. Note that XIST repeat C is exclusive of mice, while repeat D is found in humans. RSX repeat 1 is similar to XIST repeats B, C, and D, and may serve for PRC1 indirect recruitment, while RSX repeats 2, 3 and specially 4, are similar to XIST repeats A and E, which may interact with PRC2 [82]. XIST and RSX interactomes include epigenetic repressors such as PRC components, histone deacetylases, and DNA methyl binding proteins [83]. In turn, roX and MAYEX interact with RNA-binding proteins, epigenetic activators, such as proteins holding activity or related to histone acetylases, PRC antagonists, and architectural proteins [10, 84]. X chromosome is richer in repeated sequences in comparison to autosomes, which may play a role in X chromosome recognition or spreading of XCI or XCO epigenetic mechanisms.

Table 1.
Similarities and particularities of lncRNAs targeting X chromosome dosage compensation in mammals, lizards, and the fruit fly.

Since RSX was discovered a decade ago, more studies are missing to elucidate whether this lncRNA similarly configures chromatin architecture to accomplish its function. Nevertheless, a recent paper reported an antisense RNA transcribed at the *RSX* locus in early embryos of both sexes, resembling TSIX, so the authors called it XSR [80]. The previous finding points out that *RSX* locus could represent a scaffold for a particular three-dimensional chromatin arrangement favorable for XCI.

4. X chromosome overexpression in *Drosophila*

Drosophila also harbors a XY genetic sex determination system; however, the dosage compensation strategy contrasts that of mammals, in which a whole X chromosome is

inactivated in female cells. In the fruit fly, both X chromosomes are active in females and the gene dosage differences are compensated by an overexpression of the single-male X chromosome (XCO). Therefore, the transcriptional output of the single-male X chromosome equals twofold the transcriptional levels of each female X chromosome. Since *Drosophila* represents a reference animal model, and X chromosome dosage compensation is a paradigm for epigenetic regulation, vast research from decades ago has permitted to have a clear, although not yet complete, picture of XCO [4].

The *Drosophila* dosage compensation complex (DCC) is integrated by five proteins and one of the two redundant lncRNAs, roX1 and roX2, all of which were identified since its genetic perturbation leads to specific male lethality [85]. Male sex lethal 1 (MSL1) forms a dimer that serves as a scaffold for the assembly of the other protein subunits, and particularly, its binding to MSL2 is important for chromatin association [85]. MSL2 interacts with roX1/2 to recognize a GA-rich DNA motif, called the MSL-responsive element (MRE), and therefore, it targets the DCC to specific chromatin sites, referred as high affinity sites (HAS) [86]. MSL3 and MOF are relevant for the spreading of the DCC from the HAS to the rest of the X chromosome [87]. MSL3 harbors a chromodomain that reads histone methylation marks; it is possible that this subunit reads the histone mark H4K20me1 associated with active transcription to spread the complex to active genes for its upregulation [88]. H3K36me3 is a histone modification that marks transcribing gene bodies; although MSL3 does not seem to read this mark, H3K36me3 is required for DCC spreading [89]. MOF is a histone acetyl transferase (HAT) that deposits an acetyl group in the lysine 16 of histone 4 (H4K16ac) [90]; as other acetylation marks, H4K16ac is associated with chromatin relaxation and transcriptional activation, which, in this case, underline XCO. MLE is indispensable for the incorporation of roX1/2 to the DCC [91]; and the roXs play fundamental roles in the overall process of *Drosophila* dosage compensation. Noteworthy, MSL2 and roXs are the only components of DCC whose expression is restricted to males, highlighting their role in the selectivity of male XCO.

4.1 roX on the single-male X chromosome

roX1 and roX2 were first identified by two independent studies as male-specific transcripts from the X chromosome, with nuclear localization and lacking an apparent open-reading frame [8, 9]. In one of the studies, the authors were searching for sex-specific transcripts involved in sexual behavior in cDNAs libraries of female and male dorsal brains [9]. The second study aimed to identify the predicted RNA component of the DCC, and reported roX1 as an RNA molecule that specifically recognizes and coats the entire male X chromosome [8]. The roXs are expressed in the male cells from zygotic genome activation (ZGA) onward, under the control of MSL proteins [8, 92], since an MSL2 transgene activates roX transcription in females and mutations in MLE abolish roX expression in males [8]. roX1 and roX2 are functionally redundant; only a simultaneous removal of both loci results in the specific male lethality phenotype [93]. Although there are subtle spatiotemporal differences, their expression patterns mostly overlap and both lncRNAs are expressed from, localize to, and coat the entire X chromosome in male cells [93].

4.2 roX molecules

Despite of being functionally homologs, roX1 (3.7 Kb) and roX2 (0.6 Kb) show no sequence conservation, with the exception of repeated regions in tandem that are

called roX-boX (**Table 1**) [84]. These tandem repeats form stem-loop structures that interact with MLE and MSL2 [84]. Importantly, the search of stem-loop underprints revealed roX orthologous across *Drosophila* genus capable of extrinsically executing their dosage compensation function in distinct species than that of origin [94]. This is reminiscent of the *RSX* transgene inserted in mESCs triggering gene silencing in *cis* [11], and to the tandem repeats forming stem loop structures that harbor RSX and XIST to recruit a similar interactome [82]. These observations suggest that the lncRNAs involved in X chromosome dosage compensation share particular features, such as the tandem repeats that form stem loops structures.

As previously mentioned, stem loops serves for the RNA-protein interactions, and therefore, for recruiting transcriptional activators and repressors. Interestingly, similar to XIST and RSX, the roXs seem to interact with polycomb. A recent study reported a novel function of the roXs in regulating autosomal gene expression [95]. Although the roXs predominantly localize to the X chromosome, some autosomal occupancy is detected [16, 95], and loss of the repressive histone mark H3K27me3 is observed upon knocking out both roXs [95]. These results can be related to the sequence similarities between the MRE, occupied by the DCC, and the polycomb responsive element (PRE), as both are GA-rich [16]. Alternatively, polycomb subunits may have affinity for the stem loop structures on the roXs. Further studies are needed to establish whether the stem loops of lncRNAs mediating X chromosome dosage compensation share any structural similarity that facilitates interaction with polycomb subunits.

4.3 roX loci encompass high-affinity sites

A major difference between the mammalian and *Drosophila* lncRNAs is that XIST and RSX act in *cis*, while roX1 and roX2 act in *trans* (**Figure 3**). When an *XIST* transgene is inserted into an autosome, XIST coats, compacts, and silences exclusively this autosome [29]. This represents an indispensable feature, as a hypothetical action in *trans* of XIST could result in the inactivation of the two female X chromosomes (**Figure 3**). In contrast, autosomal insertions of roX have local and global effects; locally, autosomal roX recruits the DCC to the site of insertion, but the spreading of the H4K16ac and transcriptional upregulation largely depends on an active and permissive chromatin environment [8, 96]. In turn, when a roX transgene is inserted into an autosome in a double rox1/2 null background, roX still targets and decorates the X chromosome (**Figure 3**), regarding the location of its genomic locus [8, 96]. These observations emphasize that, as in other examples of experiments evaluating lncRNA function, it is important to discern between the roles of the roX1 and roX2 transcripts and that of their genomic loci.

Each roX1 and roX2 genomic locus harbors an intronic DNAse I hypersensitivity site (DHS) [92], representing a predominant HAS. MSL1 and MSL2 bind hundreds of HAS on the X chromosome, but complete incorporation of the additional subunits MSL3, MOF, MLE, and either roX1 or roX2, is needed for DCC spreading, H4K16ac deposition, and gene upregulation [96]. Therefore, HAS represents DCC nucleation points, and spreading relies on a complete assembly of the DCC, together with an active transcriptional state of neighboring genes [96]. However, the sequence composition of the HAS on the X chromosome can be also found in autosomes and how the DCC discriminates between the HAS sequences on autosomes and preferentially binds to the X chromosome has been a subject of extensive research.

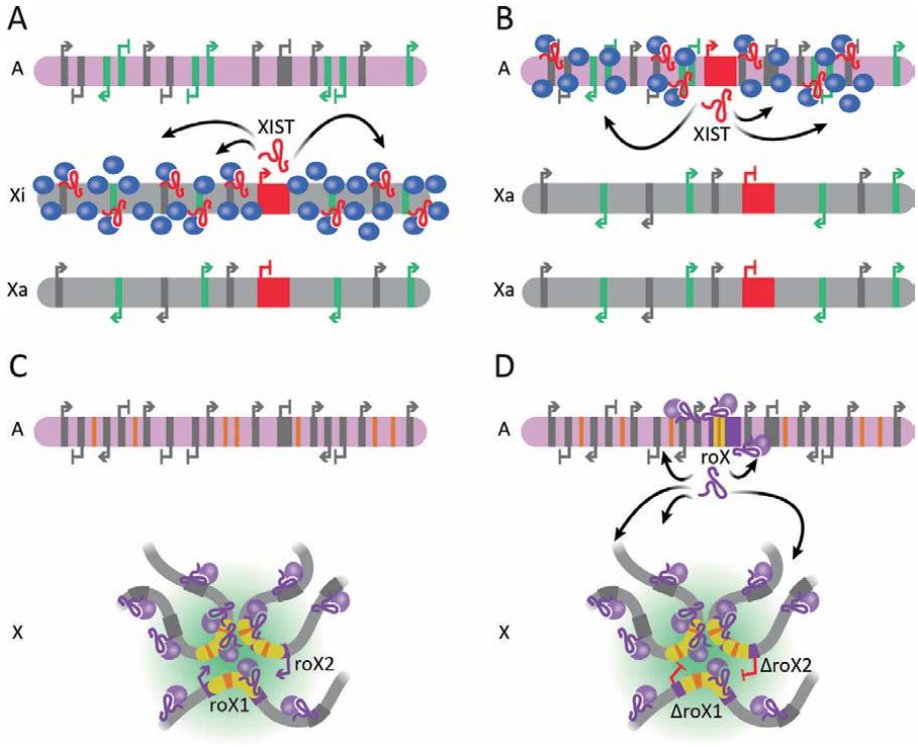

Figure 3.
XIST transcript acts in cis, while roX transcripts act in trans. *(A) XIST (in red) is transcribed from and exclusively coats the future inactive X chromosome (Xi) in mammalian females. XIST is silenced in the active X chromosome (Xa), and therefore, genes of Xa and autosomes (A) display their spatiotemporal specific expression pattern. (B) A XIST transgene is inserted into an autosome in a cell-type lacking XIST expression; for instance, naïve mouse embryonic stem cells have active their two X chromosomes. XIST is transcribed from the autosome transgene and coats exclusively this autosome, triggering its transcriptional inactivation [29]. The two X chromosomes remain active, since XIST function in cis. (C) roX 1 and roX2 comprise two predominant high affinity sites (HAS, in yellow), enclosing the MSL responsive element (MRE, in organge). HAS are in close proximity in the three-dimensional space, and a high density of clustered MRE strongly recruits MSL proteins. roX expression is upregulated and roX molecules (in purple) integrate to the dosage compensation complex (DCC, in purple), triggering its spreading to the entire single male fruit fly X chromosome and promoting global transcriptional overexpression (XCO). Autosomes harbor multiple MRE; however, roX molecules preferentially bind to the X chromosome [16]. (D) A roX transgene is inserted into an autosome of double-knockout roX1/2-/- male cells. Since roX comprises a predominant HAS [96], MSL proteins are recruited to the site of insertion, the roX transgene is upregulated, and roX molecules are integrated into the DCC. Limited DCC spreading is observed in the autosome, which relies on an active chromatin landscape of neighboring regions [96], rather than the presence of MRE. Active neighboring genes may be overexpressed. Nevertheless, most of the roX transcripts migrate to the single-male X chromosome, target the clustered HAS, spread to coat the entire X chromosome, and promote XCO.*

HAS on the X chromosome, including roX1 and roX2 genomic loci, are in close proximity in the three-dimensional space, creating a higher local density that is not observed on autosomes [97]. This particular three-dimensional conformation of the X chromosome strongly attracts the DCC and distributes it, firstly to all interacting HAS, and from there, to the entire X chromosome [97]. Indeed, a great percentage of HAS overlaps TAD boundaries on the X chromosome, in both females and males [97]. This HAS interaction network is set on prior ZGA, by DCC-independent mechanisms, which involves the chromatin-linker adaptor of MSL proteins (CLAMP) [98]. CLAMP is maternally supplied to the zygote, which recognizes GA-rich sequences on

the X chromosome as well as on autosomes of females and males [99]. Only on the X chromosome, CLAMP promotes a clustering of HAS, which is targeted by MSL2 in males upon ZGA [98, 99]. The selective targeting of the X chromosome is further supported by the presence of roX1 and roX2 and possibly by satellite repeats, which are twofold more abundant on the X chromosomes in comparison with autosomes.

5. X chromosome transcriptional output restores ancestral autosome expression in a lizard

A male heterogametic sex determination system as old as the mammalian also arose in the phylogenetic branch of the reptiles [1]. In particular, the Iguania ancestor developed the XY sexual chromosome pair, after the divergence with snakes [1]. The green anole genome was the first non-avian reptile to be sequenced, and from then, this lizard has become a reference species for studying vertebrate evolution [100]. As in the other XY species, the Y chromosome of the green anole is highly degenerated, and therefore, the XY males have a reduced gene dosage in comparison with XX females and to the ancestor of origin [1, 101]. Analysis of RNA-seq data, however, displays similar expression levels of X-linked genes between sexes throughout development and in adult tissues, evidencing mechanisms of X chromosome dosage compensation [1]. Intriguingly, the green anole developed an XCO system, similar to the fruit fly and in contrast to the XCI selected in the more phylogenetic-related mammalian species [1]. XCO achieves a twofold upregulation of transcriptional output in the green anole, balancing X-linked gene expression between sexes and also restoring the predicted expression for the ancestor of origin [1]. The molecular mechanisms of the green anole XCO share additional relevant features with those in the fruit fly, including the deposition of H4K16ac along the X chromosome, and the male-biased expression of proteins relevant for HAT activity and of a lncRNA whose locus is on the X chromosome [1, 10].

5.1 A male-specific lncRNA amplifying the expression of the X

The sex-specific RNA-seq data revealed the differential expression of two lncRNAs within neighboring loci on the X chromosome [10]. The authors called FEREX, for female-specific expressed region on the X, and MAYEX, for male-specific lncRNA amplifying the expression of the X [10]. The expression of these lncRNAs is mutually exclusive, resembling the XIST/TSIX and RSX/XSR antagonism, but restricted to females and males [10]. MAYEX is a long RNA molecule of 3.3 Kb (**Table 1**), which is conserved across various lizard species [10]. In line with a role in XCO, its interactome comprehends proteins that deposit active transcriptional histone marks, while counteracting mechanisms and histone modifications related to chromatin compaction and silencing, such as those mediated by polycomb [10]. In addition, MAYEX interacts with architectural proteins that may mediate long-range contacts with distal regions on the X chromosome [10]. Similar to the HAS enclosed at roX1/2 loci, *MAYEX* locus is engaged in long-range interactions in females and males, which may be supported by a neighboring region of repetitive sequences [10]. Nevertheless, direct contacts with some distant regions are male-specific and may rely on *MAYEX* transcription or the presence of the transcript itself [10]. Upcoming studies will provide more insights into the XCO in the green anole and will uncover the role of MAYEX in this lizard.

6. Conclusions

X chromosome dosage compensation has been widely investigated in reference animal models, partly, due its relevance in the animal biology, and partly for the evolutionary contribution of its understanding. In addition, it represents an epigenetic paradigm of coordinated transcriptional regulation at the level of an entire chromosome. The main epigenetic actors and mechanisms participate in the X chromosome dosage compensation, as DNA methylation, histone modifications, chromatin remodeling complexes, the three-dimensional chromatin architecture, and therefore, all of these as well as their interplay can be studied in this model. We have learnt, from this extensive research, that the basic strategy for regulating transcription at a whole chromosome level has common features. These include the modification of the chromatin landscape and three-dimensional conformation. The cells operated its tool box to specialize some of the pre-existent epigenetic actors and mechanisms under the control of a novel component that results to be a lncRNA transcribed from the target X.

LncRNAs can act as an interphase of the chromatin-modifying machinery and the genome. Part of the sequence of the RNA molecule engages with and reads specific DNA sequences, while the other part recruits protein complexes holding enzymatic activities. Beside the lncRNA transcripts, their genomic loci serve as a scaffold of distinct combinatorial of DNA-RNA-protein interactions. In particular, the lncRNAs involved in the X chromosome dosage compensation share similar features across mammals, lizards, and the fruit fly. These are all nuclear transcripts whose genes locate in the target X chromosome [8–11, 102]. They are transcribed only in one sex, either in females for XCI or in males for XCO. Their genomic loci are relevant regulatory targets to trigger dosage compensation. Once expressed, XIST, RSX, roX1, roX2, and MAYEX coat the whole X chromosome. Due these transcripts are particularly large, repeat rich, and enclose tandem repeats capable to form stem-loop structures, they engage in RNA-protein interactions [82, 84]. In a first layer of global transcriptional regulation, the lncRNAs associate with histone acetyl transferases, lysine methylases, histone deacetylases, and demethylases to modify the X chromosome chromatin landscape [10, 83, 84]. In a second layer, the lncRNAs interact with proteins involved in long-range contacts, such as polycomb, CLAMP, and SASH1, to strengthen a chromatin three-dimensional arrangement favorable for global dosage compensation [10, 83, 95, 99].

Evidences of global dosage compensation have been observed in other insects with genetic sex determination systems, including mosquitos and butterflies [5, 6]. Further studies will elucidate whether these animals also selected lncRNAs as orchestrators of chromosome-wide transcriptional regulation to balance the expression between males and females.

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Nomenclature

CLAMP	chromatin-linker adaptor of MSL proteins
DCC	dosage compensation complex
DHA	DNase I hypersensitivity sites
HAS	high affinity sites
HAT	histone acetylase
HDAC	histone deacetylase
hnRNPK	heterogeneous nuclear ribonucleoprotein K
lncRNA	long non-coding RNA
MRE	MSL responsive element
MSL	male sex lethal
mya	millions of years ago
Pol II	RNA polymerase II
PRC	polycomb repressive complex
PRE	polycomb responsive element
rG4	RNA G-quadruplex
roX	RNA on the X
RSX	RNA on the silent X
TAD	topologically associated domain
TSIX	XIST antisense non-coding transcription unit
Xa	active X chromosome
XCI	X chromosome inactivation
XCO	X chromosome overexpression
Xi	inactive X chromosome
XIC	X inactivation center
XIST	X-inactive specific transcript
ZGA	zygotic genome activation

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Chapter 4

The Role of Long Non-Coding RNAs in Skeletal Muscle Pathophysiology: A Therapeutic Perspective

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Abstract

Skeletal muscle, one of the largest organs in mammals, plays a pivotal role in regulating body temperature, metabolism, and movement. It is a versatile tissue that can undergo degeneration due to various factors such as disease, aging, or cancer, posing challenges to cancer therapy. Conversely, it possesses the remarkable ability to regenerate. Skeletal muscle is composed of both mononucleated cells, each with distinct functions during degeneration and regeneration, and multinucleated cells called myofibers. Recently, it was demonstrated that the transcription of mammalian cells is pervasive, highlighting the importance of junk DNA. It frequently codes for non-coding RNAs that have gained increasing importance in the pathophysiology of all tissues over the past two decades. Following the advent of COVID-19 vaccines, RNA has assumed a greater significance in therapeutic applications. In this manuscript, we will explore the impact of long non-coding RNAs (lncRNAs) on muscle degeneration and regeneration and investigate their potential therapeutic applications.

Keywords: long non-coding RNAs, circular RNAs, skeletal muscle, myogenesis, metabolic disorders, muscle atrophy, therapy, metabolism

1. Introduction

The human body is composed of over 600 skeletal muscles, which constitute approximately 40% of total body weight and contain 50–75% of all body proteins. Skeletal muscle tissue is not only involved in producing movement, maintaining body posture, controlling body temperature, and stabilizing joints, but also plays a crucial role in maintaining organisms' energy homeostasis. Notably, it can be considered a biomechanical tissue with numerous interacting elements, including vasculature used for sharing and providing components essential for energy production, such as oxygen, or myokines as brain-derived neurotrophic factor, leukemia inhibitory factor IL6, IL8, and IL15, with autocrine, paracrine, or endocrine actions and involvement in inflammatory processes [1]. Additionally, it exhibits a unique interaction with motor neurons, enabling the transmission of contracting impulses through the motor

neuron junction (NMJ). Furthermore, satellite cells contribute to the plasticity of this tissue, allowing it to adapt its structure in response to specific stimuli, such as unloading, exercise, or certain pathological conditions.

The interplay between electrical impulses, the number of blood vessels, and metabolism determines the differences in muscle types and metabolism. Generally, we can categorize two distinct muscle types: fast-contracting and slow-contracting. The former comprises myofibers that rely on glycolysis as their primary energy source, while the latter comprises myofibers that utilize mitochondrial respiration and, consequently, require more oxygen. Myofibers (containing multiple myofibrils) are the smallest complete contractile units of skeletal muscle, influencing its contraction velocity and metabolic rate. Bundles of myofibers form fascicles, and bundles of fascicles constitute muscle tissue. Each layer of muscle tissue is subsequently encapsulated by the extracellular matrix (ECM) [2] and supported by the cytoskeletal networks.

Mouse myofibers can be classified in four distinct types: Type I (slow twitching), Type IIa, Type IIb, and Type IIX (fast twitching). Each muscle fiber is characterized by the expression of a specific type of myosin heavy chain (MyHC), which in turn determines the muscle's contractility properties. A difference between rats and humans is that the latter do not have the MYH4 gene coding for myosin in type IIb myofibers [3].

A unique feature of myofibers is their multinucleated nature. Each myofiber is formed by the fusion of hundreds of myoblasts, resulting in the retention of each myoblast nucleus within the myofiber [4]. Interestingly, nuclei are arranged along the length of myofibers, in the peripheral compartment near the membrane, and express different genes based on their position. Nuclei located near the NMJ express genes coding for essential components of the NMJ while those in proximity to the myotendinous junction express genes for proteins crucial to that compartment [5]. This means that myonuclei can be functionally distinguishable according to their position within myofiber.

In the past, RNA was primarily regarded as an intermediary between DNA and proteins. However, advancements in DNA/RNA sequencing technologies have unveiled RNA's multifaceted role beyond its functional role as a mediator. The human genome is pervasively transcribed (75%), but only a very small fraction of these transcripts serve for protein synthesis (2%) [6]. This suggests that the information contained in 98% of transcribed DNA is used to produce non-protein-coding RNAs. The substantial energy expenditure associated with this extensive transcription cannot be attributed solely to random interactions between RNA polymerases and DNA. Rather, it is likely necessary for crucial functions that are not effectively performed by DNA and proteins alone [7]. In fact, considering the genome size and the fact that in complex eukaryotes as *Homo sapiens* and *Mus musculus* there are about 20,400 genes (information from Ensembl and Mouse Genome Informatics (MGI) database, respectively), the regulation of all genomic components (about 228,000 mapped genes in *H. sapiens* and 319,600 in *M. musculus*, according to the Ensembl and MGI database, respectively) is not possible only through the use of transcription factors (TFs). This is because TFs are relatively scarce, with an estimated count of approximately 1500 in mammals [8] compared to the number of genes they regulate (90% of the human genome is transcribed [9]). Chromatin immunoprecipitation and DNA sequencing experiments (ChIp-seq) permitted to estimate that a single TF regulate only 10 thousand genes [10]. Additionally, there are evidences that regulation of gene expression also occurs through the intervention of non-coding RNAs [11, 12].

The evidence presented underscores the active transcription and crucial functions of the non-protein-coding component of the human genome, thereby diminishing the significance of coding genes in genome regulation. Non-coding transcripts have emerged as pivotal entities in contemporary biology, particularly due to their involvement in a diverse range of regulatory processes and redefining the perception of non-coding regions as mere "junk DNA" [13]. To date, numerous types of non-coding RNAs have been identified. These can be categorized into short non-coding RNAs (sncRNAs) and long non-coding RNAs (lncRNAs), with the latter being the most recently studied. In this chapter, we present our comprehensive understanding of the most recently investigated lncRNAs that have significantly contributed to the advancement of the regulatory RNA field in skeletal muscle and have the potential to be further investigated as potential therapeutic target in muscle atrophy. This review delves into the intricacies of lncRNAs, elucidating their underlying mechanism of action in skeletal muscle. The primary objective of this manuscript is to position lncRNAs as potential therapeutic targets for pathologies of skeletal muscle.

2. Long non-coding RNAs

The term "long non-coding RNA" (lncRNA) encompasses a diverse group of genes or transcripts that presents a limited conservation of primary structure across species. Since this characteristic is important in gene or transcript classification, different methods of classification were proposed for lncRNAs, each basing on a distinct characteristic.

Only recently was used the term lncRNA. Initially, for the classification of non-coding transcripts were used their functions (rRNA, tRNA, and cRNA). However, the increase in the number of recognized non-coding RNAs and evidence of their abundance in the early 2000s [6] new classification approaches were introduced.

It is well established that mammalians [14] and plant organisms [15] use most of their energy to transcribe lncRNAs instead of coding RNAs. Nevertheless, only a few of these lnRNAs has been functionally characterized. Studies have demonstrated that these transcripts exhibit a wide range of roles and mechanisms of action, which are influenced by their subcellular localization or cell compartmentalization. Genomic location, subcellular localization or function can be used for the categorization of lncRNAs. Below is a description of each category, along with some recent examples of lncRNAs involved in muscle biology.

2.1 Genomic location

2.1.1 Genic lncRNAs (exonic or intronic)

LncRNAs can localize within genomic regions that overlap protein-coding genes. These transcripts are referred to as genic lncRNAs. Genic lncRNAs can be classified as intronic or exonic based on their position within protein-coding genes. The overlap with an intron of a protein-coding gene defines intronic lncRNAs, while the overlap with exons of protein-coding genes defines exonic lncRNAs. Consequently, considering the transcriptional orientation of lncRNAs, they can be classified as sense lncRNAs if they are transcribed in the same direction of protein-coding genes or antisense lncRNAs if they are transcribed in the opposite direction. Antisense lncRNAs are transcribed from the opposite strand of the closest protein-coding gene.

They can either overlap with a 5′ head-to-head divergent orientation (SINEUPs) [16–18] or with 3′ tail-to-tail convergent orientation (3′ overlapping lncRNAs). The Synaptopodin-2 (SYNPO2) intron sense-overlapping lncRNA (SYISL) is an example of intronic lncRNA that regulates myogenesis through the interaction with the enhancer of zeste homolog 2 (EZH2). EZH2 is a protein that participates in histone methylation and therefore in epigenetic gene expression regulation [19].

2.1.2 Long non-coding RNAs between coding genes (lincRNAs)

Long intergenic non-coding RNAs (lincRNAs) are located within genomic regions that encompass two coding genes, thereby excluding them from protein-coding loci. A substantial proportion (40%) of lincRNAs expressed in HeLa cells, human foreskin, and human lung fibroblasts is involved in chromatin structure modification suggesting their involvement in the regulation of gene expression [20]. The number of lincRNAs in the human, mouse, zebrafish, and sheep genomes is not limited. Indeed, 214 lincRNAs have been annotated in the human genome (Ensembl GRCh38), 390 in the mouse genome (Ensembl GRCm39), 41 in the zebrafish genome (Ensembl GRCz11), and 2020 in the sheep genome (Ensembl Oar_v3.1). These numbers indicate they may have important functions in various organisms. Furthermore, lincRNAs can be further categorized based on their transcription direction relative to the neighboring protein-coding gene. They can be classified as follows: (i) divergent when they are transcribed in the opposite direction of nearest protein-coding gene, (ii) convergent when they are transcribed in an end-to-end manner, which is the reverse of divergent, and (iii) transcribed in the same sense of the nearest protein-coding gene. A recently discussed lincRNA in muscle biology is lincMYH, which is involved in regulating the satellite cell pool [21].

2.1.3 Splice-based classification

The majority of lncRNAs are transcribed by RNA polymerase II (RNA pol II). However, some lncRNAs are transcribed by RNA polymerase III (RNA pol III) [22]. Following transcription, lncRNAs may undergo processing by the splicing machinery, resulting in various categories of lncRNAs: (i) macro lncRNAs, which originate from unspliced transcripts that are several kilobases in size; (ii) retained intron lncRNAs, which are alternative transcripts of coding genes that lose their coding properties after an intron is retained during the splicing process.

2.2 Subcellular localization of lncRNAs

The subcellular localization of lncRNAs is crucial for their functional roles. For instance, nuclear lncRNAs may interact with chromatin, while cytoplasmic lncRNAs may function as microRNA (miRNA) sponges.

2.2.1 LncRNAs within nucleus

Nuclear lncRNAs are exclusively located in the nucleus. Specific sequences within lncRNAs permit their retention in the nucleus [23]. The majority of nuclear lncRNAs function as transcriptional regulators or impact on mRNA processing. They can either activate or inhibit gene activity, for instance, by recruiting transcription factors or operating as decoys that hinder the interaction between transcription factors and

DNA. Notable examples of nuclear lncRNAs include Neat1, whose inhibition is beneficial for inducing muscle hypertrophy, but detrimental to injured muscle [24], and Malat1, whose expression decreased with age in mouse skeletal muscles [25].

2.2.2 LncRNAs in the cytoplasm

LncRNAs confined in the cytoplasm are classified as cytoplasmic lncRNAs. RNAs are synthesized in the nucleus, and their transport to the cytoplasm entails numerous distinct proteins located on both sides of nuclear pore. The RNA protein complex (RNP) composed of lncRNAs or mRNAs and the Tho complex, the RNA helicase UAP56 (or its paralog URH49), and Aly is formed in the nucleus. Subsequently, RNPs are transported through the nuclear pore thanks to its interaction with the nuclear export receptor composed of Nuclear RNA Export Factor 1 (Nxf1) and Nuclear Transport Factor 2 Like Export Factor 1 (Nxt1) (also known as TAP and p15). In the cytoplasm, RNPs undergo remodeling to recycle nuclear-associated exported factors and ultimately result in the new stable localization of the mRNA or lncRNA [26].

Cytoplasm contains processed RNAs, leading to the predominant involvement of cytoplasmic lncRNAs in post-transcriptional regulation. These lncRNAs can serve as alternative targets of miRNAs, thereby mitigating the severity of RNA interference. Alternatively, they can enhance the translation of target mRNAs without impacting mRNA transcription (SINEUPs) [27].

2.2.3 LncRNAs located within mitochondria and chloroplast

Mitochondria and chloroplasts are specialized organelles of eukaryotic cells for energy production or photosynthesis (plant cells). An endosymbiotic origin for both organelles is now generally accepted with the maintenance of their own genomes. During evolution, these genomes have undergone significant reduction, with most genes transferred to the nuclear genome. Mitochondrial DNA retains the capacity to synthesize 13 proteins (essential for certain parts of OxPhos complexes), 22 tRNAs, and two ribosomal RNAs (12S and 16S).

Mitochondrial DNA is a double-stranded circular molecule characterized by a guanine-rich strand (heavy strand) and a guanine-poor strand (light strand). The first strand is rich in genes while the latter codes only for one subunit of nicotinamide adenine dinucleotide dehydrogenase (ND), 8 tRNAs, and 3 lncRNAs. Notably, these lncRNAs regulate two genes in the heavy DNA strand (ND5; NADH dehydrogenase subunit 5 and CYTB; cytochrome b) and one in the light strand (ND6; NADH dehydrogenase subunit 6) throughout RNA complementary binding [28].

Nuclear genes coding for mitochondrial proteins regulates their expression in concert with mitochondrial necessity confirming the intercommunication among nucleus and mitochondria. In addition to lncRNAs synthesized from the light strand of mitochondrial DNA, other lncRNAs are synthesized and transported in the cell nucleus. For instance, a RNA composed of 16S mitochondrial RNA and an inverted repeat of 120 bp was located in nuclei of mouse and human sperm [29], further confirming the importance of intercommunication among these "organelles". Mitochondrial DNA also enables the synthesis of long intergenic noncoding RNA predicting cardiac remodeling (LIPCAR), which is associated with the risk of heart failure [30].

The genome of chloroplasts, such as mitochondrial one, also synthesizes for lncRNAs. Given the focus of this article on the function of lncRNA in skeletal muscle, a review on the function of long non-coding RNAs in plant organelle is provided in [31].

2.3 Classification of long non-coding RNAs based on their functional characteristics

Historically, rRNAs have been the earliest long non-coding transcripts described, primarily due to their abundance in cells. They are essential components of ribosomes and allow the interaction between ribosomes and mRNAs. Below is a classification of newer long non-coding RNAs based on their functions.

2.3.1 RNAs interacting with the chromatin

Chromosomal RNAs (cRNAs) were introduced in the late 1960s by James Bonner as a group of RNAs capable of binding chromatin [32]. LncRNAs interact with chromatin through various mechanisms, with the recruitment of the polycomb repressive complex (PRC) being the most prevalent. The PRC is an evolutionary conserved system of epigenetic gene silencer, and consequently, lncRNAs, that interact with them, are gene silencers that use an epigenetic mechanism. PRC1 and PRC2 are the two major PRCs. PRC1 components were initially characterized in Drosophila [33], and later homologs genes were identified in human: CBXs (polycomb homolog), PHC1, 2, and 3 (polyhomeotic homologs), Ring1a and Ring1b (dRING homologs), BMI1 (Polycomb Ring Finger Proto-Oncogene), and six minor others (posterior sex combs homologs) [34]. Functionally, DNA CpG density and methylation status regulate PRC2 binding to chromatin. In contrast, PRC1 may facilitate the positioning of PRC2 in unmethylated CXXC DNA domains, guiding H3K27 lysine 3 methylation-mediated chromatin silencing [35]. PRC2-associated proteins with DNA binding capabilities enable PRC2 binding to unmethylated DNA independently of PRC1 both in Drosophila and mammalian systems [36–38]. Notably, it was published a protocol to visualize the tissue-specific PcG distribution in the aorta, dorsal skin, and hindlimb muscles [39].

2.3.2 MicroRNA sponges

LncRNAs and miRNAs can interact to regulate protein expression. miRNAs are short non-coding RNAs (18–28 nt) [40] interacting with mRNAs to regulate protein expression by degrading mRNA or repressing translation [41]. LncRNAs that interact with miRNAs compete for miRNA regulatory function and for this reason are named competing endogenous RNAs (ceRNAs) or miRNA sponges [42]. One notable example of a ceRNA is the long intergenic non-coding RNA-Muscle Differentiation 1 (linc-MD1), one of the first discovered lincRNA in skeletal muscle. It interacts with miR-133 and -135 which modulate the expression of two important transcription factors that activate muscle-specific gene expression: Mastermind Like Transcriptional Coactivator 1 (MAML1) and Myocyte Enhancer Factor 2C (MEF2C) [43]. Pseudogenes are another significant class of lncRNAs that function as miRNA sponges. These are particularly significant in skeletal muscle. For example, EEF1A1P24 and TMSB4XP8 are two hypertrophy-related pseudogenes [44]. Another example of miRNA sponging RNAs are circular RNAs (circRNAs), which will be following discussed.

2.3.3 Enhancer RNAs

Enhancers can be transcribed to produce lncRNAs known as enhancer RNAs (eRNAs). Enhancers are short DNA regions (50–1500 bp), typically isolated from

coding genes, that possess the capacity to modulate gene transcription through the binding of specific protein activators [45]. eRNAs were identified in 2010 using RNA-seq and Chip-seq techniques and can augment the expression of target genes in cis [46]. Enhancers were initially discovered in 1981 by Banerji and colleagues, who observed the enhancement of the expression of a beta-globin gene by distant SV40 DNA sequences [47]. eRNAs hold significant importance in skeletal muscle development and pathology. For a comprehensive review, refer to [48]. Recently, it was evidenced that seRNA PAM-1 is involved in the regulation of skeletal muscle satellite cell and aging through trans regulation of Timp2 expression synergistically with Ddx5 [49]. Timp2 encodes for an extracellular matrix, and increased extracellular matrix proteins expressions are essential to satellite cells activation [50]. The mechanism of transcriptional enhancement exhibited by eRNAs may vary. Some eRNAs are involved in the strength of the enhancer-promoter looping interaction, while others hinder the binding of negative elongation factors (NELFs) to the promoter, thereby reducing transcriptional repression [51]. eRNAs can be categorized into two distinct types based on their transcriptional direction: 1D (unidirectional) or 2D (bidirectional). Unidirectional eRNAs are longer (>4 kb) and polyadenylated, whereas bidirectional are shorter (0.5–2 kb) and non-polyadenylated [52, 53].

2.3.4 SINEUPs

SINEUPs, already mentioned in the paragraph entitled "Genic lncRNAs (exonic or intronic)", are antisense lncRNAs with two important sequence specificities: the presence of an inverted SINEB2 sequence and a small complementarity sequence with the targeted mRNA. Due to these characteristics, SINEUPs can contact specific mRNAs by sense-antisense binding to up-regulate the translation without affecting gene expression. SINEUPs are coded by genes that overlap with the gene encoding the target protein to allow a 5′ head-to-head binding. SINEUPs can also be artificially designed and can be used to achieve the opposite effect of miRNA/siRNA translational silencing [16].

2.3.5 Target position

Certain chromatin-interacting lncRNAs, known as cis-acting lncRNAs, function by targeting the chromatin regions from which they are transcribed. The chromatin targeted by these lncRNAs is not necessarily limited to the immediate vicinity of the lncRNA. A prominent example is the well-studied lncRNA XIST, which targets the whole X chromosome through a cis approach.

Conversely, if the lncRNA target is not located in close proximity to its gene, it is classified as a trans-acting lncRNA. An example of trans-acting lncRNA is seRNA PAM-1 previously described for its involvement in the regulation of skeletal muscle satellite cell activation and aging [49].

3. Circular RNAs

The first circular RNA (circRNA) was identified in pathogens in the late 1970s [54, 55]. Until approximately 2010, their potential impact was largely underestimated. In the late 2010s, new sequencing technologies and bioinformatic pipelines were developed leading to an explosion in circRNA research. Metazoan express thousands of distinct types of circRNAs [56–58].

CircRNAs can originate from five different RNA regions: (1) exon, (2) intron, (3) exon-intron, (4) fusion gene, and (5) read-through circular RNA formed by polymerase II. Currently, three mechanisms are recognized for the formation of circRNAs. One mechanism is based on complementary pairing of intron elements, such as reverse complementary matches, intronic complementary sequences, and Alu sequences favoring the vicinity of the 5′ splice donor site of the mRNA precursor to the 3′ splice acceptor site. A second mechanism relies on RNA binding proteins that promote cyclization by binding to flanking introns of circularized exons. Last method occurs during the splicing of mRNA precursor and exon skipping, when the formation of lasso intermediate containing an intron and an exon. The exon and intron form a circular RNA. Most circRNAs are formed from exons, while a few are formed solely from introns. The majority of circRNAs are non-coding, highly conserved, and located in the cytoplasm. However, a few circRNAs are located in the nucleus [59]. CircRNAs can play regulatory roles in transcriptional [60], post transcriptional [61] regulation, and can also be translated [62].

Translated circRNAs play crucial role in maintaining skeletal muscle mass. For example, a conserved circular RNA damage-specific DNA binding protein 1 (circDdb1) exhibits significantly elevated expression in various muscle atrophy types both *in vivo* and *in vitro*, as well as in human aging muscle. CircDdb1 encodes for the protein circDdb1-867aa which interacts with and augments the phosphorylation level of eukaryotic elongation factor 2 (eEF2) at Thr56. This interaction reduces protein translation and promotes muscle atrophy [63]. Another protein encoded by circRNA (circTmeff1) that mediates pro-atrophic effects is circTMEFF1-339aa. Overexpression of TMEFF1-339aa leads to increased expression of Atrogin-1 and MuRF-1, activation of the ubiquitin proteasome system, alkaline phosphatase, and inhibition of the AKT/FOXO3A/mTOR signaling pathway [64]. All these mechanisms induced by circTMEFF1-339aa contribute to muscle atrophy.

Most studied activities of circRNAs at post-transcriptional level are their ability to sponge miRNAs. For example, by using RNA binding protein immunoprecipitation and RNA pull-down assays it was demonstrated that circMYBPC1 binds miR-23a inducing myoblast differentiation by relieving miR-23a inhibition on myosin heavy chain (MyHC) [65]. Another interesting circRNA involved in the maintenance of satellite cell population (MuSC) is circFUT10. It suppresses MuSC proliferation and differentiation by sponging miR-365a-3p and the 3′UTR of HOXA9 mRNA [66]. Several studies have indicated that HOXA9 is implicated in cell proliferation [67, 68]. Notably, one study demonstrated that HOXA9 may promote denervated muscle atrophy [69]. CircRNAs exhibit remarkable stability compared to linear RNAs [70], making them crucial actors in prolonging RNA transfection effects in therapeutic interventions. In fact, when it is necessary to modulate the expression of a specific gene, it is possible to either act or regulate mRNA degradation by sponging miRNAs that are involved in this process, or in the case that the protein is lacking, favor protein synthesis by the maintenance of mRNA within the cell. This last aspect can also be achieved by limiting mRNA degradation by favoring its circularization. For a further description of circRNAs may be useful in skeletal muscle therapy, refer to the paragraph entitled "Circular RNAs therapeutic potential in muscle atrophy".

In addition to miRNAs, circRNAs can also interact with proteins to influence muscle development. For example, over the past 5 years, two circRNAs, circSmad4 and circNFIX, have been associated with this phenomenon. Affinity pulldown followed by mass spectrometry have revealed the association of circSamd4 with

PURA and PURB, two proteins that repress myogenesis by inhibiting the transcription of the myosin heavy chain (MHC) protein family [71]. Therefore, the interaction of circRNA with PURA and PURB impacts the ability of two proteins to interact with DNA. Another circRNA that interacts with proteins is circNfix. circNfix promotes cardiac regenerative repair and functional recovery following myocardial infarction by reinforcing the interaction between Ybx1 (Y-box binding protein 1) and Nedd4l (an E3 ubiquitin ligase). This stimulates Ybx1 degradation through its ubiquitination. Indeed, Ybx1 depletion prevents pathological cardiomyocyte growth *in vitro* and promotes cardiac function *in vivo* by regulating protein synthesis [72].

4. Micro-peptides derived from linear lncRNAs

As previously described, lncRNAs are RNAs longer than 200 nucleotides that lack the ability to code for proteins. The advent of novel sequencing techniques, such as ribosomes RNA sequencing, has demonstrated the ability of ribosomes to associate with lncRNA molecules [73]. This finding supports the possibility that lncRNAs may be translated. Although ribosome profiling provides evidence of lncRNA-ribosomes interactions, it is crucial to confirm the presence of translated peptides through mass spectrometry analysis (MS). For example, mass spectrometry experiments confirmed that only 18 out of 233 lncRNAs capable to interact with ribosomes lead to the synthesis of small peptides in human cell lines [74]. Notably, many micropeptides have been functionally characterized, highlighting their significance in muscle physiology. They regulate muscle regeneration via mTOR [75], development/myogenesis [76, 77], and calcium handling [78, 79]. Furthermore, mitochondria, which are crucial organelle for the energetic and metabolic functions of skeletal muscles, are regulated by micropeptides synthesized from lncRNA translation. Human LINC00116 encodes a highly conserved muscle- and heart-enriched micropeptide that localizes to inner mitochondrial membrane. This micropeptide impacts on mitochondrial respiration and Ca2+ buffering capacities [80] leading to its designation as Mitoregulin. Another important micropeptide derived from lncRNA translation and involved in the regulation of mitochondrial function is MOXI. It enhances fatty acid β-oxidation [81]. Skeletal muscle studies regarding the synthesis of micropeptides from lncRNAs were published prior to the time period considered in this work, but we have included them to demonstrate that lncRNAs expressed from skeletal muscle also code for micropeptides. Most recent study on micropeptide and skeletal muscle was published in 2020 from Wang and colleagues [77]. They showed that lncRNA-MyolncR4 encodes a 56-aa micro-peptide, which fosters muscle formation and regeneration. Recently, the significance of micropeptides derived from lncRNA in cardiac function has been elucidated. In 2021, two distinct research publications described the topic. One study elucidated the importance of the lncRNA LINC00961-encoded micropeptide SPAAR for cardiovascular function in adulthood [82]. The other study described a way to regulate cardiomyocyte hypertrophy by using three micropeptides that modulate the activities of oxidative phosphorylation, the calcium signaling pathway, and the mitogen-activated protein kinase (MAPK) pathway [83]. For a comprehensive review on micropeptides from lncRNAs, including bioinformatic and experimental approaches for their identification see [84].

5. Extracellular lncRNAs

Skeletal muscle is a secretory organ that communicates with other tissues by secreting myokines [1]. Exosome formation is an active way of cell-cell or tissue communication. The presence of miRNAs, lipid, or proteins in exosomes derived from skeletal muscle has been extensively studied [85–87]. In contrast, there is only one study that characterized the secretion of lncRNAs in a skeletal muscle pathological condition. In 2022, Li and colleagues demonstrated that plasma exosomes of patients with dermatomyositis (DM) in addition to miRNAs contain lncRNAs [88]. DM is an immune-mediated muscle disease characterized by skeletal muscle weakness [89]. Li and colleagues identified 452 differentially expressed lncRNAs in plasma of DM patients compared with NCs. These lncRNAs were likely involved in autophagy, IFN-β production, IL-12 production, and fatty acid transmembrane transport [88]. This result suggests that lncRNAs can also be utilized from skeletal muscle for communication and that lncRNAs can also serve as pathological markers.

6. Long non-coding RNAs in muscle physiology

6.1 Myogenesis

Long non-coding RNAs (lncRNAs) have been identified in different cells playing pivotal roles in both normal muscle physiology and diseases [90]. As previously discussed, lncRNAs can either stimulate or suppress gene expression by controlling mRNA translation and stability, pre-mRNA splicing, miRNA sponging, and gene transcription [91]. Advancements in high-throughput sequencing have facilitated the identification of tens of thousands of lncRNAs in skeletal muscle tissue. However, only a selected few of these lncRNAs have been demonstrated to contribute to the development and function of skeletal muscles [92]. During embryonic development, the proliferation of mononuclear myoblasts is critical in determining the size of future skeletal muscle. As development advances, myoblasts stop dividing and differentiate into multinuclear myotubes, a phase known as myogenesis [93].

A recent study identified 385 differentially expressed lncRNAs during C2C12 differentiation, elucidating transcriptional regulation and regulatory roles of lncRNAs in skeletal muscle cell differentiation [92, 94]. Gm10561 is one of these differentially expressed lncRNAs. However, its specific role in myogenesis remains unclear. In a study by Wang et al., it was revealed that Gm10561 exhibits higher expression levels in skeletal muscle compared to other tissues and undergoes an increase during myogenic differentiation. Functional study further demonstrated that Gm10561 positively regulates the proliferation and differentiation of C2C12 myoblasts. Mechanistically, Gm10561 functions as a molecular sponge of miR-432, thereby enhancing the expression of MEF2C and E2F3. This action accelerates myoblast proliferation and differentiation. Furthermore, Gm10561 was identified as a novel positive regulator of myogenesis, potentially contributing to the elucidation of the lncRNA–miRNA–target-gene regulatory network associated with myogenesis [92]. Mechanism of Gm10561 is shown in **Figure 1**.

LncRNA Irm is another lncRNA differentially expressed during skeletal muscle morphogenesis in embryonic development, suggesting its potential involvement in myogenesis. It was reported that lrm expression is higher in the tongue and skeletal muscles during embryonic development [96]. Notably, the expression of lrm

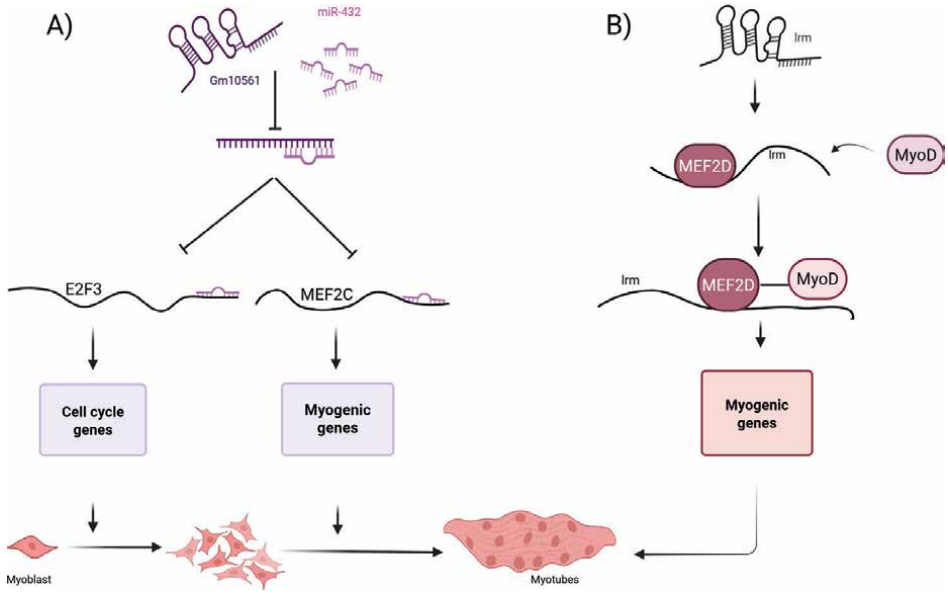

Figure 1.
(A) Gm10561 acts as a molecular sponge of miR-432 to promote myogenesis. In fact, miR-432 directly binds to the 3′UTR of E2F3 and MEF2C, which inhibits their expression. Gm10561 sponges mir-432 can increase E2F3 expression through decreasing the inhibition of miR-432 on E2F3, which leads to an increase in myoblast proliferation. Moreover, Gm10561 sponges miR-432 to enhance MEF2C expression, reduce the suppression of miR-432 on MEF2C, and thereby encourage myoblast differentiation upon differentiation [92]. (B) The lncRNA lrm plays a crucial role in regulating the expression of myogenic genes by directly binding to MyoD. This interaction enhances the assembly of MyoD/MEF2D complexes on the regulatory elements of target genes [95].

significantly increases in differentiated muscle cells, indicating its connection to active myogenesis [95]. Furthermore, using gain and loss of function experiments, Sui and colleagues demonstrated that Irm significantly promotes C2C12 differentiation. Additionally, it was shown that the absence of lrm resulted in a significant delay in the *in vivo* regeneration of injured-induced muscle. To activate its known target genes, lrm recruits MyoD/MEF2D by directly binding to MEF2D and the DNA of the promoter region. These findings revealed that lrm is a novel member of the MEF2D-regulating network that promotes myogenesis and differentiation in C2C12 cells. Furthermore, it functions as a lncRNA enhancer of MyoD/MEF2D and cooperatively controls the transcription of myogenic differentiation genes [95]. The mechanism of action of Irm is shown in **Figure 1**.

The lncRNA Has2os, situated within the coding Hyaluronan synthase 2 (Has2) gene, is another important lncRNA associated with myogenesis. Interestingly, Has2 is a gene exclusively expressed in skeletal muscles and is believed to be essential for skeletal muscle development, patterning, chondrocyte maturation, and the formation of synovial joints in growing limbs. Furthermore, myogenic differentiation requires endogenous hyaluronan synthesis, and limbal stem cell differentiation is regulated by a hyaluronan-rich microenvironment within the limbal stem cell niche. The function of lncRNAs appears to be closely linked to that of their neighboring genes through cis-acting processes [90, 97–99]. As elucidated by Cheng and colleagues, Has2os undergoes continuous accumulation during skeletal muscle differentiation. Conversely, its expression inhibition leads to the inhibition of skeletal muscle differentiation, suggesting that Has2os acts as a differentiating promoter. Additionally, Has2os plays

a role in the early stages of skeletal muscle regeneration. It has been demonstrated that Has2os contributes to these processes by controlling the JNK/MAPK signaling pathway [90]. The mechanism of action of Has2os is shown in **Figure 2**.

The lncRNA 4930581F22Rik exhibits an expression pattern comparable to lrm and Has2os, suggesting a potential role in skeletal muscle development and regeneration. During myogenesis, the expression of lncRNA 4930581F22Rik progressively increases, and its knockdown significantly impedes differentiation and myogenic

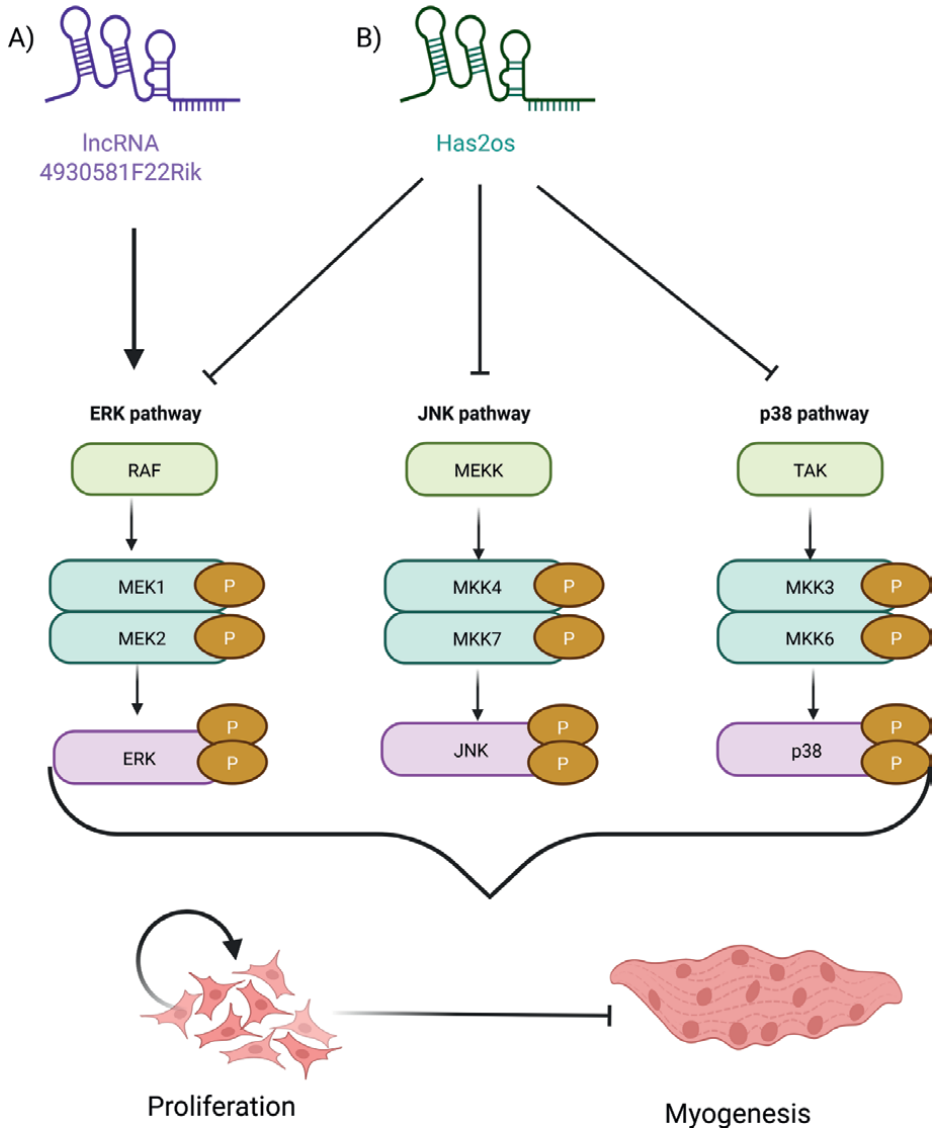

Figure 2.
(A) Expression level of lncRNA 4930581F22Rik gradually increases during skeletal muscle differentiation. LncRNA 4930581F22Rik regulates the differentiation program and the expression of myogenic markers through modulating ERK/MAPK signaling pathway [100]. (B) LncRNA Has2os is highly expressed in skeletal muscle tissue and upregulated during myogenesis. Has2os modulates myogenesis and regeneration through inhibiting the JNK/MAPK signaling pathway with three different MAPK branching signaling pathway, including JNK/MAPK, p38/MAPK, and ERK1/2/MAPK [90].

marker expression [101]. According to Chen's study, lncRNA 4930581F22Rik modulates this process by regulating the ERK/MAPK signaling pathway, which can be effectively neutralized by the ERK-specific inhibitor PD0325901. Furthermore, it has demonstrated that lncRNA 4930581F22Rik is emerging as a viable participant in the initial stages of muscle regeneration [100]. The mechanism of action of lncRNA 4930581F22Rik is shown in **Figure 2**.

To date, the physiological function of lncRNAs in muscles has been the primary focus of numerous studies [91, 102, 103]. However, there is a limited understanding of the precise mechanisms they modulate during muscle atrophy [91]. Several other lncRNAs were associated with skeletal muscle differentiation, but a limited mechanistic description was furnished. These lncRNAs are mentioned in **Table 1**.

LncRNAs	Description	Expression in myogenesis	Mechanism	Ref
Mir22hg	Mir22host gene	Increase	*Mir22hg* matures into miR-22-3p to inhibit *HDAC4* expression. This activates the downstream *MEF2C* pathway, thereby promoting skeletal muscle differentiation and regeneration.	[104]
Lnc-GD2H	*Gm13398* was named the lncRNA of growth and differentiation to histogenesis (*lnc-GD2H*).	Increase	It facilitates the expression of proliferating marker genes, including c-Myc, CDK4, CDK2, and CDK6. It establishes a feedback loop with c-Myc during myoblast proliferation. Additionally, *lnc-GD2* interacts with NACA to alleviate the inhibitory effect of NACA on Myog, thereby facilitating Myog expression and promoting differentiation.	[105]
LncMGPF	lncRNA muscle growth promoting factor (*lncMGPF*).	Increase	*lncMGPF* sponge miR-135a-5p promoting *MEF2C* gene expression. Moreover, *lncMGPF* regulates human antigen R (HuR; also known as ELAV-like RNA binding protein 1)-mediated mRNA stabilization of MyoD and MyoG genes.	[106]
Myoparr	Promoter-associated lncRNA, *Myoparr*, derived from the upstream region of the *myogenin* gene.	Increase	*Myoparr* binds Ddx17, a transcriptional coactivator of MyoD, and promotes the interaction between Ddx17 and histone acetyltransferase PCAF. *Myoparr* exerts its functions by interacting with transcriptional activator during myogenesis.	[107]
PAM	*Pax7* associated muscle lncRNA (*PAM*)	Increase	*PAM* regulates satellite cells proliferation through the interaction with Ddx5, thereby facilitating the chromatin contact between PAM SE (super enhancer) and target loci (Timp2 and Vim) during SC proliferation.	[49]

LncRNAs	Description	Expression in myogenesis	Mechanism	Ref
LncRNA-MEG3	LncRNA maternally expressed gene 3 (*lncRNA-MEG3*)	Increase	*LncRNA-MEG3* regulates muscle regeneration by sponging miR-133a-3p to regulate the expression level of proline-rich transmembrane protein 2 (PRRT2).	[108]
1700113A16RIK	A novel lncRNA, *1700113A16Rik*, that regulates muscle regeneration.	Increase	*1700113A16RIK* functions by pairing to the 3′UTR of *Mef2d* to facilitate Mef2d translation. It may help recruit ribosomes to the 3′UTR of *Mef2d* to promote translation.	[109]
Linc-MYH	*Linc-MYH* is the evolutionary conserved lncRNA, which is co-expressed with the largest myosin cluster in mammals.	Increase	*Linc-MYH* regulates the composition of the INO80 chromatin remodeler complex by directly interacting with its functional domains. Linc-MYH inhibits the INO80 complex's ability to interact with the scaffolding protein WDR5 and the transcription factor YY1. In fact, link-MYH acts as a selective molecular switch for INO80, enabling the regulation of distinct INO80 activities in MuSCs at specific stage.	[21]
2310043L19Rik	Named lnc-231	Increase	*Lnc-231* inhibits myoblasts differentiation interacting with miR-125a-5p. Inhibition activity of miR-125a-5p allows the translation of the target gene E2F3. Resulting protein promotes the cell cycle progression.	[110]
Linc-RAM	Activator of myogenesis (*Linc-RAM)*	Increase	Cytoplasmic link-RNA directly binds to glycogen phosphorylase (PYGM) and regulates its enzymatic activity, which is vital for muscle cell to differentiate.	[111]
Lnc-SMaRT	Skeletal muscle regulator of translation (*SMaRT*)	Increase	*Lnc-SmaRT* can inhibit Spire1 translation by base-pairing with its G-quadruples-forming sequence. The modulation of Spire1 expression regulates proper skeletal muscle differentiation. Furthermore, it is demonstrated that the mRNAs in the complex facilitate the indirect interaction between DHX36 and *lnc-SMaRT*.	[112]
LncMREF	LncRNA muscle regeneration enhancement factor (*lncMREF*)	Increase	*LncMREF* interacts with Smarcas5 and p300/CBP to enhance chromatin accessibility and the expression of myogenic genes. This promotes the differentiation of muscle satellite cells and muscle regeneration.	[113]

LncRNAs	Description	Expression in myogenesis	Mechanism	Ref
TUG1	Taurine-upregulated gene 1 *(TUG1)*	Increase	*TUG1* is upregulated in human skeletal muscle after exercise. Mechanistically, *TUG1* regulates transcriptional networks linked to mitochondrial calcium handling, muscle differentiation, and myogenesis.	[114]
Lnc-Malat1	Metastasis-associated lung adenocarcinoma transcript1 (*Malat1*)	Increase	Overexpression of lnc-*Malat1* competes for the binding site of miR-129-5p in the Mef2a transcription factor, thereby promoting myoblast differentiation and alleviating inhibitory effect of miR-129-5p on the same process.	[115]

Table 1.
Summary of other lncRNAs that are associated with skeletal muscle differentiation.

7. Long non-coding RNAs in muscle pathology

Dysregulated expression of lncRNAs has been implicated in various pathologies, including cancer, metabolic, and cardiovascular disorders [116, 117]. Previous studies have identified several lncRNAs, such as Linc-YY1 [118], Linc-MD1 [43, 119], Linc-RAM [120], lncRNA-Six1 [121], H19 [122], SYISL [123], lnc-mg [102], and lnc-MyoD [121], as potential regulators of myogenesis. However, with the exception of lncRNAs associated with Duchenne muscular dystrophy [DMD] [124, 125] and facioscapulo-humeral muscular dystrophy [FSHD] [125, 126], the mechanistic role of lncRNAs in skeletal muscle diseases remains largely unexplored. It has been determined that a limited number of lncRNAs, including lnclRS1, lncRNA Atrolnc-1, and lncMUMA, contribute to particular forms of muscular atrophy [127]. In agreement with the expression of skeletal muscle differentiation-related lncRNAs, such as DRR [128], DUM1 [129], linc-MD1 [43], linc-YY1 [118], LncMyod [130], Neat1 [131], Myoparr [107], Malat1 [132], and SRA [133], muscle atrophy can be categorized into two subgroups: disuse-mediated atrophy, such as denervation, casting, and tail suspension, and systemic wasting atrophy, including glucocorticoid administration, cancer cachexia, and fasting. The latter subgroup is associated with genomic imprinting-related lncRNAs, such as Gtl2, H19, and IG-DMR [134]. However, it is yet unknown whether muscle atrophy in many disease models may be mediated by a common regulatory lncRNA [127].

7.1 Metabolic disease

Skeletal muscle exhibits plasticity or metabolic flexibility, enabling it to regulate fuel oxidation in response to fuel availability. Skeletal muscle metabolism has been recognized for its significant role in both health and development as well as metabolic disorders such as obesity and muscular atrophy, which are closely associated with reduced flexibility in skeletal muscle [135]. Increased protein degradation is a defining characteristic of muscular atrophy, characterized by a reduction in muscle mass and fiber size [136]. It is more probable that lncRNAs that have typically disrupted

expression patterns in a variety of muscle atrophy disorders contribute to the role in onset or progression of muscle atrophy. However, there is limited research investigating lncRNAs that are commonly altered in various muscular atrophy diseases [134] and may represent a switcher important for therapeutic purposes.

7.1.1 LncRNAs and muscle insulin resistance

A pivotal indicator of type 2 diabetes (T2D) is insulin resistance, characterized by the pancreatic β-cells' inability to release insulin effectively and the diminished responsiveness of target tissues to normal insulin levels circulating in the bloodstream. LncRNAs may have a role in the development of insulin resistance in a number of organs [137]. Notably, lncRNA H19, an important imprinted gene expressed during embryogenesis, exhibits a significant decrease in its expression after birth in most tissues except skeletal muscle. It is worth to note that H19 expression is decreased in the skeletal muscle of patients with type 2 diabetes mellitus (T2DM) and in obese mice induced by high fat diet (HFD). Subsequently, downregulation of H19 disrupts glucose metabolism [138].

A study conducted by Alipoor and colleagues on insulin-resistant humans and rodent models revealed that H19 down-regulation increased let-7 expression (because H19 functions as sponge against let-7). This, in turn, led to the downregulation of let-7 target DUSP27, a cofactor of AMPK. Consequently, insulin signaling resistance in the muscle was observed. Furthermore, H19 was observed to block several signaling pathway components, including INSR, IRS-2, and IDE [137]. HnRNPA1 was identified as an interacting partner of H19, highlighting its involvement in various biological processes, such as glucose and lipid metabolism, mitochondrial function, and tumorigenesis. Indeed, HnRNPA1 serves as a positive regulator of lipid metabolism in muscle cells, controlling numerous genes involved in fatty acid oxidation, including CPT1b and PGC1a [137, 138]. Research by Gui and colleagues corroborated these findings that H19 recruits HnRNPA1 to CPT1P and PGC1a transcripts, thereby regulating their translation during lipid metabolism [138]. Therefore, insulin resistance may be associated with H19 downregulation and subsequent suppression of the H19/HnRNPA1 axis [137, 138]. **Table 2** provides an overview of lncRNAs and related metabolic disorders.

7.1.2 lncRNAs and cancer cachexia

Cancer Cachexia, a multifaceted metabolic syndrome, is characterized by the loss of skeletal muscle and adipose tissue mass. Its primary clinical manifestation is weight loss accompanied by anorexia, inflammation, cancer, and insulin resistance. Furthermore, cachexia has been associated with increased muscle protein degradation [141, 149, 150]. LncRNAs exert diverse effects on mRNA turnover, including acting as miRNA sponges and subsequently diminishing their regulatory functions. Notably, lncRNAs have been implicated in various diseases, including cancer and cachexia. However, there are a limited number of studies that explore the involvement of lncRNAs in cancer cachexia and elucidate their underlying mechanisms of action [141, 142].

Recent studies have elucidated the role of the lncRNA muscle anabolic regulator 1 (MAR1) in muscle differentiation and regeneration. Notably, MAR1exhibits high expression in skeletal muscle of mouse and has been positively associated with muscular growth and differentiation both *in vitro* and *in vivo*. This lncRNA impedes

Disease/ biological process	lncRNA	Description	Affected pathways	Regulatory mechanism	Ref
Diabetes	*H19*	LncRNA *H19*	PI3K/AKT pathway	Obesity and type 2 diabetes patients have less muscle mass, which impairs insulin signaling and reduces glucose absorption via the PI3K/AKT pathway because of *Let-7* miRNA.	[137, 138]
	LncRNA UCA1	Urothelial carcinoma associated 1 *(lncRNA UCA1)*	*UCA1* alleviates mitochondrial dysfunction by the miR-143-3p/ FGF21 axis.	*UCA1* upregulates the expression of FGF21 through competitively binding to miR-143-3p, thereby preventing the mitochondrial dysfunction of skeletal muscle in T2DM.	[139]
	LncASIR	Adipose-specific insulin responsive lncRNA *(lncASIR)*	Several metabolic pathways downstream of insulin signaling.	Silencing of *lncASIR* inhibits metabolic pathways downstream of insulin signaling, including PPAR and adipocytokine signaling.	[140]
Cancer cachexia	*MAR1*	lncRNA muscle anabolic regulator 1 *(MAR1)*	Wnt5a (Wnt pathway)	Serves as a sponge for mir-487b in C2C12 cells, preventing a decrease in Wnt5a expression and encouraging myogenesis. In mice, expression falls with age and muscle unloading.	[141–143]
	lncIRS1	LncRNA insulin receptor substrate 1 (*lncIRS1*)	IGF1-PI3K/Akt signaling pathway	Skeletal muscle cell proliferation and differentiation are facilitated by *LncIRS1's* regulation of IRS1 expression, which activates the IGF1-PI3K/Akt signaling pathway and promotes IGF-1 production and phosphorylates AKT	[141, 142]
	HOTAIR	HOX transcript antisense intergenic RNA (*HOTAIR*)	NF-κB signaling pathway.	The fact that HOTAIR may be an important player in cancer cachexia is sustained by its involvement in cancer progression and NF-κB signaling. However, there have not investigated on the role of *HOTAIR* and the signaling pathway involve d in cancer-induced or chemotherapy-induced cachexia.	[144]
	Malat1	Metastasis associated lung adenocarcinomatranscript1 (*Malat1*)	Wnt5a (Wnt family member 5A)	*Malat1* is linked to adipose loss in cancer-associated cachexia through regulating adipogenesis by PPAR-γ.	[145]

Disease/ biological process	lncRNA	Description	Affected pathways	Regulatory mechanism	Ref
Obesity	*Blnc1*	Brown fat lncRNA 1 (*Blnc1*)	BAT thermogenesis and mitochondrial function	Transgenic expression increases lipid metabolism and inhibits proinflammatory cytokines, while suppressing increased tissue inflammation and BAT fibrosis. WAT overexpression increases insulin sensitivity and protects against obesity brought on by an HFD.	[146, 147]
	MIST	Macrophage inflammation-suppressing transcript (*Mist*)	Interaction with histone modifiers is reliable epigenetic mechanism, which PARP1 is identified as a lead Mist-interacting partner.	*Mist* inhibits PARP1 (poly ADP-ribose polymerase-1) recruitment to inflammatory gene promoters by interacting with PARP1 in the nucleus.	[148]

Table 2.
Overview of long noncoding RNAs and related disorders.

miR-487b function by sponging it and consequently regulating Wnt family member 5A (Wnt5a). This promotes muscle differentiation and regeneration [141–143]. Furthermore, overexpression of MAR1 has been demonstrated to reduce muscular atrophy while maintaining muscle mass and strength, indicating its potential as a therapeutic target for cancer cachexia-related muscle atrophy [141].

The lncRNA insulin receptor substrate 1 (lncIRS1) is another lncRNA that demonstrated its activity as a miRNA sponge in mouse skeletal muscles. It has been demonstrated that LncIRS1 controls muscle mass and fiber sectional area *in vivo*, as well as myoblast proliferation and differentiation *in vitro* and *in vivo*. LncIRS1 can modulate IRS1 expression by acting as a sponge of the miR-15 family. Overexpression of LncIRS1 leads to the activation of the IGF1-PI3K/Akt signaling pathway, important to counteract muscle atrophy. Subsequently, this lncRNA promotes skeletal muscle cell proliferation and differentiation, leading to an increase in muscle mass and countering muscle atrophy [141, 142].

7.1.3 LncRNAs and obesity

Obesity, which is a risk factor for metabolic diseases, develops during the adipogenesis process. Obesity is inversely associated with muscle strength, activity, and hypertrophy. Obesity process involves the differentiation of precursor mesenchymal stem cells into adipocytes, their growth in size and accumulation, and their eventually fusion to enhance the storage of fat mass in white adipose tissues. Transcriptomic analyses have identified several lncRNAs that exhibit differential expression in body fat or adipose tissue (AT) in obese people. These lncRNAs play a crucial role in controlling adipogenesis and differentiation [137, 146]. The human body comprises two main types of adipose tissues: insulating energy-storing white AT (WAT) and thermogenic brown AT (BAT). Energy-burning and heat-generating BAT is situated around the shoulders and ribs, while visceral WAT, which surrounds the intra-abdominal organs, and subcutaneous WAT, located under the skin, are essential for energy homeostasis. In obesity, BAT undergoes molecular and morphological changes that lead to whitening appearance, resembling WAT. This transformation subsequently impairs metabolic function and contributes to inflammation [146].

Recent studies have identified several lncRNAs associated with the regulation of BAT, including H19 and brown fat lncRNA1 (Blnc1) [151, 152]. Studies have revealed that Blnc1 contributes to the regulation of thermogenic genes, leading to the increased expression of mitochondrial and uncoupling protein 1 (UCP1) genes [153]. Another lncRNA positively associated with the browning of adipose tissue was H19. It was inversely correlated with body mass index (BMI) [154]. In BAT, H19 has a role in regulating mitochondrial respiration, oxidative metabolism, and adipogenesis (**Table 2**). Therefore, altering lncRNA expression appears to be a promising treatment strategy for metabolic disorders. This approach may be based on the induction of browning of WAT or the enhancing of BAT activity [147]. Although lncRNAs show promise as therapeutic targets for obesity treatment, several challenges must be addressed before clinical applications can be realized. A significant obstacle lies in the lack of knowledge of the precise molecular mechanisms underlying lncRNA expression control in such different but communicating tissues as adipose tissue and skeletal muscle. Additionally, due to their size and possible off-target effects, lncRNA-based therapies remain challenging to deliver to specific tissues [137, 147].

8. Long non-coding RNAs as potential therapeutic targets in muscle atrophy

8.1 Long non-coding RNAs as therapeutic targets

New drug discovery begins with the elucidation of mechanisms underpinning physiological and pathological processes. Several studies (see long non-coding RNAs in muscle physiology and long non-coding RNAs in muscle pathology) have elucidated the role of long non-coding RNAs (lncRNAs) in skeletal muscle. Targeting lncRNAs has emerged as a promising therapeutic strategy for the treatment of a diverse range of diseases [155–157], including muscle disorders [93, 95, 104, 105, 158, 159].

Therapeutic potential of lncRNAs relies on their ability to bind complementary nucleic acid sequences. Consequently, also lncRNAs can be targeted by small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs) [155]. SiRNAs are short double-stranded RNA (dsRNA) molecules, while ASOs are short single-stranded oligonucleotides (RNA or DNA), both of which range in length from 15 to 25 nucleotides. The binding of siRNAs and ASOs to lncRNAs triggers target (lncRNA) degradation, respectively, through the action of the RISC complex and RNase H cleavage [155, 160, 161]. Recently, lncRNAs have emerged as targets of small molecules (small lncRNA-targeting molecules). In fact, the structural properties of lncRNAs enable them to interact with proteins or small molecules, leading to direct alterations in their conformation and, consequently, effects on their functions by obscuring or disrupting lncRNA interaction sites [155, 156]. Currently, CRISPR/Cas technology is used in research to modulate lncRNAs expression, but off-target effects remain a significant challenge in the therapeutic application [155, 156].

Although lncRNAs present promising opportunities in medicine, there are concerns regarding the potential adverse effects of lncRNA modulation. In fact, off-targeting may lead to undesirable outcomes. Other than this, like in other RNA-based therapies, RNA delivery systems require further development to achieve tissue specificity and mitigate immune response and toxicity issues [155, 162].

Investigation into the therapeutic potential of lncRNAs has gained prominence only in the past decade. Consequently, no lncRNA-targeting therapeutics have yet progressed to clinical trials and entered clinical translation [155].

8.2 The potential of long non-coding RNAs to treat muscle atrophy

Long non-coding RNAs have been recently studied for their role in modulating muscle atrophy, dystrophy, and regeneration (see "long non-coding RNAs in muscle physiology" and "long non-coding RNAs in muscle pathology"). In the past 5 years, numerous studies have elucidated the positive regulatory effects of lncRNAs on muscle regeneration [95, 104, 105, 163], while others have demonstrated detrimental effects [93, 107, 144, 158, 164]. Certain lncRNAs, such as Irm, Mir22hg, and GD2H, are essential for the regeneration process, and their downregulation impairs *in vivo* regeneration [95, 104, 105]. In other articles, authors have demonstrated that the downregulation of lncRNAs like Pvt1, Hotair, Mayoparr, lnc-ORA, and lncRNA-3 attenuates or rescues muscle atrophy [93, 107, 144, 158, 159, 164]. Consequently, these molecules represent promising putative targets for skeletal muscle atrophy.

Different diseases or pathological conditions resulted in muscle wasting and atrophy. Notably, the expression of non-coding RNAs could be specific to different

atrophic stimuli [103, 165, 166]. Consequently, the upregulation of lncRNAs could be beneficial or detrimental depending on the context. For instance, H19 is upregulated in amyotrophic lateral sclerosis and after denervation [158, 165], while it is downregulated in hindlimb suspension-induced [167] and fasting-induced [165] muscle atrophy. Furthermore, H19 administration alleviates dystrophy in mice models for muscular dystrophy caused by disrupting mutations of the dystrophin gene [168]. These differences linked to genetic context should be considered when a putative target is selected to develop a new drug.

Despite the substantial potential of lncRNAs as therapeutic targets, there are currently no approved drugs or clinical trials specifically focused on treating muscle atrophy and dystrophies through lncRNA modulation [155–157, 169]. However, there are a few recent examples demonstrating the potential of *in vivo* lncRNA downregulation to attenuate or even rescue muscle atrophy. For example, *in vivo* knockdown of the lncRNA Myoparr in denervated tibialis anterior (TA) muscles effectively attenuates muscle atrophy [93, 107]. Similarly, the downregulation of Pvt1 in TA muscles protects against atrophy induced by denervation [158].

Other researchers have shown that *in vivo* lncRNA downregulation impairs muscle regeneration, suggesting that these ncRNAs may promote muscle regeneration. During cardiotoxin injury-induced regeneration, downregulation of the lncRNA Irm delays TA muscle regeneration [95]. Similarly, lnc-GD2H silencing significantly impairs muscle regeneration after cardiotoxin treatment [105]. Furthermore, knockdown of Mir22hg in TA muscles inhibits and delays the regeneration of BaCl2-injured skeletal muscle [104]. This year, Zhang and colleagues investigated the role of lncRNA-3 in muscle regeneration during aging. They demonstrated that lncRNA-3 overexpression delays the skeletal muscle regeneration process, while lncRNA-3 silencing by shRNA restores muscle regenerative capacity in the gastrocnemius muscle of aged mice injured by a cardiotoxin injection [159]. The effects of long non-coding RNAs on muscle regeneration and atrophy are summarized in **Table 3**.

8.3 Circular RNAs therapeutic potential in muscle atrophy

Circular RNAs (circRNAs) are covalently closed, non-coding RNAs devoid 5′ and 3′ ends [174]. Although discovered decades ago, circRNAs gained prominence as the widespread class of RNA with the advent of high-throughput RNA sequencing (RNA-seq) and novel bioinformatic tools (see "Circular RNAs") [175, 176].

Recent studies have revealed the multifaceted biological functions of circRNAs, which can serve as miRNA sponges by binding to miRNAs, thereby preventing mRNA target downregulation. Alternatively, they can function as protein sponges and scaffolds by binding to proteins [174, 176]. The ability of circRNAs to interact with both miRNAs and proteins renders them potential therapeutic agents in a diverse range of diseases. CircRNAs play a crucial role in regulating muscle regeneration and atrophy *in vivo*. For example, the downregulation of circTmeff1 [64] and cicrDdb1 [63] prior to denervation or immobilization has been demonstrated to attenuate muscle atrophy. In addition, the overexpression of Circ2388 [170], CircARID1A [171], and CircFgfr2 [172] promotes muscle regeneration in cardiotoxin-injured muscle, while CircCPE [173] exhibits a detrimental effect on regeneration. These findings collectively demonstrate the role of circRNAs in skeletal muscle and highlight their potential as therapeutic targets for muscle atrophy. The effects of circular RNAs on muscle regeneration and atrophy are summarized in **Table 3**.

ncRNA	Model	Modulation	Effect	Ref
Long non-coding RNA				
Myoparr	Den-induced atrophy	shRNA knockdown	Attenuates atrophy	[93, 107]
Pvt1	Den-induced atrophy	Gampers knockdown	Attenuates atrophy	[158]
Mir22hg	$BaCl_2$ injury-induced regeneration	shRNA knockdown	Delay muscle regeneration	[104]
Lnc-GD2H	CTX injury-induced regeneration	siRNA silencing	Delay muscle regeneration	[105]
LncRNA-3	CTX injury-induced regeneration	Sk-lncRNA-3 knock-in	Delay muscle regeneration	[159]
		shRNA knockdown	Promotes muscle regeneration	
Circular RNA				
CircTmeff1	Den-induced atrophy	shRNA knockdown	Attenuates atrophy	[64]
	Imo-induced atrophy			
CircDdb1	Den-induced atrophy	shRNA knockdown	Attenuates atrophy	[63]
	Imo-induced atrophy			
	Angiotensin II induced atrophy			
Circ2388	CTX injury-induced regeneration	Overexpression	Promotes muscle regeneration	[170]
CircARID1A	CTX injury-induced regeneration	Overexpression	Promotes muscle regeneration	[171]
CircFgfr2	CTX injury-induced regeneration	Overexpression	Promotes muscle regeneration	[172]
CircCPE	CTX injury-induced regeneration	Overexpression	Delay muscle regeneration	[173]

Den, denervation; CTX, cardiotoxin; Imo, immobilization; shRNA, short hairpin RNA.

Table 3.
Long non-coding RNAs and circular RNA effects on muscle regeneration or atrophy in vivo.

9. Modulation of long non-coding RNAs expression in skeletal muscle cells

9.1 Skeletal muscle models

Skeletal muscle tissue is not only composed of myofibers but also satellite cells, smooth muscle cells, vascular cells, motor neurones, fibroblasts, and resident immune cells. All these cell types are organized in a highly specific architecture to form the skeletal muscle structure [177–179]. Common *in vitro* models to study skeletal muscle functions are mouse myoblasts (C2C12), rat myoblasts (L6), and primary myoblasts derived from rodent or human biopsies. C2C12 is an immortalized cell line derived from mouse skeletal muscle myoblasts. It is a commonly used model in medical research to study skeletal muscle atrophy and related diseases [180].

Primary cultures from rodents or human biopsies offer greater representation of their tissue of origin compared to immortalized cell lines such as C2C12 and L6. Primary cells retain the phenotypic and genotypic characteristics of their source tissue, making them widely used in metabolic studies [178, 180]. However, primary cultures have limited proliferative capacity, posing challenges in extensive cell usage. Consequently, efforts are being made to immortalize isolated primary myoblasts. Moreover, in the past decade, several methods have been established to differentiate myogenic cells from induced pluripotent stem cells (iPSCs) [179, 181, 182]. IPSCs derived from healthy donors or patients affected by muscular disorders overcome limitations associated with tissue availability and ethical considerations.

Bidimensional cell cultures lack a physiological extracellular environment and native skeletal muscle organization [179, 183]. Moreover, rodent myoblast cell lines (C2C12 and L6) and skeletal muscles exhibit transcriptomic and metabolic differences compared to human cells and tissue due to the species specificity [178]. To overcome limitations associated with cell lines and animal models, bioengineering human models that provide greater fidelity to native skeletal muscle tissues have been developed in recent years. Three-dimensional models are designed to replicate the complexity of the native muscle tissue and its niche in order to match the morphological and functional features of skeletal muscle [179–181, 183]. These innovative models for skeletal muscle have the potential to advance our understanding of myogenesis and muscle regeneration, but their widespread adoption in research remains limited.

9.2 Transfection of skeletal muscle cells

In the previous paragraphs (see long non-coding RNAs in muscle physiology, long non-coding RNAs in muscle pathology, and long non-coding RNAs as potential therapeutic targets in muscle atrophy), we highlighted the importance of cell and animal models for skeletal muscle to study the impact of long non-coding RNAs (lncRNAs) in this tissue. The majority of the studies presented here consider the modulation of lncRNAs expression by transfecting cells [92, 95, 115] or tissues [93, 104, 105, 107, 158, 159].

As previously mentioned, C2C12 and primary mouse myoblasts are commonly used in laboratories investigating muscle pathophysiology. However, transfecting these cells presents significant challenges [184, 185]. Over the last 10 years, C2C12 cells have predominantly been transfected using cationic-lipid transfection reagents [185], although transfection efficiency is extremely low [186]. Each transfection protocol should be optimized based on cell type being transfected (e.g., immortalized cell lines and primary muscle cells) and the nucleic acid being transfected (e.g., plasmid and siRNA) [185].

The majority of chemical transfection reagents are composed of cationic components, such as cationic polymers and lipids, which interact with negatively charged nucleic acids to form complexes. In addition to cationic reagents, alternative transfection methods can be employed for C2C12 cells (e.g., calcium phosphate [184] and gold nanoparticle-mediated transfection [187]). Regarding cationic components, in 2022, Cocchiararo and colleagues optimized and compared the transfection of C2C12 cells with small (4.15 kb) and large (8.55 kb) plasmids using five commercial transfection reagents. They assessed cytotoxic effects by evaluating cell growth, viability, and differentiation [185]. Other researchers are actively investigating the development of novel polymers to enhance muscle cell transfection efficiency while minimizing cytotoxicity [188, 189].

Studies on lncRNAs typically include cell or tissue transfections. *In vitro* knockdown of specific lncRNAs is commonly performed by transfecting C2C12 cells with small interference RNAs targeted to the lncRNA [25, 49, 90, 92, 100, 107, 127, 164, 190, 191]. Conversely, overexpression of lncRNAs is achieved by introducing in the cells plasmids carrying the lncRNA sequence [95, 110, 164]. *In vivo* modulation of lncRNAs expression in mouse skeletal muscles can be performed through electroporation of plasmids transcribing for siRNAs or the lncRNA. Alternatively, the direct electroporation of short hairpin RNAs (shRNAs) [93, 107, 110, 192] or the injection of shRNA lentiviral particles can be used [92, 104, 127]. Moreover, knock-in [159] and knock-out [106, 163] mice for specific lncRNAs were developed.

10. Conclusions

Currently, the United States Food and Drug Administration has approved six RNAi-based therapeutics: patisiran (Onpattro), lumasiran (Oxlumo), inclisiran (Leqvio), vutrisiran (Amvuttra), nedosiran (Rivfloza), and givosiran (Givlaari) [193]. The clinical application of RNA interference (RNAi) molecular mechanisms exemplifies a burgeoning class of RNA therapeutics capable of treating diseases by modulating the expression of target genes (**Table 4**). The field of RNA-based therapy emerged in the 1990s, with Martinon's pioneering work demonstrating the in vivo induction of anti-influenza cytotoxic T lymphocytes by immunizing mice with liposomes encapsulating mRNA encoding the influenza virus nucleoprotein [194].

Technique	Mechanistic summary	Common side effects	Current approved examples (agency)
Antisense Oligonucleotides (ASOs)	Bind to mRNA and induce degradation, mask splice sites, avoid translation.	Flu-like symptoms, injection site reactions	Mipomersen (Kynamro) FDA 2013 EMA No
			Tofersen FDA 2023 EMA 2024
			Fomivirsen (Vitravene®) FDA 1998 EMA 1999
			Eplontersen (Wainua) FDA 2023 EMA No
			Mipomersen (Kynamro®) FDA 2013 EMA No,
			Olezarsen (Tryngolza) FDA 2024 EMA No
			Inotersen (Tegsedi®) FDA 2018 EMA 2018,
			Volanesorsen (Waylivra®) FDA 2019 EMA 2019

Technique	Mechanistic summary	Common side effects	Current approved examples (agency)
Short inhibitory RNAs (siRNAs)	Bind to mRNA and induce degradation	Injection site reactions, hypersensitivity reactions	Patisiran (Onpattro) FDA 2018 EMA 2018,
			Nedosiran (Rivfloza) FDA 2023 EMA No
			Givosiran (Givlaari) FDA 2019 EMA 2020,
			Lumasiran (Oxlumo) FDA 2020 EMA 2020,
			Inclisiran (Leqvio®) FDA 2021 EMA 2020,
			Vuttrisiran (Amvuttra®) FDA 2022 EMA 2022
mRNA	Mimic endogenous mRNA and produces entire or part of protein	Varies depending on target and delivery method	Not yet approved
miRNA Mimics and Antagomirs	Mimic or inhibit endogenous miRNAs	Varies depending on target and delivery method	Miravirsen (Clinical trials, not yet approved)
Splice-Switching Oligonucleotide (SSO)	Bind to pre-mRNA and modulate splicing	Injection site reactions, flu-like symptoms	Nusinersen (Spinraza®) FDA 2016 EMA 2017,
			Eteplirsen (Exondys®) FDA 2016 EMA No,
			Golodirsen (Vyondys®) FDA 2019
			Viltolarsen (Viltepso®) FDA 2020,
			Casimersen (Amondys) FDA 2021
RNA Aptamers	Bind to target proteins and modulate their activity	Varies depending on target and delivery method	Pegaptanib (Macugen®) FDA 2004 EMA 2006
RNA Vaccines	Encodes antigenic proteins that stimulate an immune response	Pain at injection site, fever, fatigue	Tozinameran (Comirnaty®) FDA 2021,
			BNT162b2 Comirnaty (Pfizer-BioNTech) FDA 2021 EMA 2020
			Elasomeran (Spikevax®) FDA 2022
			mRNA-1273 Spikevax (Moderna) FDA 2022 EMA 2021

Technique	Mechanistic summary	Common side effects	Current approved examples (agency)
CRISPR-Cas9-mediated gene editing	Induces double-strand breaks in DNA, guided by RNA sequence leading to gene editing	Varies depending on target and delivery method	Not yet approved
TANGO (Targeted Augmentation of Nuclear Gene Output)	Oligo binding to exon in pre-mRNA to produce more functional mRNA that leads in increase in functional protein expression	Not yet known	In clinical trials for Dravet.

Table 4.
Classes of RNA therapeutics with the indication of approved one from Food and Drug Administration (FDA) and European Medicines Agency (EMA).

RNA-based therapy is currently focused on modulating coding genes; however, this manuscript demonstrates that also non-coding RNAs participate in skeletal pathology development. In addition to hereditary skeletal muscle pathologies, lncRNAs participate in several other hereditary disease [195], and their modulation may ameliorate therapeutic effects. An example is spinal muscle atrophy (SMA). It is caused by mutations in a gene called survival motor neuron 1 (SMN1). SMN1 deletions are typical of most SMA patients that maintain SMN2 gene as the sole SMN source. Nonetheless, a C → T substitution transforms an exonic-splicing enhancer to a silencer, triggering frequent exon7 skipping in SMN2 pre-mRNA and resulting in a trimmed protein. Patients treatment with antisense oligonucleotides correcting SMN2 splicing improves SMN expression and motor function [196]. After the discovery of a new antisense transcript from the SMN1 locus, it was demonstrated that SMN-AS1 negatively regulates SMN mRNA level recruiting the PRC2 complex. The application of antisense oligonucleotides directed at SMN-AS1, in conjunction with a splicing corrector to increase exon 7 inclusion in SMN2 mRNA, has demonstrated a two-fold enhancement of SMN2 level within neurons derived from mice that possess only the SMN2 gene [195]. This demonstrates that also lncRNA modulation may be used in therapeutical approaches. Open problems in RNA-based therapy remain. For example, delivery methods should be ameliorated to permit to target desired tissue and avoid side effects or off targeting.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Section 2

Long Non-Coding RNA's in Diseases

Chapter 5

Exploring Long Non-Coding RNAs as Therapeutic Targets for Foetal Haemoglobin Induction in β-Thalassaemia

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Abstract

Foetal haemoglobin (HbF) induction is a promising therapeutic strategy for mitigating the clinical severity of β-thalassaemia. However, a limited understanding of globin gene regulation has impeded its progress. Recently, Long non-coding RNAs (lncRNAs) have gained attention as key regulators in biological processes. While lncRNAs have been linked to a variety of diseases and developmental processes, our understanding of the mechanisms underlying their actions is still limited. There are ongoing studies on the hypothesis that lncRNAs are involved in the induction of HbF in β-thalassaemia. A limited number of well-researched lncRNAs have given us important clues, and several noteworthy functional and molecular themes have begun to emerge. Future studies aiming at determining lncRNA-mediated therapeutic targets for HbF reactivation are made possible by new understandings of the lncRNA-mediated regulatory mechanisms. This chapter explores the mechanisms underlying lncRNA function in gene regulation and their potential therapeutic applications in β-thalassaemia.

Keywords: foetal haemoglobin (HbF), β-thalassaemia, long non-coding RNAs (lncRNAs), gene regulation, therapeutic targets

1. Introduction

"β-Thalassaemia" refers to a collection of the most prevalent single-gene inherited haematopoietic disorders characterised by defects in producing β-globin chains of haemoglobin. Due to population migration, approximately 240 million people are reported to be β-thalassaemia carriers, while 15 million people suffer from a major form of β-thalassaemia [1]. The percentage of β-thalassaemia carriers in India alone ranges from 1 to 17% (average of 3.2%), meaning that 1 in every 25 Indians is a thalassaemia carrier. The primary defect in β-thalassaemia is an imbalance between the

production of α-globin and β-globin chains, caused by diminished or absent synthesis of β-globin [2]. The surplus unpaired α-globin chains aggregate during erythropoiesis, leading to increased mechanical stress and oxidative damage. The clinical severity of β-thalassaemia corresponds to the extent of this α-globin chain imbalance, resulting in variable presentations, including anaemia, chronic haemolysis, ineffective erythropoiesis, and clinical outcomes due to iron overload following repeated blood transfusions [3]. β-Thalassaemia major typically presents between 6 and 24 months of age and is characterised by growth failure and progressively enlarging liver and spleen (hepatosplenomegaly). There are numerous ways to manage β-thalassaemia major, but there are severe obstacles, especially in developing nations, such as high costs, low adherence to iron chelation medication, and restricted access to safe and sufficient red blood cell transfusions. Due to serious consequences such as iron overload, hypercoagulability, pericarditis, osteoporosis, psychological issues, and hepatocellular carcinoma, managing β-thalassaemia major is challenging [4]. Supportive care with red blood cell transfusions, iron chelation to reduce iron overload, and adjunct medicines like hydroxyurea and erythropoietin are traditional therapeutic techniques. Complications are still common and provide major obstacles worldwide despite these therapies [5].

Recent studies have extensively explored the mechanisms regulating haemoglobin switching, given the established role of inducing foetal haemoglobin (HbF) production in alleviating the clinical severity of β-thalassaemia [6]. The patients with β-haemoglobinopathies who maintain higher HbF levels often experience milder disease manifestations compared to those with lower HbF levels. HbF is the primary haemoglobin expressed during foetal development, playing a vital role in facilitating transplacental oxygen transfer. Foetal haemoglobin (HbF), which consists of two α and two γ globin chains, binds oxygen more strongly than adult haemoglobin (HbA). Following birth, definitive erythroid progenitors undergo a transcriptional switch that changes the production of haemoglobin from foetal haemoglobin (HbF, $\alpha 2\gamma 2$) to adult haemoglobin (HbA, $\alpha 2\beta 2$). This transition is governed by genetic regulation involving major transcription factors such as MYB, BCL11A, ZBTB7A, and KLF-1, leading to low HbF levels in healthy adults [7]. HbF levels drastically drop by the time a child is a year old, leaving only trace amounts to be detected. However, during the switch from HbF to HbA, persons with β-globin mutation gene may experience impaired globin chain synthesis or produce abnormal haemoglobin, as seen in β-thalassaemia [8, 9].

Non-coding RNAs (ncRNAs) have a variety of functions in different biological processes. In the human genome, ncRNAs are more prevalent than messenger RNA (mRNA) transcripts that code for proteins. Because of their regulatory influence on gene expression, long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) are gaining increasing attention in the scientific community [10]. LncRNAs can function as signals, scaffolds, guides, decoys, and transcriptional enhancers. Additionally, they can exert indirect post-transcriptional regulation by acting as competitive endogenous RNAs (ceRNAs) and attaching themselves to microRNAs. They can recruit regulatory molecules to modify gene activity or directly affect gene expression [11]. Additionally, lncRNAs can control a variety of cellular functions, potential therapeutic targets, and biomarkers for the prognosis of disease. Erythropoiesis and haematopoiesis are instances in which a large number of tissue-specific lncRNAs are expressed. Because of these crucial functions in biological processes, lncRNAs are believed to have a part in the onset and progression of numerous diseases, such as haematopoietic stem cells, malignancies, neurological disorders and other blood

disorders. However, several studies are in progress to shed light on the regulatory roles of lncRNA, which can alter the globin gene expression in β-thalassaemia and other haemoglobinopathies [12]. The targeted induction of HbF through lncRNAs that regulate γ-globin levels has become a promising area of research. However, further exploration is required to advance pharmacological approaches targeting lncRNAs for HbF induction and to identify novel strategies for γ-globin regulation. This chapter focuses on providing an updated overview of lncRNA types implicated in inducing HbF levels in β-thalassaemia.

2. What are lncRNAs?

Recently, studies using high-throughput technologies, such as next-generation sequencing (NGS) and high-resolution microarrays, have made it possible to enumerate the repertoire of non-coding genomes produced by humans over the past 10 years [13]. Approximately 21,000 genes, which is <2% of the human genome, encode proteins, indicating that most of the transcriptome comprises ncRNAs. Based on their transcript length, non-coding transcripts can be broadly classified into three primary groups: long non-coding RNAs, approximately 10,000–32,000; small non-coding RNAs, approximately 9000; and pseudogenes, 11,000 [14, 15]. There is a heterogeneous class of ncRNAs, including lncRNAs, miRNAs, small nucleolar RNAs (snoRNAs), PIWI-associated RNAs, circular RNAs (cRNAs) and small interfering RNAs (siRNAs) [16, 17]. The lncRNAs are considered the most common and functionally varied type of ncRNA. In recent years, lncRNAs have drawn much attention as a novel and important aspect of biological oversight. Although lncRNAs of all types have been linked to a range of developmental and disease processes, little is known about the mechanisms by which they work, and there is ongoing research exploring lncRNAs' function in controlling HbF levels in β-thalassaemia patients [18, 19].

2.1 Biogenesis and biological features of lncRNAs

lncRNAs have transcript lengths >200 nucleotides, and with a few exceptions, lncRNA production is comparable to mRNA synthesis. The tyrosine-phosphorylated enzyme RNA Polymerase II (Pol II) transcribes the canonical pathway of lncRNA in the opposite direction of mRNA transcription, having a polyA tail at the 3′ ends and a methyl guanosine cap at the 5′ end. The non-canonical pathway for the synthesis of lncRNA includes capping at their termini by small nucleolar RNA-protein (snoRNP) complexes, cleavage by ribonuclease P (RNase P) to generate mature 3′ ends and the creation of RNA structures that are circular. Following synthesis, lncRNA is either exported to the cytoplasm for mRNA and protein modulations or stays inside the nucleus for chromatin-gene control [20–22]. LncRNA, such as NEAT1 (Nuclear-Enriched Abundant Transcript 1) and MALAT1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1), undergo post-transcriptional modification by the enzyme Pol II to produce long RNA sequences at the 3′ end. CircRNA may be formed when intergenic lncRNA facilitates the circularisation of mRNA. A lariat loop is created when non-coding RNA breaks during mRNA methylation, but other lncRNAs, like circRNA, are immune to degradation [23, 24]. LncRNA primarily interacts with DNA, RNA, and protein to regulate gene expression at various stages, including transcriptional, translational, post-transcriptional, post-translational, and epigenetic

levels. The other biological processes of lncRNA include control of cellular functions such as nuclear transport, transcriptional activation and interference, chromosomal modification, imprinting genomic loci, allosteric regulation of enzyme activity, stem cell pluripotency, and stem cell reprogramming. The biogenesis of lncRNAs is regulated by stimuli that are specific to cell type and developmental stage [20]. lncRNA is broadly classified into six groups based on genomic origins: (1) Antisense lncRNAs (alncRNAs) transcribed from the antisense strand, (2) enhancer lncRNAs (elncRNAs), which are expressed from active enhancers, (3) sRNA-host lncRNAs (shlncRNAs), where lncRNA hosts small RNA (sRNA), such as microRNAs, (4) Intronic lncRNAs (ilncRNAs) situated within intronic regions, (5) Intergenic lncRNAs (lincRNAs) are found intergenically and do not coincide with genes that code for proteins, and (6) Pseudogene lncRNAs (plncRNAs) (**Figure 1**) [25–27].

To govern the expression of lncRNAs, epigenetic changes are essential. For instance, the Chromatin Assembly Factor 1 (CAF-1) complex regulates antisense lncRNA transcription, while H3K56 acetylation and the chromatin remodelling complex SWI/SNF increase it. Furthermore, within imprinted gene clusters, methylation of the paternal allele can dramatically suppress the transcription of lncRNA Air, activating nearby Slc22a2, Slc22a3, and Igf2r, which are protein-coding genes. The nonsense-mediated decay protein UPF1 inhibits the degradation of lncRNAs, which is carried out by exosomes in combination with the TRAMP and the nucleus, Nrd1-Nab3-Sen1 complexes or Xrn1 in the cytoplasm. These findings highlight how important epigenetic changes are to lncRNA biogenesis. However, the precise mechanisms remain unclear, especially when it comes to β-thalassaemia. To clarify how certain epigenetic changes affect lncRNA biogenesis in this situation, more investigation is necessary.

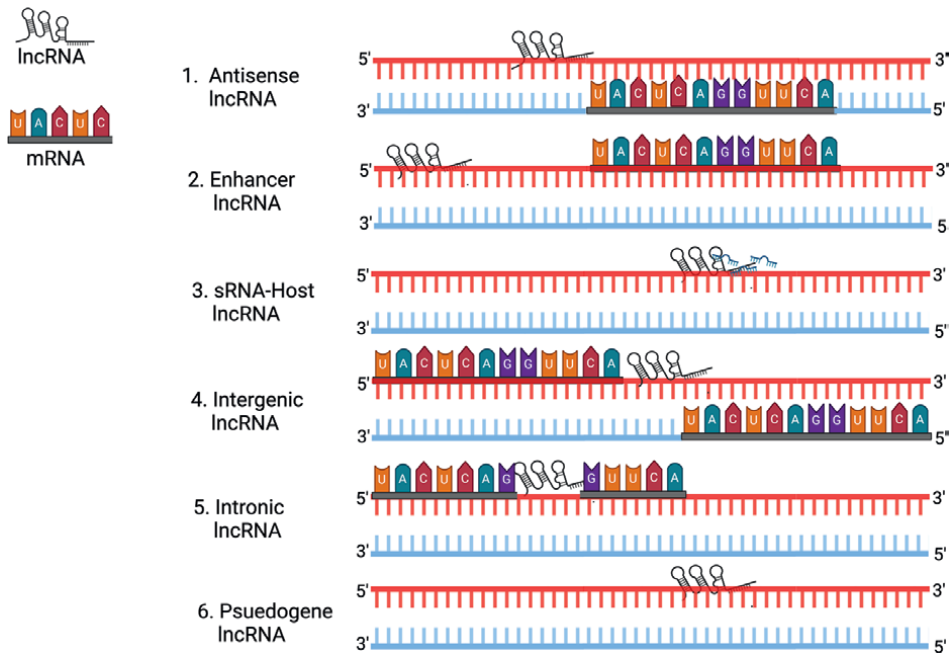

Figure 1.
Classification of lncRNAs based on their genomic location.

2.2 LncRNAs during erythropoiesis

Haematopoiesis can be defined as the continued production of red blood cells (RBC), which begins at the initial embryonic stage and extends through life. In early human embryogenesis, haematopoiesis can be categorised into the primitive phase and the definitive phase. The primitive phase occurs within the blood islands of the yolk sac. The hemangioblast, a progenitor cell precursor of endothelial cells and common myeloid progenitor cells, is the source of primitive haematopoiesis [28]. During the initial transient phase of primitive haematopoiesis, erythroid progenitors with nuclei are generated, followed by the generation of erythro-lymphoid lineages and myeloid lineages during the second transient phase of primitive haematopoiesis. Definitive haematopoiesis arises in the aorta-gonad-mesonephros (AGM) region, which also serves as the site for the generation and renewal of haematopoietic stem cells (HSCs) and their progenitor cells before the entire haematopoietic system transitions to the bone marrow and liver organs. After the embryonic stage of RBC production, in adults, erythropoiesis takes place within the bone marrow; this process includes three different phases: the initiation phase, the differentiation phase, and the maturation phase. During the initiation phase, pluripotent HSCs differentiate into erythroid lineage-committed burst-forming unit erythroid cells (BFU-E), which then progress to colony-forming unit erythroid cells (CFU-E) [29, 30]. Further CFU-E differentiate to form reticulocytes and experience significant alterations, such as a reduction in cell size, haemoglobin synthesis, condensation of chromatin and immature red blood cells without a nucleus. Erythrocytes mature from reticulocytes in the last stage of erythropoiesis, during which the cell adopts its biconcave shape and enters circulation. Mature erythrocytes travel through the bloodstream, but over time, they progressively lose haemoglobin and decrease in size; eventually, they are engulfed by macrophages in the liver or spleen [28]. In cultured mouse foetal erythroblasts, shinc-EC6 lncRNA caused Rac1 to be upregulated, activating the downstream protein PIP5K and inhibiting enucleation. This implies that the Rac1/PIP5K signalling pathway is used by shinc-EC6 lncRNA as a new modulator to control mouse erythropoiesis. LncRNAs play a key role in the regulatory network underlying erythropoiesis [31]. One such lncRNA, alncRNA-EC7, is transcribed from an enhancer and is specifically required to activate the neighbouring gene encoding BAND 3 [32].

2.3 Role of lncRNA in haematopoiesis

In recent years, multiple research teams have conducted in-depth investigations into lncRNAs associated with haematopoiesis. Broad profiling of lncRNA expression during RBC development has been performed using advanced technologies, and comprehensive data sets from RNA-sequence experiments have been obtained and validated using wet lab experiments. Over 1109 lncRNAs, comprising transcript genomes from "megakaryocytic-erythroid progenitors to lineage-committed megakaryocytes and erythroblasts", were found using mouse embryonic day 14.5 foetal liver and bone marrow cells. Among 1109 putative lncRNAs, half of them, that is, 683 lncRNAs, are transcribed in erythroblasts; among these, most of them were not annotated [33]. Another study done by Alvarez-Dominguez *et al.* showed that the transcriptomes of differentiated CFU-E and BFU-E progenitors obtained from the liver of a mouse foetus were compared with TER119-positive erythroblasts. This suggested that lncRNA expression was dynamically regulated during erythroid development, in which 96 lncRNAs were shown to be differently expressed during

erythropoiesis [32]. Additionally, RNA-sequence analyses comparing polyadenylated and non-polyadenylated transcripts were performed using TER119-positive cells. A total of 655 lncRNA genes and 9512 coding mRNA genes were found. Many functional lncRNAs have been identified, including those that are essential for the regulation of haematopoiesis. For example, by promoting the survival of erythroid progenitors, lncRNA EPS (erythroid prosurvival) controls the terminal differentiation of erythroid cells and anti-apoptotic ability mediated by erythropoietin (EPO); erythroblast enucleation is controlled by lncRNA-EC1 [33] and lncRNA-EC6 [34]. Myeloid and T cell differentiation are regulated by lncHSC-1 and lncHSC-2, respectively [35], whereas monocyte-derived dendritic cell differentiation is regulated by lnc-DC via STAT3 binding [32]. By controlling Igf2-Igf1r pathway activity [36], the oncofoetal lncRNA gene H19 plays a crucial role in regulating the equilibrium between the silencing and activation of HSCs. The endothelial-to-haematopoietic transition has been reduced when H19 fails to appear throughout the early stages of development.

Additionally, promoter hypermethylation and the concurrent down-regulation of multiple significant haematopoietic transcription factors, including Runx1 and Spi1, have been observed in pre-HSCs with H19 deficiency [36]. Chromosome 10 houses the Fas receptor gene (APO-1/CD95), within whose first intron the lncRNA Fas-antisense 1 (Saf) is transcribed from the antisense strand [37]. The maintenance of RBC production is aided by lncRNA Saf. NF-κB and the essential erythropoiesis-related transcription factors GATA-1 and KLF1 have binding sites in their promoter region. During the initial phases of erythroblast proliferation, NF-κB signalling contributes to the suppression of lncRNA Saf expression. Subsequently, at the advanced stages of RBC maturation, lncRNA Saf expression rises in parallel with increased levels of GATA-1 and KLF1, indicating that lncRNA Saf might be modulated by these transcription factors [38].

The expression of transcription factors that control haematopoiesis can be regulated by lncRNAs. By modifying the primary haematopoietic transcription factor, E2A, the silencing of two LncHSCs has had a distinct effect on lineage commitment and HSC self-renewal [11]. An antisense lncRNA has been found to regulate the PU.1 transcription factor expression. It has been demonstrated that this lncRNA inhibits PU.1 expression by controlling its translation [39]. Polyadenylated lncRNAs are expressed by mouse erythroblast cells, megakaryocyte-erythroid progenitor cells and megakaryocytes. GATA1 and TAL1 are two important transcription factors that regulate lncRNAs [33]. The CpG island that surrounds the HOXA1 gene's start site is the source of HOTAIRM1, which is the most prominent intergenic lncRNA that is overexpressed during induced granulocytic differentiation of haematopoietic cells [40]. Chromatin-related lncRNA that is expressed specifically in helper-T1 cells is called linc-MAF-4. There is an inverse relationship between its expression and that of the transcription factor MAF, which is linked to helper-T 2 [41]. 5-aminolevulinic acid synthase 2 (ALAS2) mediates the first rate-limiting step in heme biosynthesis. GATA1 stimulates transcription by binding to canonical GATA sequences found in the promoter and intronic regions of ALAS2. To create a long-range enhancer loop that promotes increased ALAS2 transcription, the GATA site in intron 1 links the intron 8 to the proximal control region of the ALAS2 promoter. The BLZ-21129 bladder carcinoma cell line is where the lncRNA known as Urothelial carcinoma-associated 1 (UCA1) was first discovered and cloned. ALAS2 mRNA stability is modulated by lncRNA UCA1. According to the study by Liu et al., UCA1 functions as a crucial RNA scaffold, facilitating the recruitment of the RNA-binding protein PTBP1 as a cofactor where GATA1 attaches to the UCA1 promoter and controls its expression. ALAS2

mRNA destabilisation and consequent impairment of heme biosynthesis resulting from the lack of UCA1 results in disruption of erythroid development at the proerythroblast stage [42].

The transcription factor Lymphoma/Leukemia-Related Factor (LRF) facilitates γ-globin gene silencing during erythropoiesis by exhibiting a high binding affinity for CpG-rich DNA sequences, thereby influencing the epigenetic transition from foetal haemoglobin (HbF) to adult haemoglobin (HbA). LncRNAs, which exhibit distinct methylation and expression profiles during terminal erythropoiesis and LRF overexpression, which correspond with the haemoglobin changeover from γ-globin to β-globin, further regulate this process. It is yet unclear how exactly this network of non-coding RNAs contributes to the last phase of haematopoiesis and the transition from the foetal to the adult stage of haemoglobin synthesis. Mice with lncRNA deletion in their blood have developed a very severe myeloproliferative disease that is defined as Leukemia and myelofibrosis. Lack of this lncRNA has caused age-dependent aberrations and aberrant maturation in HSCs [43].

2.4 Role of lncRNA in regulating HbF levels

The phrase "β-haemoglobinopathies" describes anomalies in the β-globin gene's synthesis and functioning, which are primarily inherited as an autosomal recessive pattern. β-Thalassaemia is among the most prevalent medical conditions in the human population that are linked to the Haemoglobin subunit beta (HBB). Patients with β-thalassaemia major rely on frequent blood transfusions to stay alive [44]. Therefore, it is crucial to look for possible β-thalassaemia treatments in addition to supporting and encouraging carriers to perform genetic testing and prenatal screening. Foetal haemoglobin (HbF, α2γ2) is currently being reactivated in an attempt to alleviate the symptoms of β-thalassaemia by binding to the extra α-globin chains and acting as HbA (α2β2) [45]. For β-thalassaemia major, the most effective therapeutic approach involves bone marrow transplantation (BMT) from a well-matched donor. The use of this therapeutic strategy has been restricted due to the scarcity of donors who match the human leukocyte antigen (HLA), the prolonged usage of immunosuppressive regimens, and the increased risk of immunological problems. Additionally, despite advancements in chelation therapy and transfusion procedures, numerous unresolved issues have prompted researchers to create novel therapeutic approaches such as foetal haemoglobin induction. For these attempts to be effective, it is speculated that the process of the haemoglobin switching from foetal (HbF) to adult (HbA) must be understood. Despite much research on haemoglobin flipping, some primarily epigenetic, regulatory principles remain to be clarified [46]. lncRNAs, which can act in cis or trans, have recently come to light as important regulators of gene expression. These regulators have already been demonstrated to have regulatory functions in both healthy and diseased erythropoiesis, including heme metabolism, globin switching and regulation, and erythroid cell survival [47].

The β-globin gene cluster is located on chromosome 11 at 11p15.5 in humans and consists of five genes arranged sequentially in the order of their expression during development: embryonic stage HBE (ε-globin gene), foetal stage HBG1 (γ-globin gene, Aγ) and HBG2 (γ-globin gene, Gγ), adult haemoglobin HBD (δ-globin gene), and HBB (β-globin gene). Regulatory elements like the locus control region (LCR), which is essential for controlling gene expression throughout this cluster, flank these genes. The switch from γ-globin in foetal haemoglobin to β-globin in adult haemoglobin during development is clinically significant. High HbF levels reduce

the clinical severity of β-thalassaemia. Naturally occurring mutations in the intergenic gap between the γ-globin and δ-globin genes boost the synthesis of γ-globin, indicating the existence of adverse regulators [48]. Various single-nucleotide polymorphisms (SNPs) have been discovered through genome-wide association studies (GWAS), which include the HBB gene cluster at chr11p15, BCL11A at chr2p16, and polymorphisms in the HBS1L-MYB intergenic region (HMIP) at chromosome 6q23 are quantitative trait loci (QTLs) linked to variations in HbF levels. Together, these QTLs explain around 45% of the variation in HbF among different populations. Additionally, transcription factors regulate HBG expression, including ZBTB7A, MYB, BCL11A, KLF1, NR2C1/NR2C2, KDM1α, and CHD4, as well as other cis-acting components like the intergenic region of HBD-HBBP1 [49–51].

Within the HBB gene cluster, gene expression is regulated by the HBS1L-MYB (126 kb) intergenic region, and controls erythroid cell maturation and proliferation, surrounded by erythroid-specific transcription factor binding sites RUNX1, GATA, KLF, TAL1 and LDB1, a regulatory enhancer situated approximately 84 kb upstream of MYB contains a 3-bp deletion polymorphism (rs66650371) [52]. This enhancer is most likely the functional motif responsible for the majority of the effect this QTL has on the HbF level. MYB expression was downregulated, and HBG expression was upregulated as a result of this enhancer's interaction with the MYB promoter. Additionally, using "ENCODE datasets", RNA Pol II binding and a 50-base pair RNA fragment near rs66650371 were found. This led to the identification of a new 1283 bp long non-coding RNA, HMI-lncRNA, also known as the HBS1L-MYB intergenic long non-coding RNA. γ-globin expression was dramatically increased by 200 times when HMI-lncRNA was reduced to half of its normal level, accompanied by a notable increase in γ-globin production. This impact is probably the result of less interaction between the MYB promoter and the distal enhancer, leading to reduced MYB transcription and subsequent upregulation of γ-globin expression [53].

The transcription factor Lymphoma/Leukemia-Related Factor (LRF) facilitates γ-globin gene silencing during erythropoiesis through mechanisms distinct from those employed by BCL11A [43]. LRF exerts its repressive influence on γ-globin genes by recruiting the Nucleosome Remodelling and Deacetylase (NuRD) complex. This recruitment modulates chromatin structure, leading to a reduction in activating histone markers and subsequent inhibition of γ-globin gene transcription. As an epigenetic regulator, LRF has a repressive effect on erythrocyte formation in its latter stages. The haemoglobin transition from HbF to HbA depends on this activity; this process is further modulated by lncRNAs, which exhibit different patterns of methylation and expression during terminal erythropoiesis and overexpression of LRF, which corresponds to the transition of haemoglobin from γ-globin to β-globin. The precise function of the non-coding RNA network in the late stage of haematopoiesis and the concurrent change from the foetal to the adult stage of haemoglobin production is still unknown. In HUDEP-2 cells, a human immortalised erythroid line that requires doxycycline withdrawal for terminal differentiation, the double knockout of LRF/ZBTB7A shows γ-globin expression is significantly elevated. Conversely, erythroid differentiation is delayed in human CD34+ haematopoietic stem and progenitor cells (HSPCs) that lack LRF [54].

Two regulatory regions, 1.5 kb downstream of HBG1 and 3.5 kb upstream of HBG2, are bound by ERF (Ets2 Repressor Factor), an HbF repressor. RP11-196G18.23 lncRNA interacts with the ERF promoter and facilitates the recruitment of DNMT3A (DNA (Cytosine-5)-Methyltransferase 3 Alpha), leading to DNA hypermethylation at the promoter site. This epigenetic modification suppresses ERF expression,

enhancing γ-globin production in individuals with β-thalassaemia [47]. The lncRNA Haemoglobin Subunit Beta Pseudogene 1 (HBBP1), located on chromosome 11 near lncRNA BGLT3 and adult δ-globin, plays a pivotal role in γ-globin regulation. In patients with β-thalassaemia, the single-nucleotide polymorphisms rs10128556 and rs2071348 in the second intron of HBBP1 are strongly linked to increased HbF levels [55, 56]. The transcription factor ELK1, belonging to the ETS family, a crucial modulator of γ-globin expression and HBBP1 expression, interacts with HBBP1. ELK1 attaches itself to the proximal promoter of HBBP1 to directly increase γ-globin along with its downstream effectors C-FOS and SRF [57]. The lncRNA BGLT3 facilitates the positive regulation of γ-globin genes by looping through their genomic region in a transcription-dependent manner and interacting with the MED12 subunit. Both the looping mechanism and interaction with the Mediator complex may contribute to the recruitment or stabilisation of RNA Pol II at the γ-globin genes. The increase in RNA Pol II density and transcription, facilitated by BGLT3, highlights its specific role in activating γ-globin gene expression [19, 58].

Under aberrant epigenetic environments, enzymes such as histone deacetylase 1 (HDAC1) and DNA methyltransferase 3 (DNMT3) play a critical role in chromatin landscape configuration, which in turn affects LRF's function in β-globin gene expression. Chromatin immunoprecipitation sequencing (ChIP-seq) investigations have shown that LRF interacts with BCL11A to suppress the production of γ-globin genes by binding to their promoters (HBG2/1). Furthermore, LRF targets the BGLT3 gene during erythropoiesis, encouraging the γ-δ intergenic region to produce BGLT3 lncRNA. The transcriptional transition from γ-globin to β-globin expression is facilitated by this mechanism. LRF binds to the upstream region of the BGLT3 gene and the HBG2/1 promoter regions in K562 cells, especially when haemin/EPO stimulation is present. BCL11A binding to HBG2/1 is required for HbF repression during the haemoglobin transfer from HbF to HbA, but LRF occupancy is required for complete silence. Additionally, by increasing the expression of "BGLT3-lncRNA", LRF's dual interaction with HBG2/1 and BGLT3 amplifies BCL11A-mediated silencing and facilitates subsequent access of chromatin up to the HBB gene. As an indirect chromatin regulator, LRF/ZBTB7A contributes increasingly to the HbF to HbA transition [58]. Additionally, in K562 cells, a Bcr-Abl-positive erythroleukemia model, which is marked by a chromosomal translocation between chromosomes 9 and 22 that results in the hybrid bcr-abl gene and ultimately induces apoptosis, imatinib treatment suppresses the production of BGLT3-lncRNA. According to research, Bcr-Abl is a regulator of BGLT3-lncRNA that most likely inhibits transcription by hypermethylating the DNA [18].

lncRNA, mRNA, and miRNA microarray expression profiling in reticulocytes from β-thalassaemia patients identified several dysregulated transcripts potentially linked to HbF induction. lncRNA NR_120526 and lncRNA T315543 were identified as regulators of Colony Stimulating Factors (CSF2 and CSF3), whereas lncRNA NR_001589, a lncRNA, was proposed as a regulator of HBE1. ε-globin is encoded by HBE1 and is typically active in the yolk sac of the embryo and was found to retain its expression in β-thalassaemia carriers with elevated HbF levels. The haematopoietic cell lineage pathway, critical for blood cell development, highlighted transcription factors such as CSF2 and CSF3 as significant players. These altered transcripts appear closely associated with HbF production, strongly supporting the role of lncRNA NR_001589, lncRNA NR_120526, and lncRNA T315543 as genetic modulators inducing the γ-globin gene and potential therapeutic targets to elevate HbF levels. However, additional confirmation of these findings is required [59]. MALAT1 lncRNA is a

lncRNA that is encoded by the MALAT1 gene, which is found on chromosome 11q13.1. It is extensively expressed in many normal cell types and tissues [60]. Elevated MALAT1 expression in β-thalassaemia is closely associated with an earlier onset of disease, a younger age at the initiation of blood transfusions, and an increased incidence of splenomegaly. These associations suggest a heightened rate of haemolysis, potentially contributing to reduced baseline haemoglobin levels. The lncRNA known as MIAT (Myocardial Infarction Associated Transcript) is encoded by the MIAT gene, which is found on chromosome 22q12. It is believed to interact with splicing factor 1 (SF1) via the SF1-binding motif to regulate gene expression and be involved in RNA splicing [61]. Increased severity of β-thalassaemia is linked to the early initiation of the first blood transfusion, which is indicated by higher expression of MIAT levels. The ANRIL gene, which is part of the cluster of genes CDKN2A-CDKN2B on chromosome 9p21, encodes the lncRNA known as ANRIL (Antisense Noncoding RNA in the INK Locus), which is transcribed in the antisense direction concerning the INK4B-ARF-INK4A locus. After DNA damage, the transcription factor E2F1 upregulates its expression. Additionally, ANRIL contributes to the epigenetic repression of nearby genes within the cluster through its interactions with Polycomb Repressive Complex 1 (PRC1) and PRC2 [18, 62]. In the later phases of the DNA damage response, the lncRNA ANRIL suppresses the expression of the INK4A-ARF-INK4B locus by translating to INK4b as an antisense RNA. Notably, the INK4b-ARF-INK4A locus is essential for controlling the stress response, cell division, and haematopoietic differentiation. After DNA repair is finished, ANRIL helps to restore cellular homeostasis. The long non-coding RNAs ANRIL, MIAT, and MALAT1 were notably upregulated in patients with β-thalassaemia, suggesting their potential involvement in the disease's pathophysiology and progression. This upregulation highlights their promise as novel biomarkers for assessing disease severity [63].

The lncRNA H19 is a maternally expressed and paternally imprinted carcinoembryonic gene. In studies involving erythroid differentiation, H19 overexpression resulted in a reduction of γ-globin mRNA levels in K562 cells. However, in human umbilical cord blood-derived erythroid progenitor (HUDEP-2) cells and CD34+ haematopoietic stem cells, γ-globin gene expression was drastically increased. Interestingly, H19 exhibited distinct subcellular localisation patterns: it was primarily nuclear in K562 cells, whereas it was primarily found in the cytoplasm of HUDEP-2 cells. This cytoplasmic localisation in HUDEP-2 cells raises the possibility that H19 functions as a competitive endogenous RNA (ceRNA), affecting microRNA or mRNA interactions to modify γ-globin expression [64]. Microarray profiling conducted on seven β-thalassaemia patients with elevated HbF levels revealed significantly greater levels of average expression of the lncRNA uc002fcj.1 and lncRNA NR_001589. A positive correlation was identified between these lncRNAs and HbF levels. Additional functional research of lncRNA uc002fcj.1 and lncRNA NR_001589 could provide deeper insights into the molecular mechanisms underlying β-thalassaemia. This study suggests that these lncRNAs may function as competing endogenous RNAs (ceRNAs), enhancing the expression of HBG1/2 by sponging specific miRNAs, thereby contributing to HbF induction in β-thalassaemia (**Figure 2**) [65].

The most recent studies have investigated the use of antisense oligonucleotides for miRNA suppression, "nano vector-encapsulated miRNA mimics" for miRNA restoration, and small interfering RNAs (siRNAs). Although the FDA has approved siRNA-based therapeutics, preclinical and early-phase clinical research is still being conducted to examine lncRNA-based treatments, miRNA inhibitors and mimics. Additionally, two siRNAs that target non-coding RNAs—circRNAs and

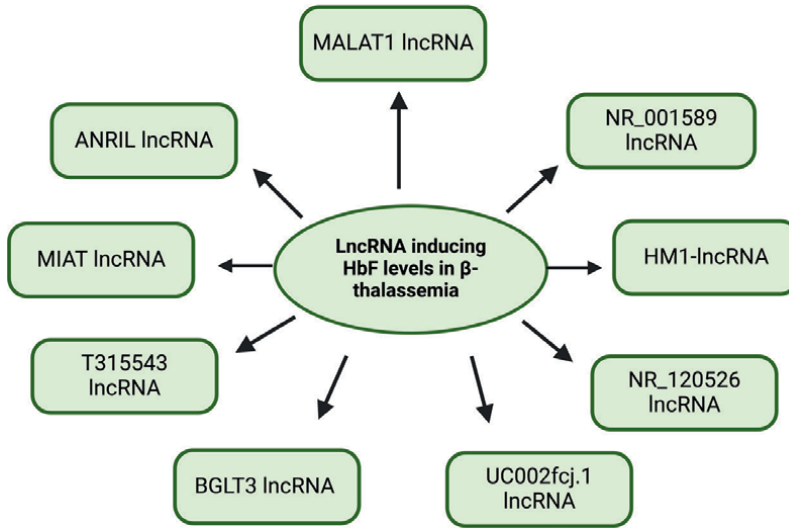

Figure 2.
List of lncRNAs involved in inducing HbF levels in β-thalassaemia.

lncRNAs—have advanced to clinical trials from preclinical research. For instance, LNP-based siRNA targeting the LINC01257 lncRNA has been suggested as a novel and secure treatment approach for acute myeloid Leukemia in the paediatric group [66]. To successfully continue into more advanced stages of clinical testing, challenges such as effective delivery, off-target effects, and safety concerns need to be addressed with further attention. The investigation of lncRNAs as biomarkers for the diagnosis, prognosis, and therapy of β-thalassaemia may advance with the help of this knowledge in the design of future studies [67].

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Chapter 6

Role of Circulatory miRNAs in Cardiovascular Disorder: Anti-Sense RNA as Therapeutic Targets

Wahid Ali, Priyanka Sharma, Nida Shabbir and Swati Kumari

Abstract

Consequently, there is a significant need for biomarkers capable of assessing the early stages of atherosclerosis and the likelihood of developing coronary artery disease (CAD). MicroRNAs (miRNAs) are brief, non-coding RNA molecules with a single strand that controls cellular processes by degrading and repressing the translation of messenger RNAs (mRNAs). miRNAs circulating in the bloodstream regulate gene expression post-transcriptionally, playing a crucial role in cell growth, differentiation, and metabolism, as well as in the pathophysiology of numerous disorders. Studies conducted both *in vitro* and *in vivo*, involving the manipulation of miRNA expression, have demonstrated its significant impact on heart function regulation, particularly in areas such as cardiac growth, hypertrophy, and failure. Earlier research has shown that individuals with heart failure (HF) exhibit lower plasma concentrations of miR-126 compared to healthy subjects, and an inverse relationship with brain natriuretic peptide (BNP) was observed, establishing miR-126 as a reliable indicator of heart failure.

Keywords: CVD, miRNA, anti-sense, RNA atherosclerosis, angiography

1. Introduction

CVDs, which is short for cardiovascular diseases, are one of the most prevalent and fatal health complications in the globe. Coronary artery disease begins with atherosclerosis, which in turn precipitates a series of pathological events that lead to significant cardiovascular events. Hence, biomarkers that can capture the first step of atherosclerosis and risk for CAD occurrence are quite valuable. Post-transcriptionally acting, short non-coding RNAs—microRNAs or miRNAs—are regarded to be suitable candidate biomarkers from this point of view. This chapter focuses on circulating miRNAs in cardiovascular diseases and considers their prospection for treatment through anti-sense RNA approaches. MicroRNAs have emerged as promising biomarkers for early detection and monitoring of cardiovascular diseases, particularly in the context of atherosclerosis and coronary artery disease. Their presence in

circulation and stability in various biological fluids make them attractive targets for non-invasive diagnostic and prognostic tools. Furthermore, the potential of anti-sense RNA approaches targeting specific miRNAs offers a novel therapeutic strategy for managing CVDs, potentially interrupting disease progression at its earliest stages. While microRNAs show promise as biomarkers and therapeutic targets for cardiovascular diseases, their clinical application faces challenges such as standardization of detection methods, identification of disease-specific miRNA signatures, and development of effective delivery systems for anti-sense RNA therapies.

2. Overview of microRNAs

2.1 Definition and function

Short, microRNAs, which are non-coding RNA molecules consisting of about 22 nucleotides in a single strand, play a role in regulating various biological functions. Their primary mechanism of action involves attaching to the 3′ untranslated regions (UTRs) of target mRNAs, subsequently causing their degradation or inhibiting their translation. Thus, miRNAs can modulate cellular functions such as proliferation, differentiation, apoptosis, and metabolism without compromising the fidelity of their input mRNAs [1, 2]. Research has shown that microRNAs play significant roles in numerous biological and pathological processes, encompassing embryonic growth, immune system responses, and the advancement of cancer. Their ability to regulate gene expression at the post-transcriptional level makes them powerful modulators of cellular behavior and potential therapeutic targets. Research shows that miRNAs exist inside extracellular vesicles enabling their use in cellular communication and their potential use as diagnostic and prognostic biomarkers.

2.2 Biogenesis of miRNAs

DNA transcription by RNA polymerase II starts the formation of pri-miRNA, which becomes the primary miRNA. The microprocessor complex, which includes Drosha and DGCR8, functions within the nucleus to transform pri-miRNAs into pre-miRNAs. Full development of miRNAs leads them to the RNA-induced silencing complex (RISC), which includes Argonaute proteins [1, 3]. Between these stages, Dicer modifies pre-miRNAs and produces mature miRNAs. In RISC assembly, the miRNA receives direction toward its specific mRNA target, so it properly attaches in the 3′ untranslated region. Through the binding of miRNA to its target sequence, the level of compatibility determines mRNA destabilization and translation suppression [3]. The specific section of the seed functions by detecting similar sequences on target mRNA molecules for proper attachment. Several aspects determine how well miRNA controls gene expression including the number and placement of binding sites found in target mRNA as well as which RNA-binding proteins enhance or disrupt miRNA binding.

3. Role of circulating miRNAs in cardiovascular disorders

Scientists have recently identified circulating microRNAs (miRNAs) as crucial elements in cardiovascular disease study. Research indicates that these small non-coding

ribonucleic acid molecules are vital in controlling cellular and genetic operations inside the cardiovascular system. The stability of miRNAs circulating in body fluids and blood makes them attractive as biomarkers for cardiovascular disease diagnosis and management purposes. Scientific research demonstrates how unique miRNA profiles can determine the development of multiple heart conditions, starting from myocardial infarction to heart failure and atherosclerosis. Circulating miRNA detection requires no invasive procedures, which makes them suitable for medical applications so healthcare providers may do without more complex diagnostic measures. Toward better treatment of cardiovascular diseases, scientists are currently studying how to manage miRNA expression through interventions to produce beneficial outcomes [4].

3.1 Circulating miRNAs as biomarkers in cardiovascular disorders

Many diseases including cardiovascular disorders can be diagnosed using body fluid miRNAs due to their stable nature in serum and plasma. Certain levels show that a cardiovascular system suffers from pathology. HF patients show decreased plasma concentrations of miR-126 when compared to people who do not have heart failure. Research indicates that miR-126 shows an inverse relationship with brain natriuretic peptide BNP, which serves as a primary marker of heart failure therefore making it a promising diagnostic and prognostic biomarker for HF [4]. Scientific findings indicate that miR-126 has value as a biomarker substitute for BNP in heart failure clinical evaluation. The potential role of miRNAs extends to cardiovascular biomarker detection of myocardial infarction and atherosclerosis, apart from heart failure. Further research is needed to verify miRNA utility as diagnostic indicators and develop standard evaluation techniques for implementing their use in cardiovascular disease management. The use of miRNAs as biomarkers shows promise in diagnosing and predicting multiple cardiovascular diseases as well as heart failure cases. Scientists conduct present-day research to determine the diagnostic accuracy of miRNA tests for tracking cardiovascular disease progression at multiple stages. Research shows that integrating microRNA biomarkers into healthcare practice would advance patient-specific treatment methods for people dealing with cardiovascular diseases.

4. Mechanisms of action in cardiovascular pathophysiology

Cardiac development together with hypertrophy and heart failure functions under the regulation of these miRNAs. These miRNAs place them in a successful position to manage pathways significant for cardiovascular disease progression including inflammation and apoptosis with fibrosis development. The microRNA miR-21 has been associated with cardiac hypertrophy, according to studies that show its capacity to inactivate phosphatase and tensin homolog (PTEN) while promoting survival signaling pathways [5]. Medical tests indicate miR-21 expression control shows promising results for treating cardiac hypertrophy together with heart failure patients. The cellular activities of cardiomyocytes are influenced by additional microRNAs, which have been proven to control both their proliferation and differentiation processes. New therapies for heart conditions show promise because researchers have observed that microRNAs form complex relationships with cardiovascular disease mechanisms.

Three miRNAs have been identified to affect cardiac remodeling alongside miR-21: miR-1, miR-133, and miR-208. Scientists determined that MiR-1 maintains control of

cardiomyocyte proliferation and apoptosis and miR-133 blocks cardiac hypertrophy by targeting muscle growth signaling pathways [6]. Scientists have discovered that miR-208 serves as a regulator of cardiac stress responses by controlling intronic part within α-myosin heavy chain gene found at Ref. [7]. Research data demonstrates how miRNAs sustain complex relationships with multiple cellular processes found in cardiac tissue. Multiple scientific laboratories across the world study these miRNAs to develop them as potential therapeutic targets against cardiovascular diseases. Cancer pathologies affect miR-499 expression levels in cardiac cells, where this microRNA maintains an essential role in cardiac differentiation and remodeling [6]. Single miRNAs do not function alone in heart regulation because they perform their complex regulation through collective mechanisms that precisely control both gene expression and cellular responses throughout cardiac tissue. Understanding the complex networks of interactions and cardiac function effects will support the future development of novel therapeutic solutions for heart disease treatment. The research shows that MiR-499 has essential functions during cardiac differentiation and remodeling while displaying altered expression levels in numerous cardiovascular diseases [6]. The discovery of miR-177-3p increases the complexity of miRNA regulatory mechanisms within heart tissue. The collective function of multiple miRNAs that control gene expression patterns requires thorough research into molecular interactions.

5. *In vitro* and *in vivo* studies

Researchers have proven the regulatory effect of specific miRNAs on cellular stress responses by testing cardiomyocyte cell lines *in vitro*. The research showed that the overexpression of miR-21 in cardiomyocytes made them become resistant to apoptosis under oxidative stress which indicates therapeutic potential [5]. At the same time, research showed miR-1 and miR-133 controlled how cardiomyocytes responded to hypertrophy and cardiac remodeling under stress conditions [6]. The stress response mechanism of the heart and its fibrotic development are both influenced by miR-208a [7]. The identified potential of using miRNA-based treatments to manage cardiac stress reactions and boost heart performance becomes more promising through both lab-based research and animal testing. Knockout mice without specific miRNAs display modified heart functions and stress reactions, which helps researchers better understand cardiovascular molecule functions during health. Clinical tests have initiated research to evaluate miRNA-based treatments for patients with heart failure and other cardiac conditions and the assessments yield promising indicators.

Researchers have further proved the roles of miRNAs in disease progression using *in vivo* heart failure models in animal studies. The cardiac fibrosis regulatory function of miR-29a makes it a potential therapeutic target for treating heart failure, according to Van Rooij et al. [7]. Research about miRNAs in heart failure development has sparked new interest in creating miRNA-based therapeutic strategies for heart failure treatment. Multiple research studies involving animals have demonstrated successful results when targeting specific microRNA molecules in order to enhance cardiac performance and lower fibrotic issues. Effective clinical treatments based on these findings face significant barriers because delivery strategies and unintended consequences must receive proper attention. New research investigates different strategies to develop miRNA-based heart failure therapeutic options despite existing

implementation hurdles. Nanoparticle delivery systems show great potential to enhance the accuracy of miRNA modulator transport to cardiac tissue. Research investigates combination therapy approaches to target multiple miRNAs that impact heart failure development for possible synergistic treatment effects.

6. Anti-sense RNA as therapeutic targets

6.1 Therapeutic potential of anti-sense RNA

Anti-sense RNA possesses technology that uses designed oligonucleotides to match specific miRNAs, which results in their functional inhibition. The method shows potential to reverse established pathological processes of cardiovascular disorders. Through its inhibition mechanism, anti-sense RNA can stop disease-related miRNAs from expressing while restoring normal gene expression patterns, which leads to improved cardiac function.

Research data indicates promising results from this new approach because it successfully controls specific miRNA targets that contribute to cardiac reshaping and scarring. Scientific trials at present examine anti-sense RNA treatments as a therapeutic approach for patients who have heart disorders such as congestive heart failure and artery plaque buildup. The successful implementation of these therapies should transform cardiovascular disease management by enabling doctors to treat specific molecular pathways for each patient. Custom miRNA inhibition capabilities present clinicians with a strong tool to create treatments that match individual patients' genetic compositions. Anti-sense RNA technology has potential benefits compared to conventional pharmaceutical treatments by minimizing side effects as well as improving long-term care results for patients who have cardiovascular conditions.

Research into anti-sense RNA therapies for cardiovascular conditions has established a major breakthrough for precision medical care. Anti-sense RNA therapies show promise to develop better treatments that produce fewer systemic side effects than traditional treatments. The ongoing research will likely result in higher numbers of anti-sense RNA-based treatments entering clinical trials which will eventually become accessible in the market for cardiovascular diseases. The emergence of anti-sense RNA therapeutic approaches for cardiovascular diseases stands as a major achievement in precision medicine discipline. The treatment method can achieve better results offering patients reduced systemic side effects above traditional therapeutic methods. The field of anti-sense RNA research continues to advance so it is expected more therapies in this category will undergo clinical trials before reaching the market for cardiovascular treatments [8].

7. Application in cardiovascular disease

Researchers have studied different cardiovascular environments to apply therapeutic anti-sense RNA that targets specific miRNAs. The study of anti-sense oligonucleotides directed against miR-21 leads to substantial reductions in cardiac hypertrophy along with fibrosis in animal subjects [8]. The therapeutic application of miRNA expression regulation through this method resulted in reduced cardiac fibrosis and better heart performance [7]. Animal cardiovascular health at a systemic

level may improve through anti-sense oligonucleotide treatment of blood miRNA, which reduces circulating levels of these molecules. Medical experts interpret these results as evidence that anti-sense RNA therapy, which targets particular miRNAs, presents promising possibilities for cardiovascular disease treatment. More investigations are necessary to discover complete mechanisms of action behind these therapies and enhance their clinical delivery efficiency. Improved cardiovascular patient care will result from using more specific anti-sense oligonucleotides that deliver better therapeutic effects and produce minimal side effects. Doctors are performing clinical trials to establish safety measures and effectiveness rates of anti-sense RNA treatments for human cardiovascular patients. Scientists use these studies to convert successful animal model results into applications in human patients. The research seeks to combine anti-sense RNA therapy with standard cardiovascular treatments in order to deliver better therapeutic results for patients.

8. Challenges and considerations in therapeutic development

Although the potential of anti-sense RNA as a therapeutic strategy for cardiovascular disease appears promising, challenges must be met. The first problem is a delivery issue as anti-sense oligonucleotides still need to reach the tissues where they are going to work. The stability and uptake of these molecules in the cardiovascular system into the desired target site are necessary for the therapeutic efficacy. They must be delivered through effective delivery systems. Secondly, off-target effects and by products need to be carefully watched for. Most miRNAs target many mRNAs and so inhibition of a particular miRNA could have unintended consequences with respect to other pathways. Extended preclinical investigations must occur before using anti-sense oligonucleotide therapies in cardiovascular medicine since they need evaluation in terms of safety and treatment performance abilities. The potential immunological reactions to these foreign therapeutic molecules require thorough consideration. Scientists must find ways to match appropriate timing and proper dosage levels of delivery to obtain therapeutic benefits and avoid unwanted side effects. A comprehensive study program should establish both effect duration and delayed responses following extended anti-sense RNA therapy applications in cardiovascular medicine. Research on anti-sense RNA therapies for cardiovascular diseases requires ongoing development due to their remaining challenges. Research translation of promising anti-sense RNA treatments requires combined work from academic establishments together with pharmaceutical businesses and regulatory organizations to address current obstacles. Forthcoming innovations in drug delivery methods combined with better oligonucleotide chemistry research plus advanced preclinical model development will enable the complete realization of this therapeutic potential.

9. Future directions

9.1 Biomarker development

Research into circulating miRNAs represents a key academic field for developing biomarkers that detect cardiovascular conditions early and assess cardiovascular

risks. Two modern technology platforms, namely Next Generation Sequencing and high-throughput quantitative PCR, enable scientists to discover cardiovascular-related novel miRNAs. Standardization of biomarkers must occur before they become practical for daily clinic use, while researchers must determine strong associations between biomarkers and clinical situations. Scientific research must identify and confirm the clinical worth of particular miRNA biomarkers which would serve in cardiovascular disease detection and risk evaluation. The confirmation of these biomarkers' performance characteristics as testing tools requires extensive studies conducted on various patient demographics. Standardization in miRNA measurement methodologies alongside reference value creation forms the basic requirement for applying these biomarkers in clinical environments. The clinical value of using particular miRNA biomarkers for cardiovascular disease diagnosis requires more research to confirm its applications. Mass-scale prospective studies of these biomarkers are essential to measure their performance rates when dealing with different patient demographic groups. Society will benefit from normalized miRNA measurement approaches alongside baseline reference definitions to achieve broad clinical acceptance of these biomarkers.

9.2 Therapeutic innovations

Future work should look to optimize anti-sense RNA techniques to make them more specific and delivery methods. Alternatively, nanotechnology and lipid-based carriers may provide effective delivery of oligonucleotides to target tissues, improving therapeutic outcomes in cardiovascular diseases. These advancements in delivery methods could potentially enhance the efficacy of anti-sense RNA therapies by increasing their bioavailability and reducing off-target effects. Additionally, combining anti-sense RNA techniques with other emerging technologies, such as CRISPR-Cas9 gene editing, may offer synergistic benefits in treating complex cardiovascular disorders. Further research is also needed to elucidate the long-term safety and potential side effects of these novel therapeutic approaches in clinical settings. Longitudinal studies should be conducted to assess the long-term efficacy and safety profiles of anti-sense RNA therapies in cardiovascular patients. Moreover, personalized medicine approaches could be explored to tailor these therapies based on individual genetic profiles and disease characteristics. Finally, investigating the potential of anti-sense RNA techniques in preventing cardiovascular diseases, rather than just treating existing conditions, could open up new avenues for preventive cardiology.

9.3 Personalized medicine

Research into circulating miRNAs represents a key academic field for developing biomarkers which detect cardiovascular conditions early and assess cardiovascular risks. Two modern technology platforms namely Next Generation Sequencing and high-throughput quantitative PCR enable scientists to discover cardiovascular-related novel miRNAs. Standardization of biomarkers must occur before they become practical for daily clinic use, while researchers must determine strong associations between biomarkers and clinical situations. Scientific research must identify and confirm the clinical worth of particular miRNA biomarkers which would serve in cardiovascular disease detection and risk evaluation. The confirmation of these

biomarkers' performance characteristics as testing tools requires extensive studies conducted on various patient demographics. Standardization in miRNA measurement methodologies alongside reference value creation forms the basic requirement for applying these biomarkers in clinical environments. The clinical value of using particular miRNA biomarkers for cardiovascular disease diagnosis requires more research to confirm its applications. Mass-scale prospective studies of these biomarkers are essential to measure their performance rates when dealing with different patient demographic groups. Society will benefit from normalized miRNA measurement approaches alongside baseline reference definitions to achieve broad clinical acceptance of these biomarkers.

10. Conclusion

Circulating miRNAs represent a novel class of biomarkers with great potential to contribute to cardiovascular disorder assessment and management. Little is known about their roles in regulating key biological processes, but they are of special interest as they play central roles in the pathophysiology of diseases like heart failure and atherosclerosis. Through the development of anti-sense RNA therapy to modulate specific miRNAs, this strategy promises to modulate disease processes and benefit cardiovascular patients. Our findings should spawn continued basic research in this area in order to translate them to clinical practice, and thereby enhance our ability to diagnose, treat, and prevent cardiovascular disease. The circulating miRNAs have demonstrated potential for early identification and tracking of heart-related conditions, possibly allowing for more prompt interventions and tailored treatment approaches. Additional research must be conducted to clearly understand the intricate regulatory circuits and signaling networks which result from these miRNAs in cardiovascular disease pathology. Future advancement in miRNA study will enable researchers for creating new therapeutic options that could employ miRNA regulation to control disease progression while improving patient results. Specific miRNA biomarkers require validation through major clinical experiments involving various patient demographics for diagnostic and prognostic applications. The clinical application of miRNA profiling requires standardized methods for specimen handling before testing along with analytical protocols to reach high precision reliability. The translation of miRNA research into clinical settings could transform cardiovascular medicine by providing exact diagnostic methods and well-targeted therapeutic strategies.

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Conflict of interest

The authors declared no conflict of interest.

Appendix and nomenclature

miRNAs	microRNAs
BNP	brain natriuretic peptide
HF	heart failure
CVD	cardiovascular disease
RISC	RNA-induced silencing complex
pre-miRNAs	precursor miRNAs
PTEN	phosphatase and tensin homolog

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Section 3

Long Non-Coding RNA's in Biotechnology

Chapter 7

CRISPR Technology: Mechanisms and Applications in Genome Editing and Long Non-Coding RNA Functional Analysis

Poonam Ranga, Varsha Ranga and Anita Mann

Abstract

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has become a groundbreaking tool in genome editing, providing unmatched precision for DNA modification. The system is composed of two main elements: Cas (CRISPR-associated) protein, typically Cas9, and a guide RNA (gRNA). Together, they enable the targeted alteration of specific genomic regions, allowing for gene knockouts, insertions, deletions, and corrections with high accuracy. While methodologies to study non-coding regions, including long non-coding RNAs (lncRNAs), have historically lagged behind those for coding genes, CRISPR has introduced novel techniques for investigating these largely unexplored regions. CRISPR-mediated loss-of-function approaches allow for the disruption or repression of lncRNA transcription, while gain-of-function strategies enable the insertion of custom sequences and activation of transcription. LncRNAs, which make up a significant portion of the non-coding genome, are known to play crucial roles in a variety of biological processes, but their specific functions remain poorly understood due to limited genetic tools. The introduction of CRISPR/Cas9 technology has greatly expanded the ability to screen for novel lncRNAs and study their functional roles. This chapter aims to offer a comprehensive overview of CRISPR-Cas9 technology in genome editing and explore how it can be utilized to gain new insights into the biology of long non-coding RNAs (lncRNAs).

Keywords: genome editing technology, CRISPR/Cas9, workflow of CRISPR/Cas, applications, long non-coding RNA function

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) systems have revolutionized the field of genome editing by providing unprecedented precision, versatility, and efficiency. Originally

discovered as part of the adaptive immune system in prokaryotes, CRISPR/Cas systems enable bacteria and archaea to fend off invading genetic elements such as viruses and plasmids. The ability to repurpose this natural mechanism for targeted genome editing has opened new frontiers in biological research, biotechnology, and medicine. CRISPR loci were first identified in *Escherichia coli* in 1987, where repetitive DNA sequences were observed but their function remained unclear [1]. Subsequent studies revealed that these loci are part of a defense system that incorporates fragments of foreign DNA (spacers) into the host genome, which are then transcribed into CRISPR RNAs (crRNAs) [2, 3]. When a similar invader reappears, crRNAs guide the Cas proteins to recognize and cleave the target DNA, providing sequence-specific immunity [4]. The most widely used CRISPR/Cas system in genome editing is CRISPR/Cas9 from *Streptococcus pyogenes*. It relies on two main components: a single-guide RNA (sgRNA), which is engineered to match the target sequence, and the Cas9 nuclease, which introduces double-strand breaks (DSBs) at the specified genomic location [5]. Following DSB formation, cellular repair mechanisms such as non-homologous end joining (NHEJ) or homology-directed repair (HDR) can be harnessed to introduce desired genetic modifications.

The specificity of CRISPR/Cas9 is dictated by the sgRNA and the protospacer adjacent motif (PAM) sequence, ensuring precise targeting of the genome. Innovations such as high-fidelity Cas9 variants and modified sgRNAs have further minimized off-target effects, enhancing the precision of this technology [6]. CRISPR technology is highly versatile, supporting applications beyond simple gene knockout. It has been adapted for base editing, where single-nucleotide changes are introduced without generating DSBs [7]; prime editing, which allows for precise insertions, deletions, and substitutions [8]; and epigenome editing, where transcriptional activity is modulated without altering the DNA sequence [9]. Additionally, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) systems enable gene repression or activation, respectively, providing powerful tools for functional genomics [10–14].

2. Non-coding RNAs in genomics

Non-coding RNAs (ncRNAs) are increasingly recognized as pivotal players in the regulation of genomic processes, transcending their initial categorization as "junk" or "passive" transcriptional byproducts. Among the ncRNAs, long non-coding RNAs (lncRNAs) have garnered significant attention due to their versatile roles in gene expression regulation, chromatin organization, and cellular signaling pathways. Their involvement spans a wide range of biological processes, including development, differentiation, and stress responses, underscoring their essential functions in maintaining cellular homeostasis (**Figure 1**). The discovery of ncRNAs began with the identification of transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) in the mid-twentieth century, which were essential for protein synthesis. However, the advent of high-throughput sequencing technologies unveiled a vast landscape of ncRNAs, revealing their abundance and diversity in eukaryotic genomes [15]. LncRNAs, typically defined as transcripts longer than 200 nucleotides with limited protein-coding potential, constitute a significant subset of these ncRNAs [16].

Long non-coding RNAs (lncRNAs) exert their regulatory effects through a variety of mechanisms, including chromatin remodeling and epigenetic regulation. For example, lncRNAs such as XIST and HOTAIR interact with chromatin-modifying complexes, influencing histone modifications and DNA methylation. These interactions can lead to either transcriptional activation or repression [17]. LncRNAs also

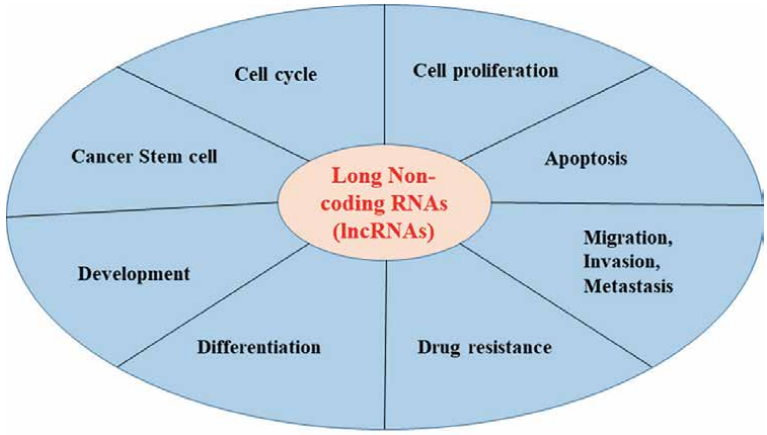

Figure 1.
Biological functions of long non-coding RNAs (lncRNAs).

play a crucial role in transcriptional regulation by functioning as enhancers, scaffolds, or decoys. In these capacities, they modulate the activity of transcription factors and RNA polymerase, thereby influencing the recruitment of these factors to target loci [18]. Beyond transcription, lncRNAs contribute to post-transcriptional regulation. They impact mRNA stability, splicing, and translation by binding to RNA-binding proteins or forming RNA-RNA duplexes, essential for fine-tuning gene expression at the post-transcriptional level [19]. Furthermore, certain lncRNAs act as molecular sponges for microRNAs, regulating their availability and activity within cellular signaling networks [20].

The functional diversity of lncRNAs extends to critical biological processes such as embryogenesis, organogenesis, and immune responses. However, dysregulation of lncRNA expression has been linked to various diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases [21]. For instance, lncRNAs like MALAT1 are involved in metastasis regulation in cancer, while others, such as MEG3, function as tumor suppressors by activating p53. Thus, lncRNAs are integral to both normal cellular processes and disease pathogenesis.

3. CRISPR-Cas9: A revolutionary tool for studying lncRNAs

CRISPR-Cas9 technology has revolutionized the study of long non-coding RNAs (lncRNAs) by providing precise and efficient tools for manipulating their expression and function. One of its key advantages is targeted gene editing, which allows for precise knockouts of lncRNA genes to assess their biological roles. By introducing small indels at specific loci, researchers can disrupt transcription or alter regulatory sequences to evaluate the functional consequences [22].

CRISPR interference (CRISPRi) uses a catalytically inactive Cas9 (dCas9) fused with repressive domains, enabling the transcriptional silencing of lncRNAs without altering the underlying DNA sequence. This method is particularly useful for studying lncRNAs that overlap with protein-coding genes [23]. In contrast, CRISPR activation (CRISPRa) employs dCas9 fused to transcriptional activators to upregulate lncRNA expression, offering insights into their gain-of-function effects in cellular processes [24].

High-throughput CRISPR screens have also been employed to identify lncRNAs involved in specific pathways, such as cancer progression or drug resistance [23]. Additionally, CRISPR-based tools like Cas9-targeted RNA editing enable precise modifications or tagging of lncRNA transcripts, facilitating the study of their localization, structure, and interactions [25]. These approaches are transforming lncRNA research by providing detailed insights into their regulatory roles and biological significance.

3.1 Functional analysis of lncRNAs

CRISPR-Cas9 offers a robust platform for functional analysis of lncRNAs, allowing researchers to integrate gene editing with transcriptomic and proteomic approaches. For instance, selectively targeting enhancer-associated lncRNAs can reveal their impact on enhancer activity and downstream gene expression [26]. Furthermore, CRISPR-Cas9 facilitates the creation of isogenic cell lines with lncRNA-specific alterations, enabling controlled studies of their contributions to complex phenotypes.

3.2 Emerging tools and applications

Advances in RNA sequencing (RNA-seq) and CRISPR technologies have accelerated the functional annotation of lncRNAs. Techniques such as CRISPRi and CRISPRa enable precise modulation of lncRNA expression, facilitating their study in specific biological contexts [27]. Given their broad functional spectrum and involvement in health and disease, lncRNAs represent a promising frontier in genomic research. The integration of CRISPR-Cas9 technology into this field provides unparalleled opportunities to uncover their roles and translate these findings into clinical applications, such as therapeutic interventions and diagnostic biomarkers.

4. Mechanisms of CRISPR/Cas systems

4.1 Structure and components

The CRISPR/Cas system, derived from a bacterial adaptive immune system, comprises two primary components: the CRISPR-associated protein (Cas) and guide RNA (gRNA). In the CRISPR/Cas9 system, Cas9 functions as an endonuclease that introduces double-strand breaks (DSBs) at specific DNA sites, while the gRNA directs Cas9 to the target sequence with high precision.

4.2 Cas9 protein

Cas9 is a multidomain protein with nuclease activity. It consists of two main nuclease domains: the RuvC and HNH domains. The RuvC domain cleaves the non-target DNA strand, while the HNH domain cleaves the target strand. The protein also contains a PAM-interacting domain, which ensures the correct targeting by recognizing the protospacer adjacent motif (PAM) sequence.

4.3 Guide RNA (gRNA)

The gRNA is a synthetic RNA molecule that combines two components found in the natural bacterial system: the CRISPR RNA (crRNA) and the trans-activating

crRNA (tracrRNA). The crRNA contains a spacer sequence complementary to the target DNA, while the tracrRNA stabilizes the complex and facilitates Cas9 binding. In genome editing, a single-guide RNA (sgRNA) is commonly used, which combines these two components into one engineered molecule. The precision of DNA targeting in the CRISPR/Cas9 system is determined by the complementarity between the gRNA spacer sequence and the target DNA, alongside the presence of a PAM sequence immediately downstream of the target site.

4.4 CRISPR/Cas9 workflow

The CRISPR/Cas9 genome editing process involves several steps, each critical for ensuring specificity and efficiency. The workflow for CRISPR/Cas9 is illustrated in **Figure 2**.

a. Design and Synthesis of gRNA: The first step is to identify the target DNA sequence and design a complementary spacer sequence in the gRNA. Bioinformatics tools aid in selecting target sites with minimal off-target effects.

b. Construction of CRISPR/Cas9 Complex: The synthesized gRNA is combined with the Cas9 protein to form a ribonucleoprotein complex. This complex acts as a molecular machine to locate and cleave the target DNA.

c. Target Binding: The gRNA directs the Cas9 complex to the target DNA through base-pairing interactions. The presence of a PAM sequence (e.g., 5′-NGG-3′ for SpCas9) downstream of the target site is essential for binding and activation of the nuclease.

d. DNA cleavage: Cas9 introduces a double-strand break at the target site. The cell's repair machinery then resolves this break through one of two pathways: (i) non-homologous end joining (NHEJ): Error-prone and often results in insertions or deletions (indels) and (ii) Homology-Directed Repair (HDR): Precise repair using a donor DNA template.

The importance of the PAM sequence cannot be overstated, as it serves as a critical checkpoint for Cas9 activity, preventing off-target binding and cleavage in regions lacking the correct PAM motif.

4.5 Alternative Cas systems

CRISPR technology extends beyond Cas9 with the discovery of alternative Cas proteins, broadening the potential for genome and transcriptome engineering. Cas12 proteins, including Cas12a (Cpf1), target DNA using a single RNA molecule and generate staggered double-stranded breaks (DSBs) with sticky ends, in contrast to the blunt ends produced by Cas9. Cas12a recognizes a distinct PAM sequence (e.g., 5′-TTTV-3′) and has a simpler gRNA architecture, making it especially useful for multiplexed genome editing. On the other hand, Cas13 proteins, such as Cas13a (C2c2), target RNA instead of DNA, enabling RNA-specific manipulation. Cas13 proteins cleave single-stranded RNA in a sequence-specific manner, with applications such as RNA knockdown, where specific RNA transcripts are

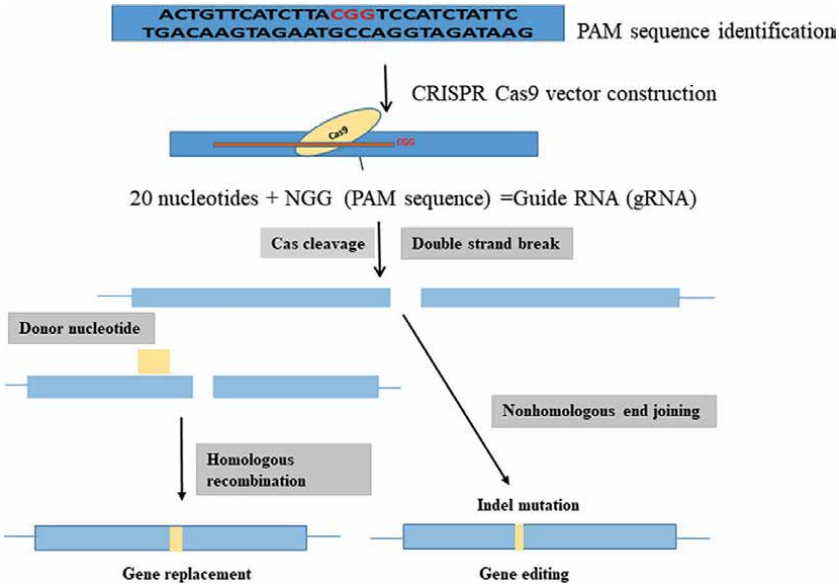

Figure 2.
Workflow of CRISPR/CAS9 editing.

degraded, and RNA editing, which allows for base editing or nucleotide modification in RNA molecules. Additionally, emerging Cas variants like Cas14 and CasPhi offer unique capabilities, such as targeting ultra-small genomes or non-canonical sequences, further enriching the CRISPR toolkit for genetic studies and applications.

5. CRISPR/Cas9 in genome editing-coding regions

The CRISPR/Cas9 system has revolutionized genome editing, particularly in coding regions, and has made significant advancements in functional genomics and biotechnology. One of its key applications is gene knockout, where CRISPR/Cas9 introduces indels through non-homologous end joining (NHEJ), effectively silencing the target gene. This approach is widely used to study gene function and disease mechanisms across various organisms [28]. Additionally, CRISPR/Cas9 facilitates gene insertion through homology-directed repair (HDR), enabling the precise integration of desired sequences at specific loci. This capability is instrumental in developing disease models and therapeutic applications [29]. The system can also introduce point mutations *via* HDR, allowing researchers to mimic disease-causing mutations or correct genetic defects, advancing personalized medicine and functional analysis of protein-coding genes.

CRISPR/Cas9 offers several advantages over traditional genome editing techniques, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). One major benefit is its high precision, achieved through the complementarity between the guide RNA (gRNA) and target DNA, which minimizes off-target effects when properly designed. The system's simplicity and rapid implementation contribute to its efficiency, making it suitable for generating mutations in a wide range of cell types and organisms. Moreover, CRISPR/Cas9 is highly scalable,

enabling high-throughput genome editing and supporting large-scale functional genomic studies and multiplexed editing strategies. Its versatility is another advantage, as it can induce various genetic modifications, ranging from simple knockouts to complex alterations like large insertions or chromosomal rearrangements.

Genome-wide CRISPR screens have emerged as a powerful tool for identifying essential genes and regulatory elements. Pooled libraries of sgRNAs targeting thousands of genes can be used to perform loss-of-function or gain-of-function screens, facilitating the discovery of genes critical for survival, drug resistance, or disease pathogenesis and aiding in the identification of therapeutic targets [30]. Furthermore, advances in single-cell sequencing technologies now allow for detailed analysis of gene perturbations at the single-cell level, offering new insights into cellular heterogeneity and lineage determination. Together, these CRISPR-based approaches are transforming our understanding of gene function and driving innovations in medicine and biotechnology.

5.1 CRISPR/Cas9 applications in lncRNA functional analysis

CRISPR/Cas9 technology has significantly advanced the study of long non-coding RNAs (lncRNAs), which play crucial roles in gene regulation, chromatin modification, and cellular differentiation. Unlike protein-coding genes, lncRNAs exhibit diverse and often tissue-specific functions, making them essential for understanding complex biological processes. For instance, lncRNAs like XIST regulate gene expression through transcriptional, post-transcriptional, and epigenetic mechanisms, as seen in X-chromosome inactivation [31]. Additionally, lncRNAs such as HOTAIR recruit chromatin-modifying complexes like Polycomb Repressive Complex 2 (PRC2) to specific genomic loci, leading to gene silencing through histone modification [32]. LncRNAs like TUG1 and NEAT1 are also involved in cellular differentiation processes such as neuronal development and cancer progression [33, 34]. Despite their importance, much of the functional understanding of lncRNAs remains limited due to their context-dependent roles.

CRISPR/Cas9 provides a powerful platform to investigate lncRNA functions through knockout, repression, activation, and tagging strategies. Using CRISPR-mediated knockout, researchers can introduce targeted loss-of-function mutations or complete deletions of lncRNAs, such as MALAT1, by disrupting key functional domains [35]. Complete deletions of lncRNA loci, such as HOTAIR and XIST, can also be achieved using paired guide RNAs flanking the target region [36]. Another approach, CRISPR interference (CRISPRi), utilizes a catalytically inactive Cas9 (dCas9) fused to transcriptional repressors to inhibit lncRNA transcription without causing DNA breaks. This method has been used to study lncRNAs like NEAT1, revealing their role in paraspeckle formation and stress responses [23]. CRISPRi offers specific, reversible repression of lncRNAs without the risk of off-target mutagenesis associated with DNA cleavage.

Conversely, CRISPR activation (CRISPRa) allows researchers to upregulate lncRNA transcription by using dCas9 fused to transcriptional activators. This technique has been applied to upregulate oncogenic lncRNAs such as LINC00116, providing insights into their roles in cellular processes and diseases [37]. Furthermore, CRISPR/Cas9 facilitates the insertion of functional tags or reporter genes into lncRNA loci to track their expression and localization. For example, inserting fluorescent tags like GFP allows researchers to study the dynamics of lncRNAs in live cells [38], while reporter genes like luciferase enable the measurement of transcriptional activity under different conditions, as demonstrated with GAS5 in glucocorticoid receptor signaling [39].

Overall, CRISPR/Cas9 has revolutionized the study of lncRNAs, providing valuable tools to uncover their roles in gene regulation, chromatin dynamics, and cellular differentiation. As CRISPR technology continues to evolve, it will further enhance our understanding of lncRNA biology and its implications in various health and disease contexts.

5.2 Challenges and limitations in CRISPR editing

While the CRISPR/Cas9 system offers immense potential in genome editing, it is not without challenges. One of the primary limitations is the risk of off-target effects. These occur when the CRISPR-Cas9 system binds to unintended genomic regions, leading to unintended edits. This issue arises because the guide RNA (gRNA) used to direct Cas9 can tolerate mismatches between its sequence and the target DNA, especially in regions distal to the PAM (Protospacer Adjacent Motif) site. This tolerance increases the likelihood of off-target binding to similar, but unintended, sequences elsewhere in the genome [40, 41]. Off-target effects can lead to mutations in unrelated genes, potentially disrupting essential genes or activating oncogenes, posing a significant risk in therapeutic applications [42].

To address these concerns, several strategies have been developed to enhance CRISPR/Cas9 specificity. High-fidelity Cas9 variants, such as eSpCas9, SpCas9-HF1, and HypaCas9, exhibit reduced off-target activity by improving the precision of gRNA-DNA pairing [43]. Improved gRNA design, aided by computational tools, can also minimize sequence similarity with non-target sites, further reducing off-target binding [44]. Another approach involves using paired Cas9 nickases, which introduce single-strand breaks rather than double-strand breaks. This requires two nickases to act near each other, reducing off-target effects [45]. Additionally, chemically modifying gRNAs or using truncated versions (tru-gRNAs) can enhance specificity and reduce unintended interactions [46].

5.3 DNA repair mechanisms

After CRISPR/Cas9 induces double-strand breaks, cellular DNA repair mechanisms dictate the outcome of the editing process. However, controlling these pathways remains a significant challenge. The predominant repair pathway, non-homologous end joining (NHEJ), is error-prone and often introduces insertions or deletions (indels) at the break site. While useful for gene knockout applications, NHEJ limits the precision of genome editing [47]. In contrast, Homology-Directed Repair (HDR) enables precise DNA sequence modifications using a homologous DNA template. However, HDR is less efficient than NHEJ and is most active during the S and G2 phases of the cell cycle, limiting its applicability in non-dividing or slowly dividing cells [48].

Efforts to bias repair toward HDR, such as synchronizing the cell cycle or using small-molecule inhibitors of NHEJ (e.g., SCR7), have shown promise but remain inconsistent across different cell types and conditions [49]. Enhancing HDR efficiency while avoiding cellular toxicity is an ongoing challenge in therapeutic applications [50].

5.4 Delivery challenges

Efficient delivery of the CRISPR/Cas9 system to target cells or tissues is crucial for successful genome editing. Various delivery methods come with their own sets of challenges.

- *Viral delivery methods*: Adeno-associated viruses (AAVs) are commonly used due to their safety and ability to infect a broad range of cell types. However, AAVs have a limited packaging capacity (~4.7 kb), which may be insufficient for delivering both Cas9 and gRNA, particularly when using larger Cas9 variants like SpCas9 [51]. Lentiviruses and retroviruses offer stable integration of CRISPR components, but the risk of insertional mutagenesis and long-term side effects in therapeutic contexts remains a concern [52].

- *Non-viral delivery methods*: Lipid nanoparticles (LNPs) can encapsulate CRISPR components and facilitate their delivery into cells *via* endocytosis. While LNPs are non-integrative and can be tailored for tissue-specific targeting, their efficiency can vary depending on the formulation and cell type [53]. Electroporation, a method that uses electric pulses to introduce CRISPR components into cells, is highly efficient in ex vivo applications but can cause significant cell damage, making it less suitable for *in vivo* use [54]. Physical delivery methods such as microinjection and hydrodynamic injection are labor-intensive and impractical for large-scale or systemic applications [55].

In summary, while CRISPR/Cas9 offers transformative potential for genome editing, overcoming challenges related to off-target effects, DNA repair mechanisms, and efficient delivery remains essential for its widespread and effective application in research and therapy. Continued advancements in protein engineering, delivery systems, and repair pathway modulation will be pivotal in overcoming these limitations and realizing the full potential of CRISPR in research and therapeutic contexts.

5.5 Ethical and regulatory considerations in CRISPR research

CRISPR-Cas9 technology has revolutionized genetic research and molecular biology, offering precise and efficient genome editing capabilities. However, its application raises several ethical concerns, especially in human genome editing and the editing of non-coding regions with unclear functions. Human genome editing, particularly germline editing, has sparked significant debate. Key ethical concerns include the potential risks of off-target mutations, mosaicism, and unforeseen long-term effects, which can be difficult to predict. Additionally, there are challenges regarding consent, as it is impossible to obtain consent from future generations who may be affected by these modifications. Issues of equity also arise, as access to genome editing technologies may be limited by socioeconomic factors, raising the potential for genetic inequality or the creation of "designer babies" that cater only to the wealthy. Furthermore, over-reliance on genome editing could reduce genetic diversity, leading to unforeseen vulnerabilities in human populations. The idea of editing non-disease traits, such as intelligence or physical appearance, could also lead to ethical dilemmas surrounding human enhancement and eugenics. Editing non-coding regions, once considered "junk DNA," presents additional concerns due to the unclear functionality of many of these sequences. The full roles of these regions in gene regulation and chromatin structure are still being uncovered, and modifying them without a comprehensive understanding could lead to unintended consequences, including disruptions in essential regulatory pathways and long-term developmental issues.

In addition to the ethical challenges, the regulatory landscape surrounding CRISPR research varies across countries and focuses on ensuring safety, transparency,

and ethical oversight. Regulatory agencies like the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the World Health Organization (WHO) oversee the safety and approval of CRISPR-based therapies. Preclinical studies, often using animal models, are required to evaluate potential off-target effects and genomic stability before CRISPR interventions can proceed to clinical trials. Clinical trials, conducted under strict ethical oversight, are necessary to assess the safety and efficacy of these therapies in humans, ensuring that patient consent and long-term effects are carefully monitored. Transparency in CRISPR research is also crucial for maintaining public trust, with regulatory bodies pushing for clear reporting of research findings, including off-target effects and unintended genetic modifications. Open data sharing and ethical review boards are essential components of this transparency, helping to ensure the reproducibility and reliability of research. Finally, CRISPR-based therapeutic applications, such as gene editing for inherited diseases, must undergo rigorous regulatory approval processes, including FDA evaluation and international collaboration to harmonize standards across the globe. These measures aim to ensure that CRISPR technologies are used safely and responsibly, minimizing risks while maximizing their potential benefits.

CRISPR technology holds immense promise in medicine, agriculture, and beyond, but it also raises significant ethical and regulatory concerns. The ethical challenges, especially regarding human genome editing and the modification of non-coding regions, require careful consideration of safety, consent, and the long-term implications of genetic modifications. Simultaneously, the regulatory landscape must evolve to ensure that CRISPR-based interventions are safe, transparent, and ethically sound. As research progresses, it is crucial that regulatory agencies, scientists, and ethicists work together to guide the responsible use of this powerful technology.

5.6 Future directions

The transformative potential of CRISPR-based approaches in lncRNA research is immense, with evolving technologies opening up exciting possibilities for the future. One key area is personalized medicine, where CRISPR tools could be used to target disease-associated lncRNAs. Many diseases, especially cancers, are linked to dysregulated lncRNA expression, and CRISPR could precisely edit or silence these non-coding RNAs to create tailored therapeutic strategies. This approach could go beyond protein-coding gene correction, allowing for the reprogramming of entire non-coding RNA networks to address specific genetic conditions. Additionally, CRISPR-based therapies hold promise for treating diseases caused by lncRNA dysfunction, offering a direct solution where traditional gene-editing methods may struggle.

Another promising avenue is the role of lncRNAs as modulators of epigenetic regulation. LncRNAs play pivotal roles in chromatin modifications and gene silencing, influencing cellular processes such as differentiation, pluripotency, and cancer metastasis. With CRISPR, researchers can manipulate lncRNA expression or function to investigate how these non-coding RNAs interact with epigenetic regulators, which could lead to novel epigenetic therapies targeting diseases like cancer or developmental disorders.

In vivo functional studies of lncRNAs are also becoming more feasible with CRISPR technology. The ability to manipulate lncRNAs directly in living organisms using CRISPR-based tools like CRISPRa and CRISPRi allows researchers to gain a deeper understanding of how these molecules influence gene expression across

various tissues and disease models. This will provide valuable insights into their roles at both the transcriptional and post-transcriptional levels.

Overall, CRISPR technology has significantly impacted functional genomics by enabling precise modifications not just in coding genes but also in the realm of non-coding RNAs, such as lncRNAs. Once considered merely transcriptional "noise," lncRNAs are now recognized as critical regulators of gene expression, chromatin remodeling, and key cellular processes like differentiation, development, and disease progression. By allowing targeted knockouts or activation/inhibition of specific lncRNAs, CRISPR has revolutionized our ability to explore these roles in greater detail. This has far-reaching implications for understanding cellular processes and advancing clinical applications, especially in areas like immune response and adaptation, as seen in ongoing research on Drosophila's immune pathways. As CRISPR continues to evolve, its integration into lncRNA research will likely uncover new therapeutic strategies and enrich our understanding of the complex regulatory landscape of the genome. This growing body of research will continue to shape the future of functional genomics, leading to better models of gene regulation and improved disease treatment strategies.

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Chapter 8

Long Non-Coding RNAs in Stem Cell Regulation and Regenerative Medicine: Stemness, Differentiation, and Therapeutic Innovation

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Abstract

Long non-coding RNAs (lncRNAs) have emerged as critical regulators in stem cell biology, influencing cellular functions such as pluripotency, differentiation, and self-renewal. Their unique ability to modulate gene expression at multiple levels—epigenetic, transcriptional, and post-transcriptional—makes lncRNAs powerful tools for controlling cell fate. In regenerative medicine, understanding the roles of specific lncRNAs can enhance therapeutic approaches, particularly in stem cell-based tissue repair and engineering. By modulating lncRNA activity, researchers can potentially direct stem cell differentiation toward desired lineages, facilitating the development of functional tissues for clinical applications. This chapter explores how lncRNAs influence stem cell states, highlights current research in regenerative therapies, and discusses potential future applications where lncRNA-based interventions could drive advancements in tissue engineering and regenerative medicine.

Keywords: long non-coding RNA (lncRNA), stem cell biology, regenerative medicine, pluripotency, bioinformatics

1. Introduction

The human transcriptome is remarkably complex, with a substantial proportion transcribed into non-coding RNAs (ncRNAs) that lack protein-coding potential. These ncRNAs constitute a heterogeneous group with key roles in regulating transcriptional and post-transcriptional processes, including X-chromosome inactivation, epigenetic modulation, genomic imprinting, and mRNA splicing [1–3]. NcRNAs are broadly categorized into two groups based on their size, small ncRNAs (18–200 nucleotides) and long non-coding RNAs (lncRNAs, >200 nucleotides). While small ncRNAs like microRNAs (miRNAs) and small interfering RNAs (siRNAs) have been

widely studied in their gene expression ability, lncRNAs remain less understood but increasingly recognized for their pivotal regulatory roles [2]. Long non-coding RNAs (lncRNAs) can be classified in several ways, with one common method being based on their genomic organization. This classification divides lncRNAs into four main categories according to their positions relative to protein-coding genes: (1) intergenic lncRNAs, also called lincRNAs, which are transcribed from regions of DNA located between two protein-coding genes and often play regulatory roles; (2) intronic lncRNAs, derived from the introns of protein-coding genes and sometimes involved in splicing or transcriptional regulation; (3) overlapping lncRNAs, which are transcripts that partially or entirely overlap with known protein-coding genes, suggesting potential interactions with their overlapping counterparts; and (4) antisense lncRNAs, transcribed in the opposite direction of a protein-coding gene, often influencing gene expression through complementary base-pairing or chromatin modification [2, 4–8].

LncRNAs are transcribed by RNA polymerase II and exhibit features such as 5′ capping, splicing, and polyadenylation, akin to mRNAs [1]. Functionally, lncRNAs participate in chromatin remodeling, transcriptional regulation, and post-transcriptional processing, acting as essential regulators in embryonic stem cell pluripotency, development, differentiation, and tumorigenesis. Long non-coding RNAs (lncRNAs) exert significant regulatory influence across transcriptional, post-transcriptional, and translational processes and alternative splicing. Transcriptionally, lncRNAs can affect transcriptional complexes or DNA elements (e.g., promoter). In this regard, LncRNA "PANDA" regulates transcription by interacting with the transcription factor NF-YA. This interaction sequesters NF-YA away from its target gene-associated chromatin [9]. LncRNAs can also transcriptionally control gene expression by acting as scaffolds for histone-modifying enzymes, affecting the expression of genes [10]. HOTAIR, a well-studied lncRNA, alters histone H3K27 methylation patterns by interacting with the PRC2 complex. This modification silences specific genes, facilitating tumor cell invasion and metastasis [11]. Post-transcriptionally, they regulate mRNA stability, such as Linc-RoR stabilizing c-Myc by interacting with AUF1 and hnRNP I [2, 12]. LncRNAs can also modulate alternative splicing by cooperating with hnRNPs [13]. MALAT1 influences serine-arginine proteins [13]. At the translational level, lncRNAs like Linc-RoR suppresses p53 translation by blocking hnRNP I interactions with p53 5′ UTR [14], while others, such as Uchl1, enhance translation through UTR-mediated interactions [15]. Despite their functional diversity, lncRNAs are generally expressed at low levels, exhibit high cell-type specificity, and are often dysregulated in diseases, including cancer.

Stem cells represent a unique cellular state characterized by self-renewal and the ability to differentiate into specialized cell types, and lncRNAs play a pivotal role in maintaining this delicate balance. LncRNAs such as H19, TUNA, and linc-ROR are central to regulating pluripotency and lineage commitment. For example, Linc-ROR modulates the transcription factors OCT4, SOX2, and NANOG, which are essential for maintaining the pluripotent state [16]. Moreover, lncRNAs like MALAT1 and MEG3 are involved in cell fate determination, influencing differentiation pathways through epigenetic and transcriptional regulation [17]. These molecules act as critical modulators of stem cell identity, responding to developmental cues and environmental signals to drive specific differentiation trajectories. Dysregulation of stem cell-associated lncRNAs has been implicated in developmental disorders and cancer, further underscoring their importance in cellular homeostasis and disease. Understanding the role of lncRNAs in stem cell biology offers exciting prospects for regenerative medicine and the development of targeted therapies.

Advancements in high-throughput sequencing and bioinformatics have facilitated the identification and characterization of thousands of lncRNAs, revealing their roles as master regulators of gene expression [18]. LncRNAs can exert their regulatory functions through various mechanisms, including acting as molecular scaffolds, decoys, or guides, forming complex interactions with proteins, RNA, and DNA. While some lncRNAs, such as XIST and H19, have been studied for decades, the field continues to uncover their intricate involvement in health and disease. As lncRNAs are increasingly implicated in cancer biology and stem cell regulation, understanding their mechanisms and functional diversity holds immense potential for novel therapeutic interventions, including precision medicine and regenerative therapies.

2. LncRNAs in stemness and differentiation

Long non-coding RNAs (lncRNAs) are emerging as pivotal regulators of stem cell biology, playing crucial roles in maintaining stemness and differentiation. Stem cells, characterized by their unique ability to self-renew and differentiate into specialized cell types, are central to tissue development, repair, and homeostasis. The regulation of these processes involves intricate molecular networks, where lncRNAs act as key modulators by influencing gene expression, epigenetic landscapes, and signaling pathways [19]. Through interactions with transcription factors, chromatin remodelers, and microRNAs, lncRNAs fine-tune the balance between maintaining a pluripotent state and committing to lineage-specific differentiation [20]. Understanding the role of lncRNAs in these processes sheds light on fundamental aspects of stem cell biology and opens new avenues for therapeutic applications in regenerative medicine and disease treatment.

2.1 LncRNAs in embryonic stem cells

Long non-coding RNAs (lncRNAs) are pivotal regulators in maintaining pluripotency and the differentiation of embryonic stem cells (ESCs). Genome-wide studies in mouse ESCs (mESCs) identified lncRNAs such as AK028326 and AK141205, which Oct4 and Nanog transcriptionally regulate. AK028326, for example, functions as a coactivator of Oct4, while AK141205 is repressed by Nanog, indicating the direct involvement of these lncRNAs in the pluripotency network [21]. Additionally, lncRNAs (lincRNAs) play roles in pluripotency and proliferation, many of which are directly regulated by key transcription factors such as Sox2, Oct4, and Nanog [19]. In human ESCs (hESCs), lncRNAs like RMST and SOX2OT have been implicated in maintaining pluripotency and regulating differentiation. RMST interacts with SOX2 as a transcriptional co-regulator [22], while SOX2OT modulates SOX2 expression [23]. Furthermore, cytoplasmic lncRNAs, such as linc-RoR, act as microRNA sponges, shielding mRNAs of key transcription factors from degradation and supporting ESC maintenance [24]. These findings underscore the dual nuclear and cytoplasmic roles of lncRNAs in ESC biology.

The functional significance of lncRNAs extends to lineage-specific differentiation and chromatin regulation. The majority of lincRNAs in ESCs influence gene expression, with 26 lincRNAs directly affecting Nanog expression [25]. Moreover, lineage-specific differentiation involves the activity of numerous lincRNAs, such as those associated with endoderm, ectoderm, and mesoderm formation. The involvement of pluripotency-associated transcription factors, including Oct4, Sox2, and Nanog, in

regulating these lincRNAs shows a possible tightly interconnected regulatory network [21]. Additionally, the association of 30% of lincRNAs with chromatin-modifying proteins highlights their role in remodeling chromatin to maintain ESC identity [21]. Gas5, for instance, maintains pluripotency by regulating OCT4 and SOX2 through the TGFβ pathway, while Digit influences endoderm differentiation *via* SMAD3 [26, 27]. Similarly, Terra promotes self-renewal through the WNT/β-catenin pathway, and its overexpression prevents ESCs from exiting pluripotency [28].

X-chromosome inactivation (XCI) is a critical process in female ESC differentiation with lncRNA involvement. XCI is regulated by non-coding RNAs like Xist, Tsix, and RepA, which are themselves targets of pluripotency transcription factors [29]. For example, Oct4 and Nanog repress Xist expression in mESCs, maintaining two active X chromosomes, a hallmark of pluripotency [30]. Upon differentiation, Xist expression increases, leading to XCI [30]. Furthermore, NEAT1 plays a role in the transition from pluripotency to differentiation. NEAT1 is a nuclear lncRNA absent in undifferentiated ESCs, facilitating paraspeckle formation in differentiated cells [31, 32]. Other lncRNAs, such as Lncenc1, regulate ESC metabolism by interacting with RNA-binding proteins like PTBP1 and HNRNPK to control glycolytic gene expression [33].

Collectively, lncRNAs have different roles in ESC biology. They act as molecular scaffolds, transcriptional regulators, microRNA sponges, and modulators of chromatin and signaling pathways. While significant progress has been made in understanding their functions, many questions remain about the precise mechanisms and interacting partners of these lncRNAs. Future research is needed to explore the dynamic regulation of lncRNAs and their potential uses in regenerative medicine and cell-based therapies.

2.2 LncRNAs in induced pluripotent stem cells

Research about the roles of lncRNAs in induced pluripotent stem cells (iPSCs) can shed light on the molecular mechanisms underlying reprogramming. Several studies have identified distinct sets of lncRNAs that are differentially expressed in iPSCs compared to ESCs, suggesting their functional relevance in achieving and maintaining pluripotency. For instance, 26 lncRNAs have been identified to be overexpressed in both human ESCs and iPSCs, with some showing elevated levels specifically in iPSCs [25]. Notably, lncRNAs, such as Meg3, Meg9, and Rian, located in the Dlk1-Dio3 gene cluster, are typically expressed in ESCs but often silenced in iPSCs [21]. Similarly, transcriptional changes in the X-inactivation center, including the biallelic expression of Tsix and activation of Xite, are important in reactivating both X chromosomes during the transition to the pluripotency state [34, 35].

Furthermore, lncRNAs such as lincRNA-SFMBT2, lincRNA-VLDLR, and lincRNA-ST8SIA3 are linked to the reprogramming process. They have been found to interact with core pluripotency transcription factors Oct4, Sox2, and Nanog [36]. Knockdown studies have demonstrated that these lncRNAs are essential for efficient colony formation, with lincRNA-ST8SIA3 showing strong involvement in regulating p53-mediated apoptotic pathways during reprogramming [37]. LincRNA-RoR (Regulator of Reprogramming), functions as a competing endogenous RNA [38]. Silencing linc-ROR expression in stem cells prompts their exit from the stem cell state, accompanied by elevated levels of ectodermal markers, including SOX1 and vimentin, further highlighting its role in maintaining the pluripotent state [17]. Global epigenetic remodeling and the dynamic expression of over 400 lncRNAs at defined reprogramming stages show their complex regulatory mechanisms [39]. Collectively,

lncRNAs are critical in transcriptional and epigenetic regulation which are necessary for iPSC generation and maintenance, offering new strategies for stem cell-based therapies and personalized medicine.

2.3 LncRNAs in mesenchymal stem cell

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into various lineages, including mesodermal, ectodermal, and endodermal fates. MSCs support hematopoietic stem cells and can differentiate into bone, cartilage, and adipose tissues. MSCs are also present in various other tissues such as adipose tissue, umbilical cord blood, and the dental pulp of baby teeth [40]. Due to their capacity for multipotent differentiation, MSCs hold significant promise for regenerative medicine. Research has highlighted the important regulatory roles of lncRNAs in MSCs and their promising potential in disease and therapeutic goals. Notably, lncRNAs like ANCR, MALAT1, and H19 have been shown to regulate MSC differentiation processes, influencing cell fate decisions across different lineages. For example, ANCR has been implicated in inhibiting the trans-differentiation of adipose-derived stem cells (ADSCs) into endodermal lineages [41], while MALAT1 plays a role in the trans-differentiation of bone marrow-derived stem cells (BMSCs) into hepatocytes [41]. Furthermore, lncRNAs are involved in regulating MSC differentiation into ectodermal lineages, including neural cells. Recent studies have shown that specific lncRNAs modulate the neural differentiation of BMSCs, such as the negative regulation of neural-like differentiation by H19 through the miR-675-IGFR axis [41].

2.3.1 LncRNAs in adipogenic differentiation

In adipogenesis, MSCs differentiate into adipocytes. It involves complex regulatory mechanisms, including the pivotal role of long non-coding RNAs (lncRNAs). MSCs differentiate into both brown adipose tissue (BAT) and white adipose tissue (WAT), with specific transcription factors, such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα), driving adipogenesis [42]. RNA sequencing has shown that lncRNAs are dynamically expressed during adipogenesis. For example, 175 lncRNAs are associated with the differentiation of BAT and WAT [43]. Among these, the brown fat lncRNA 1 (Blnc1) is essential for brown adipogenesis, forming a ribonucleoprotein complex with EBF2 to regulate thermogenic gene expression and promoting a feed-forward regulatory loop [44]. The lncRNA SRA is known for the promotion of adipogenesis by enhancing PPARγ expression, which results in improving insulin sensitivity while inhibiting inflammatory pathways [45]. Another lncRNA, ADINR, positively regulates PPARγ and C/EBPα by recruiting the PA1 complex to the CEBPA promoter, affecting histone modifications to facilitate adipogenic gene expression [46]. Such findings highlight the central role of lncRNAs in adipogenic differentiation and energy metabolism.

The process of adipogenesis is divided into two phases: lineage commitment, where MSCs commit to the adipocyte lineage, and terminal differentiation, where preadipocytes mature into lipid-storing adipocytes [47]. Numerous intracellular and extracellular signals, such as TGF-β, Wnt, and Hedgehog signaling pathways, are present in this process by regulating transcription factors like PPARγ and C/EBPα [47]. LncRNAs have been shown to influence these pathways, often functioning as competing endogenous RNAs (ceRNAs) or interacting with chromatin to modulate gene expression. For instance, during adipogenesis, the levels of the lncRNA GAS5

gradually decrease and impair adipogenesis. It regulates the MSC's adipogenic differentiation by sponging miR-18a, co-suppressing CTGF protein translation [48], while HOTAIR reduces adipogenesis through DNA methylation *via* a triple helix structure [49]. lncRNAs such as MEG3 and ADNCR act differently for suppressing adipogenesis. MEG3 inhibits adipogenic differentiation by sponging miR-140-5p, a microRNA that upregulates PPARγ and C/EBPα [50], while ADNCR functions as a competing endogenous RNA (ceRNA) for miR-204 to promote SIRT1 expression, suppressing PPARγ activity and adipocyte differentiation [51]. These findings show the potential opportunities for targeting lncRNAs for therapeutic applications in obesity and related metabolic disorders.

2.3.2 LncRNAs in osteogenic differentiation

Osteogenesis is a dynamic process involving the differentiation of mesenchymal stem cells (MSCs) into osteoblasts and subsequent bone tissue formation. It is regulated by long non-coding RNAs (lncRNAs), which modulate key signaling pathways, transcription factors, and epigenetic modifications, playing crucial roles in lineage commitment and maturation during osteogenic differentiation. For instance, the lncRNA H19 enhances osteogenesis by sponging miR-141 and miR-22, resulting in promoting the Wnt/β-catenin signaling pathway, which is essential for osteoblast development [52]. Similarly, Linc-ROR activates the Wnt/β-catenin pathway with upregulating osteogenic genes by interacting with miR-138 and miR-145 [53]. In contrast, inhibitory lncRNAs such as MIAT and DANCR suppress osteogenesis by targeting specific pathways. MIAT inhibits osteogenic differentiation by sponging miR-150-5p and modulating AKT activity [54], while DANCR interferes with p38 MAPK signaling and downregulates osteogenic markers [55]. Additionally, lncRNA ANCR maintains the undifferentiated state of MSCs. It represses the osteogenic transcription factor Runx2 through EZH2-mediated H3K27me3 modifications [56]. Beyond these, lncRNAs such as MEG3 and AK141205 enhance osteogenesis *via* BMP4 activation and CXCL13 expression [57]. Given their critical involvement in osteogenic differentiation, lncRNAs represent potential therapeutic targets for treating bone-related disorders such as osteoporosis and osteogenesis imperfecta.

2.3.3 LncRNAs in cardiomyocyte differentiation

Long non-coding RNAs (lncRNAs) are emerging as critical regulators of cardiomyocyte differentiation and cardiovascular development by orchestrating gene expression, chromatin dynamics, and lineage specification. Fendrr and Braveheart (Bvht) are two identified lncRNAs among the several differentially expressed lncRNAs during cardiac differentiation, which play pivotal roles in heart development. Fendrr, expressed in the lateral mesoderm, interacts with the PRC2 and/or TrxG/MILL complex to regulate chromatin signatures at cardiac transcription factor promoters, ensuring proper mesodermal differentiation and heart formation [58]. Its loss leads to severe myocardial dysfunction and embryonic lethality [58]. On the other hand, Braveheart drives cardiomyocyte lineage specification. Studies showed its ability to interact with SUZ12 and CNBP, with its AGIL motif being indispensable for its function in promoting cardiomyocyte differentiation [59, 60].

Other lncRNAs such as HBL1 and CARMEN add further complexity to this regulatory network. HBL1 acts as a suppressor of cardiomyocyte differentiation by sponging miR-1 and downregulating critical cardiac genes [61], while CARMEN, a

super-enhancer-associated lncRNA, promotes cardiac progenitor cell (CPC) differentiation into cardiomyocytes and enhances the expression of cardiac markers like GATA4 and NKX2.5 [20, 62]. Additionally, novel lncRNAs such as yylncT have been implicated in mesodermal commitment during cardiac differentiation, regulating the expression of BRACHYURY by inhibiting DNMT3B activity [63]. Emerging evidence also points to the roles of TTN-AS1, ALIEN, and PUNISHER in cardiovascular differentiation, with functions ranging from modulating extracellular matrix remodeling to enhancing endothelial cell maturation [20, 64].

Collectively, studies show the diverse and stage-specific mechanisms that lncRNAs regulate cardiomyocyte differentiation and cardiovascular development. Their ability to modulate transcriptional programs, chromatin dynamics, and lineage specification highlights them as a promising therapeutic target for treating cardiovascular diseases and advancing regenerative medicine strategies. Further research into these molecules promises to unveil novel cardiac repair and regeneration approaches.

2.3.4 LncRNAs in myocyte differentiation

Myogenesis, the highly regulated process of muscle formation, is orchestrated by a dynamic network of epigenetic regulators, transcription factors, and long non-coding RNAs (lncRNAs). Muscle-specific lncRNAs play crucial roles at various stages of myoblast proliferation, differentiation, and myotube formation. Linc-MD1, one of the first identified muscle-specific lncRNAs, is activated during myoblast differentiation and acts as a competing endogenous RNA (ceRNA). It sponges miR-133 and miR-135 and enhances the expression of MEF2C and MAML1, two key transcription factors driving muscle-specific gene expression [65]. Similarly, lncRNA H19 is expressed during myoblast differentiation and enhances skeletal muscle differentiation through multiple mechanisms, including encoding miR-675 to suppress BMP signaling and Cdc6 expression [65]. It also promotes Igf2 expression, a critical myogenic stimulator [66]. H19 also regulates the SIRT1/FoxO1 axis, further underscoring its involvement in muscle-specific pathways [67].

The lncRNAs LncMyoD and MUNC demonstrate their importance in regulating MyoD expression and early myogenic differentiation. LncMyoD, located upstream of the MyoD gene, inhibits myoblast cell cycling and facilitates terminal differentiation by directly interacting with the IMP2 protein [68]. In contrast, MUNC acts as an enhancer RNA (eRNA) transcribed from the MyoD regulatory regions, driving the expression of key myogenic transcription factors like MYOGENIN and MYH3 [36]. Notably, silencing of MUNC delays muscle regeneration, highlighting its pivotal role in muscle repair [69]. Other lncRNAs such as Dum and Yam-1 also illustrate the diversity in lncRNA functions. MyoD induces Dum and facilitates myogenic differentiation by repressing Dppa2 through DNA methylation at its promoter [70]. Conversely, Yam-1 inhibits myogenesis by modulating miR-715 expression, which targets Wnt7b and impairs Wnt/β-catenin signaling [71].

Interestingly, lncRNA Malat1, initially identified in non-muscle contexts, inhibits skeletal muscle differentiation by regulating the miR-181a-Malat1-MyoD/Suv39h1 axis [72]. Malat1 suppression accelerates myoblast differentiation and enhances muscle regeneration which can be used as a therapeutic target in future research. Additionally, SRA (steroid receptor RNA activator) emerges as a coactivator of MyoD in myogenesis [73]. While its non-coding RNA form enhances myogenic transcriptional activity, alternative splicing generates a protein counterpart, SRAP, which antagonizes the RNA's coactivator function, demonstrating a finely tuned regulatory

mechanism during muscle differentiation [74]. Collectively, lncRNAs are central regulators of skeletal muscle development and regeneration, offering their potential therapeutic uses for muscle-related disorders.

2.4 LncRNAs in hematopoietic stem cells and hematopoiesis

The hematopoietic system is a complex biological framework that produces blood cells and immune system components. This system plays a critical role in maintaining homeostasis in the human body. Red blood cells (RBCs) are vital for oxygen transportation, platelets are essential for blood coagulation, and white blood cells (WBCs) act as defenders against pathogens, serving as a part of the immune system [75]. In the early 1960s, Till and McCulloch revealed the existence of hematopoietic stem cells (HSCs), which are responsible for blood regeneration. These multipotent stem cells can self-renew and differentiate into all blood cell types [76]. The clinical significance of HSCs is profound, with applications in treating leukemia, lymphoma, and genetic disorders such as anemia and thalassemia. Bone marrow transplants, often derived from HSCs, are a cornerstone of these therapies, replacing a patient's defective blood cells with healthy ones from a compatible donor.

Recent advancements in understanding the regulatory mechanisms of hematopoiesis have highlighted the pivotal role of long non-coding RNAs (lncRNAs). lincRNA-EPS is among the first lncRNAs identified in erythropoiesis (red blood cell production). Hu et al. [77] conducted RNA-seq analysis on embryonic liver cells, with active erythropoiesis, and identified over 400 lncRNAs involved in the process of erythropoiesis. Of these, 163 were upregulated and 42 were downregulated during erythropoiesis. Their work focused on lincRNA-EPS, which was shown to be crucial for erythroid differentiation. Functional studies revealed that lincRNA-EPS depletion led to increased apoptosis and proliferation reduction of erythroid progenitors, even in the presence of erythropoietin. The anti-apoptotic role of lincRNA-EPS was confirmed by the overexpression studies of lincRNA-EPS in erythroid progenitors. Mechanistically, lincRNA-EPS represses the pro-apoptotic gene Pycard, which activates caspase-mediated apoptosis and ensures the survival of erythroid progenitors during differentiation. Another analysis [78] identified approximately 1100 lncRNAs expressed during the erythro-megakaryopoiesis (development of red blood cells and platelets) process in both murine and human models. Intriguingly, the chromatin immunoprecipitation sequencing (ChIP-seq) showed that about 75% of these lncRNAs were transcribed from promoter regions of genes, while 25% originated from enhancer regions. Key transcription factors, such as GATA1 and TAL1, were found to occupy these lncRNA loci. Knockdown experiments revealed that several lncRNAs, including LINCRED1, ERYTHRA, and SCARLETLTR, were critical for the terminal maturation of erythroblasts into reticulocytes. Eosinophils, another lineage derived from myeloid progenitors, play essential roles in parasitic immunity and allergic responses. The lncRNA EGO (eosinophil granule ontogeny) was discovered within the intronic region of the Itpr1 gene and found to be upregulated upon stimulation of CD34+ hematopoietic stem cells with IL-5, a cytokine specific to eosinophil development [79]. The expression of eosinophil proteins such as major basic protein (MBP) and eosinophil-derived neurotoxin (EDN) was weakened through silencing EGO. This indicates that EGO is essential for normal eosinophil differentiation [80]. Another well-studied lncRNA is HOTAIRM1 (HOX antisense intergenic myeloid 1), specifically expressed during granulocytic differentiation [81]. Induction of HOTAIRM1 is lineage-specific and regulated by the transcription factor PU.1, a key

player in myeloid differentiation [82]. Silencing HOTAIRM1 impairs myeloid cell maturation. The absence of its activity suppresses the expression of granulocytic markers, including HoxA1 and HoxA4, as well as integrins such as CD11b and CD18 [81]. The lncRNAs also regulate T-cell differentiation and maturation. LincRNAs like LincR-Ccr2-5′AS regulate chemokine receptor expression, influencing T-cell migration during immune responses [83].

Emerging research continues to show the roles of lncRNAs in regulating hematopoiesis, providing deeper insights into their potential as therapeutic targets for hematological disorders. These non-coding RNAs not only regulate lineage-specific differentiation but also ensure the functional integrity of blood and immune cells, paving the way for innovative approaches in regenerative medicine and disease treatment.

2.5 LncRNAs in neural stem cells and neurogenesis

LncRNAs play crucial roles in the complex network governing neural differentiation and neurogenesis, mediating key processes in embryonic and adult neural stem cells (NSCs). Transcriptomic studies have revealed numerous lncRNAs with differential expression patterns during neural differentiation. For instance, genome-wide shRNA screening identified ~20 essential lncRNAs for NSC pluripotency such as TUNA and lncRNA-1604 [84]. They are highly expressed in the central nervous system (CNS). TUNA promotes neural differentiation by recruiting multiprotein complexes to gene promoters, enhancing histone modifications (H3K4me), and activating pluripotency factors like Nanog and Sox2 [84]. Similarly, lncRNA-1604 interacts with miR-200c to suppress ZEB1/2 expression, thereby facilitating ectodermal differentiation and upregulating neural progenitor markers like Sox1 and Nestin [85].

Another pivotal lncRNA, MEG3, is derived from the DLK1-DIO3 locus, which controls neural marker expression and modulates neural lineage differentiation rates [86, 87]. Evf2, transcribed from the Dlx5/6 intergenic region, is critical for hippocampal development, regulating GABAergic neuron production through topological and transcriptional mechanisms [88, 89]. Moreover, RMST collaborates with SOX2 to drive neurogenesis by enabling SOX2's binding to neurogenic transcription factor promoters [90]. PAUPAR and DALI are other lncRNAs that have different functions in NSC maintenance and differentiation. PAUPAR regulates Pax6 expression and gene networks involved in synaptic activity [91], while DALI modulates neuronal differentiation *via* cis and trans-regulatory mechanisms, influencing chromatin organization and DNA methylation [92].

There are several lncRNAs with inhibitory roles in neuronal differentiation. Expressed in NSCs, Pnky interacts with PTBP1 to maintain stem cell states, repressing premature differentiation into neurons. Pnky is critical in balancing neural stem cell self-renewal and differentiation, and its knockdown leads to increased neurogenesis [20, 93]. Collectively, these findings reveal that lncRNAs act as both positive and negative regulators in the intricate orchestration of neural differentiation, offering insights into their potential as therapeutic targets for neurodevelopmental disorders and regenerative medicine.

2.6 LncRNAs in spermatogonial stem cells and differentiation

Spermatogenesis is a highly regulated physiological process that generates millions of mature spermatozoa daily from spermatogonial stem cells (SSCs). These

progenitor cells originate from primordial germ cells (PGCs), which migrate to the gonadal ridges during embryonic development and enter mitotic arrest until birth [94]. Recent studies have highlighted the pivotal role of long non-coding RNAs (lncRNAs) in regulating various stages of spermatogenesis, from SSC maintenance to spermatid differentiation.

The MRHL lncRNA (mouse recombination hotspot locus) plays a critical role in SSC physiology by negatively regulating the Wnt signaling pathway. MRHL physically interacts with the helicase protein p68/DDX5, restricting the nuclear translocation of β-catenin and thereby modulating downstream Wnt signaling [95]. Genome-wide occupancy studies revealed that MRHL binds to over 1400 chromatin loci, including 37 genes involved in spermatogenesis and differentiation [95]. Furthermore, Wnt signaling downregulates MRHL expression *via* CTBP1-mediated repressive histone modifications, highlighting a feedback loop in SSC differentiation [96]. LncRNA 033862 is transcribed in an antisense direction from exon 9 of the Gfra1 locus; it physically associates with Gfra1 chromatin and regulates SSC self-renewal and maintenance. Its knockdown leads to apoptosis, reduced expression of self-renewal genes (Bcl6b, Ccnd2, and Pou5f1), and diminished SSC colony size. These findings underscore the critical role of 033862 in maintaining SSC integrity and its regulation by GDNF signaling [97, 98].

TSX, identified through transcriptomic studies, is a testis-specific X-linked lncRNA that exhibits dynamic expression, peaking in pachytene spermatocytes during meiosis [99]. TSX knockout leads to increased apoptosis of germ cells at this stage, suggesting its role in facilitating meiotic progression [100]. Similarly, high-throughput analyses have highlighted lncRNAs AK011429 and AK00574, which correlate with protein-coding genes critical for spermatogonial renewal and differentiation [101]. While many of these lncRNAs remain functionally uncharacterized, their stage-specific expression patterns and chromatin interactions suggest they play intricate roles in germ cell development and testicular homeostasis.

3. LncRNAs in cancer stem cells and cancer

lncRNAs have become significant regulators in various biological processes, including cancer development and progression. Cancer stem cells (CSCs), which possess self-renewal and differentiation capabilities, are key contributors to tumor initiation, metastasis, and chemoresistance. LncRNAs regulate key pathways associated with CSCs, such as stemness, epithelial-mesenchymal transition (EMT), immune escape, and epigenetic reprogramming, highlighting their potential as therapeutic targets. This review explores the roles of lncRNAs in CSCs, their mechanistic involvement in cancer, and their clinical significance.

3.1 LncRNAs in tumorigenesis and CSC maintenance

Long non-coding RNAs (lncRNAs) are increasingly recognized as crucial regulators in cancer stem cells (CSCs), impacting tumor initiation, progression, and metastasis. CSCs are a small subset of cells within tumors capable of self-renewal, differentiation, and resistance to conventional therapies, contributing to tumor recurrence and chemoresistance. LncRNAs influence these functions through various mechanisms, including regulation of stemness, epithelial-mesenchymal transition (EMT), and interaction with the tumor microenvironment. Several lncRNAs are

upregulated in CSCs and are associated with regulating critical stemness transcription factors such as Sox2, NANOG, and Oct4. By modulating these pathways, lncRNAs help maintain the CSC phenotype and enhance tumorigenicity [102].

3.1.1 LncRNAs and stemness regulation in CSCs

LncRNAs maintain the stem-like properties of CSCs by regulating key signaling pathways involved in self-renewal and differentiation. For example, HOTAIR, MALAT1, and H19 are among the most studied lncRNAs in CSCs [102]. These lncRNAs promote the expression of stemness-related genes by having interactions with other factors. LncRNAs function as "miRNA sponges," a well-known mechanism that prevents miRNAs from binding to their target mRNAs [103]. This regulatory process can increase the expression of stemness-related transcription factors such as Sox2 and ZEB1/2 [102]. For instance, MALAT1 promotes the activation of the Wnt/β-catenin signaling pathway, which is essential for the maintenance of stemness in various cancers, including liver cancers [104]. Similarly, the lncRNA H19, which is involved in the regulation of miR-675, modulates the expression of stemness factors in various CSCs [105].

3.1.2 LncRNAs in epithelial-mesenchymal transition (EMT) in CSCs

EMT is a crucial process for CSCs giving them migratory and invasive properties, contributing to metastasis. LncRNAs are key regulators of EMT in CSCs, and their expression levels are often linked to cancer progression. For example, HOTAIR, MALAT1, and MEG3 promote EMT by enhancing the expression of key transcription factors such as ZEB1/2 and Twist. These transcription factors drive the loss of epithelial markers and the gain of mesenchymal markers, which are hallmarks of EMT [106]. LncRNA HOTAIR is upregulated in CSCs and induces EMT. It promotes tumorigenesis and metastasis [107, 108]. Additionally, lncRNAs such as linc00617 have been identified as critical regulators of EMT, acting through the activation of Sox2 transcription and promoting cancer progression and metastasis [109].

3.1.3 LncRNAs and chemoresistance in CSCs

CSCs are key factors of chemoresistance. Chemoresistance remains one of the major challenges in cancer treatment. LncRNAs have been implicated in the regulation of chemoresistance by modulating the expression of genes involved in drug efflux, apoptosis resistance, and DNA repair. For example, lncRNAs such as H19 and MACC1-AS1 are effective in drug efflux, thus promoting resistance to chemotherapeutic agents. They enhance the expression of ATP-binding cassette (ABC) transporters [110]. Additionally, lncRNAs like LET, MEG3, and GAS5 counteract chemoresistance by regulating apoptosis and reducing stemness [102]. In bladder cancer, for instance, the lncRNA LET is downregulated by TGF-β1 signaling, leading to enhanced stemness and resistance to gemcitabine [111]. These findings underscore the potential of targeting specific lncRNAs to overcome chemoresistance in CSCs.

3.1.4 LncRNAs and tumor microenvironment interactions

The tumor microenvironment (TME) plays a critical role in the regulation of CSC behavior, and lncRNAs are critical in the interaction between CSC and TME.

Several lncRNAs, including HOTAIR, MALAT1, and H19, are upregulated in CSCs by secreted factors from the TME, such as TGF-β1 [112]. This cytokine, secreted by various cell types within the TME (e.g., cancer-associated fibroblasts and macrophages), promotes stemness, EMT, and metastasis [102]. In gastric cancer, TGF-β1 secreted by mesenchymal stem cells induces the expression of MACC1-AS1, which enhances CSC properties and chemoresistance [110]. Additionally, lncRNAs such as H19 and Sox2ot, which are packaged into exosomes, facilitate communication between CSCs and other cells in the TME, resulting in the support of stemness and EMT [113]. These findings highlight the intricate interplay between CSCs and the TME and the potential of targeting lncRNA-mediated pathways to disrupt CSC functions.

3.1.5 LncRNAs and immune escape in CSCs

CSCs are known to regulate immune suppression within the TME. LncRNAs contribute to immune escape by regulating immune checkpoint molecules, cytokine production, and antigen presentation. CSCs express immune checkpoint molecules such as PD-L1 and Galectin-3, suppressing T-cell activity and promoting immune tolerance [114]. Additionally, lncRNAs like TUC339 and AFAP1-AS1 have been shown to modulate the immune response by influencing the function of immune cells such as myeloid cells and T cells [115, 116]. The release of lncRNAs in exosomes from CSCs further contributes to immune suppression by dampening the inflammatory response. As a result, lncRNAs represent potential therapeutic targets for overcoming immune escape and improving the efficacy of cancer immunotherapies.

3.2 Oncogenic and tumor suppressor LncRNAs

Depending on their cellular context, lncRNAs can act as either oncogenes or tumor suppressors. HOTAIR, a well-known oncogenic lncRNA, promotes breast cancer metastasis by enhancing tumor cell invasion. Overexpression of HOTAIR is associated with poor prognosis in breast cancer, and its knockdown significantly inhibits invasiveness [11]. On the other hand, lncRNAs such as Xist function as tumor suppressors. Knockdown of Xist in hematopoietic cells led to aggressive myeloproliferative neoplasm and myelodysplastic syndrome, suggesting that Xist plays a vital role in preventing genomic instability and cancer development [117]. lincRNA-p21, regulated by the tumor suppressor p53, also is a tumor suppressor lncRNA. It interacts with hnRNP-K to mediate gene repression, which is essential for maintaining cellular homeostasis in response to DNA damage [118].

3.3 LncRNAs in epigenetic reprogramming in cancer

Epigenetic changes have a key role in tumorigenesis and cancer, and lncRNAs have been identified as critical regulators of chromatin remodeling and gene expression. LncRNAs such as HOTAIR and ANRIL are involved in silencing tumor suppressor genes by recruiting polycomb repressive complexes (PRC2) to chromatin. HOTAIR, for instance, promotes tumorigenicity in breast cancer by altering PRC2 localization, which correlates with increased cell invasiveness [119]. Similarly, ANRIL targets the p15 locus, inhibiting its expression and contributing to cancer cell proliferation [120]. SChLAP1, another lncRNA, promotes metastasis by antagonizing the SWI/SNF chromatin-remodeling complex, the importance of lncRNAs in the epigenetic reprogramming of cancer cells [121].

3.4 LncRNAs in microvascular invasion and angiogenesis

The formation of new blood vessels known as angiogenesis, is essential for tumor growth and metastasis. Recent studies have highlighted the role of lncRNAs in promoting angiogenesis. MVIH, a lncRNA associated with microvascular invasion in hepatocellular carcinoma, is overexpressed in tumors and correlates with poor prognosis [122]. MVIH facilitates angiogenesis by inhibiting phosphoglycerate kinase 1, a key enzyme involved in energy metabolism, thus enhancing tumor growth [120, 123]. Additionally, MEG3 has been shown to regulate angiogenesis through p53 signaling [124]. Loss of MEG3 contributes to tumor progression by enhancing the migration and invasion [125].

3.5 Clinical implications of LncRNAs in cancer therapy

LncRNAs are considered potential therapeutic targets. Several lncRNAs have been linked to poor prognosis, chemoresistance, and metastasis in various cancers, making them promising biomarkers for early detection and monitoring. Targeting lncRNAs with antisense oligonucleotides (ASOs) or CRISPR/Cas9 technology has shown promising results in preclinical models, suggesting that lncRNA-based therapies could inhibit CSC functions and improve the treatment process [126–130]. Additionally, the stability of lncRNAs in body fluids, such as serum and plasma, makes them excellent candidates for non-invasive biomarkers [131]. Identifying and targeting specific lncRNAs involved in CSC biology may lead to the development of novel therapeutic strategies that target the root causes of cancer progression and resistance.

4. Conclusions

Long non-coding RNAs (lncRNAs) have emerged as essential regulators in stem cell biology, modulating gene expression at epigenetic, transcriptional, and post-transcriptional levels. Their ability to interact with chromatin, transcription factors, and RNA-binding proteins underscores their functional diversity and specificity. As critical modulators of pluripotency and differentiation, lncRNAs like H19, MALAT1, and linc-ROR exemplify their roles in maintaining stem cell identity and directing lineage commitment. Despite their immense potential, much remains to be uncovered about their context-specific mechanisms and therapeutic applications. Emerging tools like single-cell, spatial transcriptomics, and CRISPR-based functional studies are promising in deepening our understanding of lncRNA biology. Furthermore, therapeutic approaches of lncRNA-targeting technologies, including antisense oligonucleotides and synthetic lncRNAs, could make a great progression in regenerative medicine and cancer therapies. Addressing challenges in delivery, specificity, and safety will be crucial for clinical translation. By integrating bioinformatics, advanced technologies, and collaborative efforts, lncRNAs can unlock new opportunities for precision medicine and advancements in stem cell research and therapeutic uses.

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Author contributions

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Conflict of interest

The authors declare no conflict of interest.

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Section 4

Long Non-Coding RNA's in Plants

Chapter 9

Perspective Chapter: Long Non-Coding RNAs in Plants – Unlocking Hidden Regulators for Stress Tolerance and Crop Resilience

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Abstract

Long non-coding RNAs (lncRNAs) are emerging as crucial regulators in plant biology, particularly under abiotic and biotic stress conditions. These molecules, which lack protein-coding potential, exhibit diverse roles in regulating gene expression at transcriptional, post-transcriptional, and epigenetic levels. Recent advancements reveal their involvement in critical stress responses, including drought, salinity, extreme temperatures, and heavy metal toxicity, highlighting their potential in enhancing crop resilience amidst changing climatic conditions. Additionally, lncRNAs influence chromatin remodeling, histone modifications, and DNA methylation, underlining their role in epigenetic regulation. High-throughput sequencing technologies and computational tools have significantly advanced the identification and functional annotation of lncRNAs across various plant species, paving the way for innovative strategies in crop improvement programmes. This chapter explores the evolution, classification, functional mechanisms, and stress-specific roles of lncRNAs, providing a comprehensive understanding of their applications in sustainable agriculture.

Keywords: lncRNAs, abiotic stress, biotic stress, gene regulation, climate resilience

1. Introduction

In the twenty-first century, the global challenge of food scarcity persists and is exacerbated by the profound effects of climate change [1]. It is estimated that the world population will grow to 10.6 billion people by 2050, increasing concerns about food security [2]. However, severe unfavorable climatic conditions such as drought, high temperatures, unpredictable rainfall, salinity, and the increase in CO_2 levels in the atmosphere are the main causes of impeded crop growth and reduced food production. These stress factors lead to changes in plant physiology, including altered photosynthetic efficiency, nutrient uptake, and water-use efficiency, which in turn lead to lower yields [3, 4].

The increase in atmospheric CO_2 levels and temperature leads to a decrease in various essential nutrient compositions such as the protein, vitamin, and mineral content of crops, resulting in malnutrition, which affects human health and is the cause of various diseases [5]. In the current scenario, several studies have found that cultivation of staple crops such as wheat, rice, and maize under various abiotic stress conditions leads to deterioration in the composition of various essential nutrients such as protein, zinc, and iron by reducing the photosynthetic process and down-regulating the expression of various genes in the plants that are necessary for increasing the nutrient content in the food crops. The decline in nutrient quality poses a significant risk to global food shortages.

To counteract the disadvantages of climate change, the scientific community has focused on developing new molecular genetic approaches to climate change resilience. There are many unanswered questions about how plants adapt to stress conditions by altering the expression of different genes in a spatio-temporal manner by regulating biological functions. In this context, various genes have been altered by overexpression and silencing using gene transformation and gene modification to improve their ability to regulate stress. In addition, unknown intergenic spaces between protein-coding genes are found by analysing unclear "experimental artefacts" or "junk DNA" using sequencing technologies such as transcriptome analysis [6].

The non-coding RNAs or non-protein-coding RNAs represent a diverse class of transcripts that differ in their mechanism and genetic genealogy and include both regulatory non-coding RNAs and housekeeping RNAs [7]. Among regulatory ncRNAs, two major types are classified based on nucleotide length: (i) long noncoding RNAs (lncRNAs), which are predominantly over 200 nucleotides in length, and (ii) small noncoding RNAs, which are approximately 50–200 nucleotides in length, including small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), and microRNAs (miRNAs) [8, 9]. The functional significance of lncRNAs is still uncertain because, in contrast to the well-characterized mRNAs and miRNAs, they show only low expression and are poorly conserved in different species [10, 11]. In plants, long non-coding RNAs (lncRNAs), which help to increase plant production and improve nutrient content by regulating the expression of various genes at different levels, including transcription, epigenetics, and post-transcription, play a role in plant survival by combating various stress conditions [12–14]. Nevertheless, extensive research has uncovered the pivotal roles lncRNAs perform in numerous essential biological processes in plants, including the maintenance of cellular structural integrity, heat shock response, cell cycle progression, genomic imprinting, regulation of splicing, translation, and transcription. Under condition of climate change, these lncRNAs plays vital role for crop improvement programmes in plants with several facets of plant biology by examining complex regulatory networks of stress-responsive lncRNAs adaptation to a dynamic and challenging environment.

2. History/discovery for evolution of lncRNA in plant

In RNA biology of eukaryotes, plant biology has a long history of contributing important findings (**Figure 1**), such as the first reports of stress-induced RNA-protein cytoplasmic aggregates and posttranscriptional gene silencing. During the era of 1990s and 2000s, to evaluate the function of lncRNAs researchers have significantly used approaches such as traditional gene targeting. During the early of 1991, the X Inactive Specific Transcript (XIST) plays a role in controlling the inactivation of the

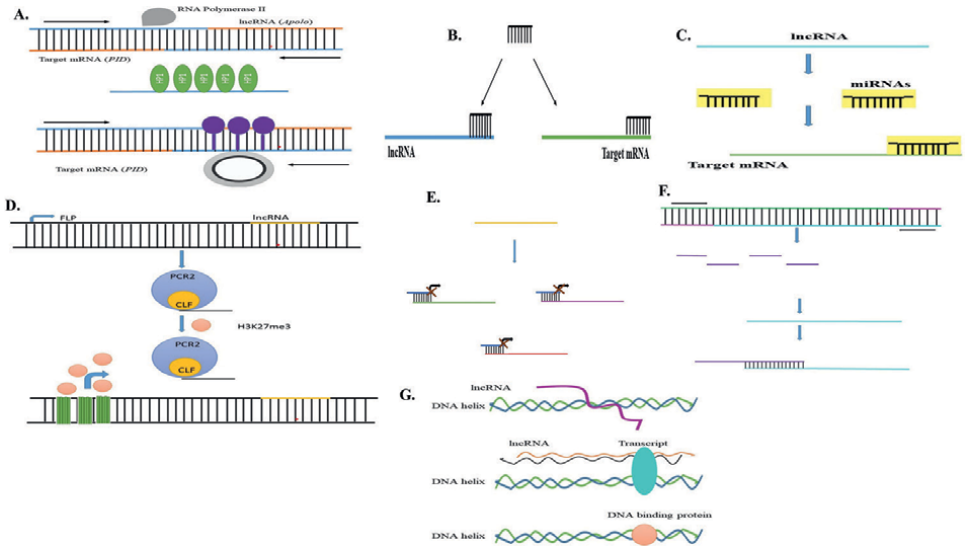

Figure 1.
Biotic and abiotic stress responses and the regulatory functions of pant lncRNA (A) DNA methylation, (B) miRNA mimics, (C) histone modification, (D) miRNA precursor, (E) cis-acting factor, (F) trans-acting factor, and (G) chromatin organization modifications.

X chromosome the first lncRNA, which was discovered in animal cells [15]. In 1999, Kouchi et al. discovered that rice (*Oryza sativa* L.) has the first lncRNAs found in plants [16]. This led to the discovery of numerous lncRNAs in other plant species, including Arabidopsis (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*) [17].

Many long non-coding RNAs (lncRNAs) had already been recognized as essential regulators in a wide range of cellular processes before the genomics era and the historic sequencing of the *Arabidopsis thaliana* genome in 2000 (Arabidopsis Genome Initiative 2000). Currently, among 1239 organisms, the NONCODE v3.0 database, which compiles lncRNAs documented in scientific literature, has identified 73,370 lncRNAs. However, only a small fraction fewer than 200 have known functions [18]. The discovery of the initial long non-coding RNAs (lncRNAs) was made possible by advancements in molecular biology and genetic analysis tools. Of ncRNAs, these groundbreaking innovations empowered researchers to uncover previously neglected genomic elements that do not code for proteins but are pivotal in orchestrating gene expression, modulating chromatin dynamics, and regulating fundamental cellular processes. During the year of 2000, researchers identified lncRNA in different five plant spp. such as *Arabidopsis*, tomato, tobacco, cucumber and *Medicago truncatula* related to genes such as *GENE WITH UNSTABLE TRANSCRIPT 15 (GUT15)*, *TOMATO PHOSPHATE STARVATION INDUCED 1 (TPSII), GENE WITH UNSTABLE TRANSCRIPT 15 (GUT15)*, *CYTOKININ REPRESSED 20 (CR20),* and *EARLY NODULIN 40 (EN40)*, respectively. Among these genes related to lncRNAs, some lncRNAs such as CR20 and GUT15 are conserved among angiosperms but have some limitations. These lncRNAs have been identified as alternatively spliced and regulated by hormones [19, 20]. In addition, scientists have also identified lncRNAs in legumes that are related to ENOD40, which play an important role in root nodulation [21]. Root nodulation in legumes is influenced by ENOD40, the first lncRNA to be independently identified in both soybean (*Glycine max*) and *M. truncatula*. The

first lncRNAs identified under environmental stress by transcriptomic approaches analysed their widespread expression. Two lncRNAs, namely MT4 and TPSI1, which respond to phosphate starvation and are conserved in dicotyledonous plants, have been identified by researchers in response to nutrient stress [22]. These studies, particularly in model plants, marked the beginning of research into lncRNAs in plants and their role in regulatory networks.

3. Type of LNCRNAs

Long non-coding RNAs (lncRNAs) function as *riboregulators*, a diverse class of RNA transcripts transcribed by RNA polymerase II or III. Unlike mRNAs, lncRNAs lack protein-coding potential, are typically over 200 nucleotides in length, and are found within the cytoplasm or nucleus. These molecules are integral to various regulatory processes, influencing gene expression, chromatin structure, and cellular signaling pathways [23, 24]. The progenitors of small interfering RNAs (siRNAs) are Pol IV lncRNAs [25]. Both strands of the protein-coding locus can produce Pol V-dependent lncRNAs, which help to modify the local chromatin loop, expressing themselves in a "tissue-specific" way [26, 27]. Long noncoding RNAs (lncRNAs) originate from dedicated promoters as well as from promoters shared with various other transcriptional elements. These origins provide lncRNAs with versatile regulatory potential, allowing them to modulate gene expression networks through diverse transcriptional landscapes [28].

LncRNAs are majorly divided into three categories based on mechanism of action, location of genome, and effect of DNA. Based on their genomic positioning relative to nearby protein-coding genes, lncRNAs can be broadly classified into: (i) long intergenic non-coding RNAs (lincRNAs), (ii) circular long non-coding RNAs (circRNAs), (iii) intronic non-coding RNAs (incRNAs), (iv) natural antisense transcripts (NATs), and (v) each category reflects distinct biogenesis pathways and functional roles, enabling lncRNAs to orchestrate complex layers of gene regulation across the genome [12, 29]. Moreover, according to their mode of action, these lncRNAs can further be categorized based on their specific roles in gene regulation. Some lncRNAs function by interacting with chromatin to modulate gene expression, while others regulate RNA processing or act as scaffolds for protein complexes that influence cellular processes.

Additionally, lncRNAs can influence how pre-mRNAs are spliced into different forms. They can also act as natural decoys, competing with various miRNAs, and can serve as sources for siRNAs and miRNAs [30, 31]. In contrast, the back-splicing of internal exons within pre-mRNA culminates in the generation of circular RNAs (circRNAs), a relatively rare and distinct class of RNA molecules that exhibit exceptional stability and pivotal regulatory functions. Due to their covalently closed-loop structure, circRNAs are impervious to exonuclease degradation, thus conferring them a remarkable resilience. These unique molecules are integral to a variety of biological processes, including the fine-tuning of gene expression, modulation of splicing events, and the formation of complexes with RNA-binding proteins, thereby influencing cellular homeostasis and signalling pathways [32]. These circular lncRNAs serve diverse functional roles within cellular processes also known as scaffolding backbones, sponge molecules, guiding agents, molecular decoys, and signaling molecules. Natural Antisense Transcripts (NATs), a distinct category of lncRNAs, exhibit both cis- and trans-regulatory functions. These transcripts are derived from complementary DNA strands of sense-coding regions, where they exert profound influence on gene expression through mechanisms such as

transcriptional interference, epigenetic modulation, and post-transcriptional regulation [33]. Additional functions of lncRNAs encompass modulating the alternative splicing of pre-mRNAs, serving as endogenous target mimics (eTMs) that compete with various miRNAs, and acting as precursors to miRNAs and siRNAs [26]. From the different regions, intronic regions were used for transcribe incRNAs, whereas intergenic regions developed LincRNAs. These generated lncRNAs engage in trans-regulatory functions, modulating distant genes, and display tissue-specific expression patterns. They are polyadenylated but exhibit inefficient splicing [34].

Based on their genomic positioning, these lncRNAs are primarily classified into five subcategories: bidirectional lncRNAs, antisense lncRNAs, sense lncRNAs, intergenic lncRNAs, and intronic lncRNA. In the space between two genes, intergenic lncRNA is developed also known as large intervening ncRNAs (lincRNAs) or large interventional ncRNAs [35]. The transcription initiation site of the protein-coding gene on the antiparallel strand, oriented in the reverse direction, lies in striking proximity to the commencement site of bidirectional lncRNA transcription [36]. The space between two genes produces intergenic lncRNA. Meanwhile, the mRNA precursor sequence or secondary transcript's intron region is the source of intronic lncRNAs. The exon stages of another protein-coding gene on the opposite or same strand coincide with antisense or sense lncRNAs, respectively [37].

Wang and Chekanova [38] delineated lncRNAs into four primary archetypes based on their modes of action: (1) guide molecules: RNA-binding protein (RBP) complexes can be recruited to particular genomic loci by lncRNAs that coordinate their localisation to specific target sites or interact with chromatin remodeling complexes to modify chromatin dynamics [36]; (2) signaling molecules: The expression of numerous genes is orchestrated by specific long non-coding RNAs (lncRNAs) that function as pivotal regulators in signalling pathways that govern these intricate regulatory mechanisms. These lncRNAs, often referred to as signalling lncRNAs, play a crucial role in modulating gene expression by acting as molecular beacons or mediators within cellular communication networks; (3) skeleton molecules: By interacting with protein complexes or other effector molecules, lncRNAs can function as molecular scaffolds, facilitating intricate molecular interactions that fine-tune the precision of signalling processes. These lncRNAs play a critical role in modulating the specificity and efficacy of signalling pathways, acting as essential mediators that influence cellular responses and ensure the accurate transmission of regulatory signals across biological systems; and (4) bait molecules: By binding several proteins such as RNA binding protein to particular targets, these LncRNAs that are involved in the recruitment of RNA-binding proteins act as crucial modulators in cellular processes [39].

4. Features and characteristics of lncRNA

An alternative site for splicing, a 3′ polyadenylated tail, and a 5′ cap m7G caps are structural traits that mRNAs and lncRNAs have in common. These characteristics of lncRNA highlight the diversity between these RNAs in terms of function and allow them to play a variety of functions in gene regulation. The cytoplasm and nucleus are sites for synthesis of lncRNAs, which have a significant impact on cellular homeostasis and signaling pathways by influencing RNA processing, control of transcriptional process, and post-transcriptional regulation [40]. In contrast to mRNA, lncRNA has been identified more abundantly expressed, less conserved, and fewer site of exons. In contrast to mRNA, lncRNA exhibits higher levels of expression, reduced

evolutionary conservation, and a more limited number of exonic regions [41]. Their unique features of lncRNAs show their role in protein coding and reflect their adaptability in orchestrating complex gene regulatory networks and modulating cellular processes across diverse biological contexts [42, 43]. Cellular organization and regulation, encompassing processes such as cell growth, RNA transcription, DNA replication, cell differentiation, and protein translation, is significantly influenced by lncRNAs [44]. These molecules often exhibit the capacity to adopt highly stable secondary and tertiary structures, enabling them to act as critical modulators within these intricate biological systems. Several researchers have observed that the folding of the secondary structure of long non-coding RNA (lncRNA) is significantly influenced by environmental conditions and cellular positioning. This intricate process occurs with remarkable efficiency, requiring minimal energy expenditure [45]. The enhanced nuclear retention of lncRNAs is closely associated with an extended distance between the 3′ splice site and the branch point, coupled with attenuated internal splicing signals [46]. Furthermore, the accumulation of lncRNAs within the nucleus is driven by additional factors, including differential expression of key splicing regulators, which intricately modulates the splicing machinery and its efficiency [47]. Distinct patterns of lncRNA distribution have been documented across the genomes of various plant species. In grapevines, lncRNAs exhibit a notably uneven chromosomal distribution, contrasting with their uniform distribution across the maize genome [48]. In maize, however, a marginally reduced density of lncRNAs is observed on chromosome 1, reflecting subtle genomic variation [49]. Several types of RNA molecules such as ceRNA, mRNA, and miRNA can also interact with lncRNAs, another kind of post-transcriptional regulation. According to ceRNA's prior description, several kinds of lncRNA can interact to change the amount of gene expression or transcription isoforms [50].

5. Techniques for identification of lncRNA in plant

The high-throughput sequencing technologies and computational bioinformatics approaches used by researchers to identify role of several genes, and transcription factors plays vital role in plant growth and development [51]. In genome sequence, RNA transcripts are used for identification of lncRNAs after those with large open-reading frames and/or are eliminated, along with those that exhibit traits of mRNAs and other ncRNAs (such as and snRNA) filtered and remaining non-coding RNAs in transcriptomics sequence identified as a lncRNAs [52]. In current scenario, the biotechnological approaches such as the lncRNA microarray technique, RNA-sequencing (RNA-Seq), and degradome sequencing are widely used by scientific community for identification of molecular mechanism behind the plant behavior in stress condition [53]. Among these approaches, the use of next-generation sequencing techniques for transcriptomic or RNA-sequencing is highly used for the finding of lncRNAs [54]. These approaches not completely identify all lncRNAs in plants due to their several unique features such as due to the intricacy of these molecules to code several peptides or lengthy open-reading frames that are not translated [55, 56]. The advancement of biological experimentation hinges on the precise prediction of non-coding peptides (ncPEPs), as this greatly conserves both time and resources. Accurate ncPEP identification streamlines experimental workflows, enabling targeted approaches that optimize efficiency and reduce expenditure, while minimizing the potential for erroneous classification of these molecules as coding genes [55]. For example, earlier

Type of modification	No. of plant species located and identified lncRNAs	Identified monocot plant species	Total identified Eudicotes
AlnC	678	21.7%	69.6%
GreeNC v2.0	93	32.3%	57%
PLncDBv2.0	80	20%	65%
CANTATAdb v2.0	39	—	—
LncPheDB	9	55.6%	44.4%
NONCODE v6.0	23	21.7%	69.6%

Table 1.
Statistics of different database for lncRNA in plants.

Type of modification	Modification site	Transcriptional effect	Group
Acetylation	H2A/H2B, H3, H4	Activation	Methyl
Biotinylation	H4, H3, H2A	Repression	Biotin
Sumoylation	H2A, H4, K17	Repression	SUMO
Phosphorylation	H3	Activation	Phosphate
Ubiquitination	H2A and H2B	Repression and activation	Ubiquitin
Acetylation	H2A/H2B, H3, H4	Activation	Methyl

Table 2.
Effect of transcription through various ways of alteration to histones.

studies anticipated that, within the database of the model plant *Arabidopsis thaliana* (Araport11), certain protein-coding transcripts were misidentified [57, 58]. Notably, the auxin-regulated *APOLO* (Auxin-Regulated Promoter Loop Induced by Phosphate Starvation) [59] and *IPS* (lncRNA IPS), initially classified as protein-coding, were later confirmed to be long non-coding RNAs (lncRNAs) [60, 61]. Despite these challenges, for accurate prediction of lncRNAs in plants from next-generation sequencing data, it is essential to collect diverse transcriptome or RNA sequencing data to capture plant response changes across time, developmental stages, and species-specific regulation, ensuring robust and comprehensive identification [62].

In plant species, two primary strategies are predominantly employed for the annotation and localization of long non-coding RNAs (lncRNAs): (1) prediction of non-coding conserved regions and (2) comparative genomics [10]. A novel and promising approach involves leveraging machine learning to detect lncRNAs through various bioinformatics tools, such as LncADeep [63], CPAT [64], PLEK [65], CPC2 [66], LncFinder [63], and CNCI [67] (see **Table 1**).

These tools leverage sequence and structural features of transcripts, employing advanced computational models such as support vector machines (SVMs) and logistic regression algorithms to robustly assess coding potential and distinguish between coding and non-coding RNAs [68]. For the advancement of lncRNAs prediction in plants need of developing robust ensembles techniques and retraining popular algorithms. A wide array of publicly accessible databases and web-based analytical platforms are available to facilitate advanced research on long non-coding RNAs (lncRNAs), encompassing curated datasets from over 900 plant species, each featuring transcripts exceeding 200 nucleotides in length (see **Table 2**).

6. Modes of action of lncRNAs in plants

By developing complex systems to coordinate exact temporal and spatial patterns of gene expression in response to both internal developmental cues and external environmental stimuli, plants have effectively adapted to their ecological niches [69]. Epigenetic regulation, encompassing mechanisms and the actions of noncoding including histone variants, chromatin remodeling, histone modifications, and DNA methylation, represents a cornerstone of intricate and finely tuned biological processes. In recent decades, plant epigenetics has emerged as a pivotal research frontier, driven by its profound implications for tackling global challenges such as food security and agricultural productivity [70]. In plants, researchers identified involvement of long noncoding RNAs (lncRNAs) in a diverse range of biological processes over the last 10 years [31, 71]. In plants, lncRNAs play a crucial role in combating the challenges posed by climate change such as salinity, temperature fluctuation, and drought stress as well as regulating growth and developmental processes from germination to fruit development [72, 73]. In climate resiliencies, sustainable agriculture, and food scarcity, plant epigenetics study plays a vital role for gained prominence [74]. The interplay between epigenetic modifiers and other epigenetic factors in regulating gene transcription and long non-coding RNAs (lncRNAs) in plants remains insufficiently understood [75]. This gap in knowledge highlights the need for further research to unravel the intricate network of interactions that govern plant gene regulation. lncRNAs involved in various epigenetic regulation process include lncRNA-RNA interactions, chromatin remodeling, splicing regulation, R-loop formation, and DNA methylation [76].

6.1 DNA methylation

In plants, the enzyme DNA methyltransferases (DNMTs) involved in process of DNA methylation an essential epigenetic modification [77, 78]. In tobacco plants, the phenomenon of DNA methylation mediated by RNA molecules was first documented in 1994. This pivotal discovery revealed the intricate interplay between RNA and epigenetic regulation, highlighting RNA molecules as key mediators in guiding sequence-specific DNA methylation. This process also known as RNA-directed DNA methylation (RdDM) plays a vital role in transfer of genetic information from DNA to RNA [79]. RNA-directed DNA methylation (RdDM) plays a pivotal role in regulating a multitude of mechanisms that enhance a plant's adaptability and resilience to fluctuating environmental conditions and pathogen attacks. By directing sequence-specific DNA methylation, RdDM modulates gene expression, fortifies genome integrity, and activates defense pathway [80].

In the process of DdRPs, many enzymes are involved to regulate the process such as Dicer-like protein 3 (DCL3), RNA-dependent RNA polymerase 2 (RDR2), Pol IV, and Pol V [81, 82]. The ncRNA-directed DNA methylation (RdDM) pathway plays a pivotal role in orchestrating *de novo* DNA methylation, encompassing the regulation of asymmetric CHH methylation. This sophisticated epigenetic mechanism employs non-coding RNAs to target specific genomic loci, enabling the establishment and maintenance of precise methylation patterns [83]. Both distinct yet interdependent roles are used in the synthesis of siRNA and ncRNA scaffolds, with Pol II mediating critical steps in this intricate process. Within this framework, long non-coding RNAs (lncRNAs), derived from siRNA precursors, function as precise molecular guides to facilitate targeted DNA methylation in plants [84].

Both Pol V and Pol IV play distinct yet interdependent roles in the biogenesis of lncRNA and siRNA 24 nt, with Pol II mediating critical steps in this intricate process. Within this framework, long non-coding RNAs (lncRNAs), derived from siRNA precursors, function as precise molecular guides to facilitate targeted DNA methylation in plants [85]. Double-stranded RNA (dsRNA) is meticulously synthesized from single-stranded RNA (ssRNA) templates through the synergistic interplay of RDR2 and Pol IV. The strong production of dsRNA molecules is the result of this complex process, which begins with Pol IV starting the transcription of ssRNA and is subsequently continued by the catalytic accuracy of RDR2 to produce complementary RNA strands [86, 87]. The hypothesized ATP-dependent chromatin remodelling factor CLSY1 (CLASSY1) is likely to mediate or catalyse this process. Following DICER-LIKE 3 (DCL3) protein cleavage of this dsRNA, 23 nt and 24 nt siRNAs are produced in which 23 nt siRNA acts as passenger strand and subsequently loaded onto an ARGONAUTE protein, specifically AGO4 (sometimes AGO6) to develop the complex of AGO4-siRNA [88].

IN DE NOVO 2 (IDN2) protein is hypothesized to stabilize and reinforce the duplex formation between siRNA and lncRNA, with its engagement transpiring subsequent to the assembly of the AGO4-siRNA complex. The *de novo* methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) recruitment efficiency is enhanced by this stabilization process. In all cytosine settings (CG, CHG, CHH; where H stands for A, T, or C), DRM2 is in charge of putting methylation marks at particular genomic locations [89].

6.2 Chromatin remodeling by lncRNA

The course of the cell cycle in plants, which is intricately regulated by developmental and environmental cues, relies on chromatin dynamics. In the cell nucleus, the genetic material is derived from chromatin, a complex of DNA and histone proteins that intricately organize into chromosomes during cell division [90]. For proper molecular mechanism, processes such as recombination, plant transcriptional silencing, genome replication, and DNA repair rely on the chromatin structure [91]. The previous studies highlighted the critical role of lncRNAs as key regulators of protein-coding genes, expression of genes, and functioning as influence of both trans- and cis-acting activities [92].

It has been determined that about 38% of the cell's lncRNAs play a crucial role in chromatin remodeling. They accomplish this by coordinating transcriptional suppression and epigenetic regulation by building complexes with the polycomb repressive complex 2 (PRC2) or chromatin-modifying proteins such as SMCX and CoREST [93]. Priming, intricately linked to chromatin modifications and capable of persisting independently of active gene transcription, equips plants with an extraordinary ability to "memorize" prior stress experiences. This adaptation enables them to withstand extreme environmental fluctuations by orchestrating more robust and efficient responses when re-exposed to similar stressors. The three dynamic features of nucleosomes reconstruction, covalent alteration mediated by enzymes, and repositioning are essential for the fundamental mechanism of chromatin remodeling [94]. The remodeling of chromatin mainly relies on nucleosome properties repositioning, reconstruction, and enzyme-induced covalent modification. During chromatin reconstruction, the SWR1 histone-exchange complex facilitates the incorporation of histone variants by replacing canonical histones. The newly formed variant then recruits certain unique regulators (Reg) to regulate a number of biological processes, such as centromere construction and gene activation.

The process of chromatin remodeling occurs mainly *via* two ways (1) ATP-dependent chromatin remodeling and (2) covalent histone modification. The process of chromatin remodeling occurs mainly *via* three ways: (1) ATP-independent chromatin remodeling, (2) ATP-dependent chromatin remodeling, and (3) covalent histone modification. Chromatin remodelling can also occur through ATP-independent mechanisms, which do not rely on energy from ATP hydrolysis. In such cases, histone dissociation from chromatin is facilitated by histone chaperones or DNA-binding transcription factors that aid in nucleosome reorganization.

In ATP-dependent chromatin remodeling, structure of chromatin altered by the use of hydrolysed ATP'S energy and ATPase includes *BRG1*, *BRM*, *CHD1*, *CHD3*, *CHD4*, *Mi-2*, *SWR1*, *SNF2L*, and *SNF2* [95]. The process begins when the complex identifies a specific target region on chromatin, often guided by transcription factors or DNA-binding proteins [96]. Through the activity of ATPase subunits such as Snf2, the complex disrupts histone-DNA interactions, enabling three main outcomes: nucleosome sliding (to modulate accessibility by repositioning nucleosomes along DNA), nucleosome ejection (removing nucleosomes entirely), or histone exchange (replacing histones with variants) [97]. These modifications are pivotal for regulating fundamental nuclear processes such as DNA accessibility, facilitating or repressing transcription, replication, DNA repair, or recombination. In this process, nucleosomes repositioning regulated by complex of remodelers use hydrolysed ATP's energy (12–14 kcal mol^{-1}). In addition, this process also involves the replacement or removal of H2A–H2B dimers, as well as the potential ejection of entire histone octamers from nucleosome core [98]. Such modifications significantly influence the chromatin landscape, dynamically balancing the interplay between chromatin compaction and accessibility, thereby ensuring precise and context-specific regulation of genomic functions.

6.3 Histone modification mediated by lncRNAs

The fundamental structure of chromatin is established through the intricate coiling of nucleic acids (DNA) around nucleosomes that are protein complexes consisting of histone octamers. These octamers comprise two copies each of the histones H2A, H2B, H3, and H4. Numerous stages of plant development, including seed germination, hypocotyl elongation, and the onset of flowering, are intricately regulated by dynamic alterations in histone modifications. These chromatin-based changes play a pivotal role in controlling gene expression, thus driving the precise timing and coordination of key developmental processes [90]. The modifications of histone are essential in the regulation of gene expression because they influence the structure of chromatin, the material that makes up chromosomes [99]. Chromatin structure is a key factor in determining whether a gene is accessible for transcription or not. Histones are proteins around which DNA is wrapped, and chemical modifications to histones can either loosen or tighten this wrapping, affecting the ability of transcription factors and other regulatory proteins to access the DNA [100]. By altering chromatin structure and controlling the recruitment of transcriptional complexes, histone alterations coordinate the transcription of genes. The particular histone variation, the exact amino acid residue targeted, and the type of chemical modification used all influence the transcriptional result, whether it be activation or repression [101]. Under the catalytic influence of specialized enzymes, histones are subject to a broad spectrum of post-translational modifications, including acetylation, phosphorylation, methylation, and ubiquitination (see **Table 2**) [102]. These intricate and

dynamic alterations are indispensable for orchestrating gene expression, either by facilitating transcriptional activation or enforcing repression, while simultaneously governing chromatin architecture with precision.

7. Functional implications of lncRNAs in climate resilience for plants

LncRNAs are increasingly recognized for their role in enhancing plant resilience to climate change-induced stresses. By regulating key genes involved in drought, metal stress, salinity, and heat tolerance, lncRNAs allow plants to adjust their physiology by regulating heat shock proteins, controlling ion transporter genes enhancing efficiency of water uptake, and maintaining osmotic balance protecting cellular machinery of plant to improve various resilience.

7.1 lncRNAs associated with abiotic stress in plants

Climate change is a critical global challenge that significantly impacts crop nutritional quality, stunts growth, and hampers development, thereby exacerbating issues of food scarcity and malnutrition. Adverse climatic conditions, such as extreme temperatures, heavy metal toxicity, drought, salinity, cold stress, and nutrient deficiencies, drastically reduce crop yields. By surviving abiotic stresses such as drought, temperature swings, salt, and heavy metals, the algal lineage started terraforming the terrestrial ecosystem about half a billion years ago, allowing green life to thrive in a wide variety of habitats [103]. In response to these environmental stressors, plants activate intricate defense mechanisms, including the production of specialized proteins and enzymes, to mitigate damage and sustain growth. Furthermore, ncRNAs are essential for influencing how plants react to biotic and abiotic stresses, allowing for resilience and adaptation in harsh environmental circumstances. Their diverse roles highlight their importance in systems of stress tolerance and development [104]. In plants, role of lncRNAs as biological regulators helps to adapt biotic and abiotic stressors identified by previous studies and noted involvement of several transcription factors [105].

7.1.1 Response of LncRNAs in condition of temperature stress

Global warming has precipitated profound and unprecedented temperature anomalies across the globe. Recent summers have witnessed record-shattering heat, marking the highest temperatures observed in decades. Conversely, certain regions have endured exceptionally frigid winters, with temperatures plunging to historic lows. These temperature changes affect growth and development of plant and reduce year the round production.

7.1.1.1 Cold stress

An essential abiotic stressor that hinders plant growth and productivity is low temperature stress, which causes freezing and chilling injury. In plants, stress memory has evolved as a sophisticated adaptive strategy, enabling them to withstand recurring stressors with heightened resistance or tolerance. Following an initial exposure to environmental challenges such as drought, salinity, or pathogen attacks, plants undergo molecular, physiological, and epigenetic changes that "imprint" the

stress experience. To identify the molecular mechanisms behind plant tolerance to low temperatures, researchers are focusing on the discovery of quantitative trait loci (QTLs) and the expression profiling of genes under cold stress, leveraging advances in high-throughput sequencing technologies [14].

Cold stress is major reason to cause oxidative stress in plants by affecting antioxidant enzyme activity due to damage of cell membrane by the extravasation of osmotic substances, which affects the function of membrane localization proteins and downstream. By reducing the activity of antioxidant enzymes, cold stress significantly contributes to oxidative stress in plants. This happens because osmotic material leakage damages the cell membrane, impairing the integrity and functionality of proteins that are located in the membrane [106]. Plant health and resilience are further jeopardized as a result of this cascade's detrimental effects on downstream physiological and biochemical processes.

To ensuring the survival of plant in adverse environments, a diverse array of regulatory microRNAs (miRNAs) and transcription factors such as *C-repeat Binding Factors* (CBFs) regulate the expression of *Cold-Regulated* (COR) genes, essential for bolstering cold tolerance and synergistically enhancing the plant's resilience to chilling and freezing conditions [107]. Plant gene expression under cold stress is intricately regulated by transcription factors, notably those within the C-repeat Binding Factor (CBF) pathway. Key players, such as the inducer CBF expression 1 (ICE1), bind to specific promoter regions, initiating the transcription of cold-responsive genes [108]. To provide tolerance under cold stress conditions, signaling pathway of CBF plays vital role activated by activity of calmodulin-binding transcriptional activators (CAMTA3 and CAMTA5) and further regulated by CdWRKY2, which directly targets the CdCBF1 promoter that exerts regulatory control by directly binding to the CdCBF1 promoter [109]. To help plants endure freezing conditions, Plant U-Box 25/26 targets the R2R3-MYB transcription factor, a MYB domain protein 15, for degradation. This protein, which plays a role in the ICE-CBF pathway, acts as a negative regulator of transcription [110].

Furthermore, cold resilience in plants regulated by hormones and the reports noted that to enhance plant ability to survive in condition of cold stress. The genes COR47 and RD29A, associated with hormonal pathways, are instrumental in conferring cold tolerance [111]. Their expression is regulated by the binding of specific cis-elements, CACTTG and CACGTG, which are variants of the E-box motif. These motifs serve as binding sites for transcription factors such as Brassinazole-Resistant1 (BZR1) that plays a pivotal role in modulating the activity of cold-responsive promoters like CBF1 and CBF2. This intricate regulatory network underscores the interplay between hormonal signaling and cold stress responses. These coordinated mechanisms synergistically enhance the expression of cold-responsive genes, fortifying plant resilience against low-temperature stress.

7.1.1.2 lncRNAs in high temperature

Global warming becomes worse by industrialization and the overuse of fertilizers and pesticides, which causes temperatures to rise sharply worldwide. The rising temperate and heat wave retard crop productivity by negatively impact on its growth and development. To provide tolerance to stress by changes expression of genes and biochemical pathway, plants adapt several mechanism [112]. Heat stress causes extensive damage to plant cells by compromising membrane integrity and inducing protein denaturation by altering the synthesis of metabolites,

phytohormones, activity of enzymes, heat shock proteins (HSPs), and of heat shock transcription factor (HSFs) [112].

To provide tolerance towards varying temperature conditions, HSPs play a major role to and its expression regulated by the HSFs. HSFs are classified into three categories (1) HSFs class A; (2) HSFs class B, and (3). HSFs class C [113]. Notably, HSFs in classes C and B lack transcriptional activators functions. In contrast, transcriptional activation is primarily orchestrated by HSFA1, attributed to the presence of conserved amino acid motif residues. This activation profoundly influences the expression of other transcription factors, including MULTIPROTEIN-BRIDGING FACTOR 1C (MBF1C), DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A (DREB2A), and various heat shock transcription factors such as HSFBs and HSFAs (e.g. HSFA7 and HSFA2) [114]. These factors are integral to the heat stress response, initiating a cascade of gene expressions to mitigate cellular damage and ensure survival under thermal stress.

There are many studies available identified role of lncRNAs to provide tolerance against high temperature by regulating several molecular pathways. In response to high temperatures in this system, the lncRNA TCONS_00016454 displayed antagonistic expression patterns with its target, a BES1/BZR1 homolog, suggesting that it may be involved in providing heat tolerance *via* BR signaling in Chinese cabbage. Wang et al. identified the role of hormones specifically BR-related lncRNA TCONS_00016454 in Chinese cabbage providing tolerance against heat stress with targeting a homolog BES1/BZR1 [115].

In wheat heat stress is a major issue in pollen development due to affecting cell division at meiosis stage and recuing percentage of maturation of pollen and reducing wheat productivity. In wheat heat stress-responsive lncRNAs identified transcriptomic data by Babaei et al. and study noted stress-responsive miRs such as miR156, miR159, miR160, miR167, miR399, miR408, and miR444 and miR1122 [116]. Future advancements to develop engineering heat-tolerant crop plants could be significantly facilitated by strategically manipulating the overexpression or silencing by RNAi and CRISPR strategy of lncRNAs that regulate genes critical to the heat stress response. This targeted approach holds promise for enhancing plant resilience under elevated temperatures.

7.1.2 lncRNAs in metal toxicity

Plants require different nutrients and metal elements for growth and development. In proper amounts, these improve enhance biological and molecular functioning in plants resulting enhancing plant productivity. The metal enhances crop yield and biochemical attributes play a key role by carrying out as cofactor/activation agent in various process such as DNA replication, processing of protein, electron transport, and biosynthesis of chlorophyll [117].

Globally, the indiscriminate discharge of industrial waste and the excessive use of fertilizers and pesticides severely compromise soil fertility and diminish soil properties, ultimately reducing the yield of agricultural and horticultural crops [118]. Unfortunately, this activity done by humans leads to retard crop productivity. Additionally, these contaminants including toxic heavy metals such as lead, copper, arsenic, nickel, and mercury accumulate in the soil and water, infiltrate the food chain, and pose significant risks to human health [119]. The crop production near industrial areas or metal accumulated soil and water sites causes negative impact on human body due to metal accumulation in different body organs or tissue.

To adapt or provide tolerance in plants by molecular mechanism plays a key role to cope with stressors by regulating molecular mechanism following heavy metal movement from soil. In order to lessen the harmful consequences of heavy metals, lncRNAs have also become important molecular actors, intricately modifying a wide range of cellular pathways. Plants have evolved sophisticated regulatory mechanisms to ensure precise homeostasis of essential metal ions, encompassing their absorption, efflux, translocation, and storage [120]. This intricate machinery reflects the critical need to maintain optimal metal ion concentrations for proper physiological and biochemical functions. The excess or overuse of metal ions causes toxicity disrupt the cell and cell membrane and change structure of protein resulting in negative impacts on biochemical and metabolic activity by the production of reactive oxygen species (ROS) [121].

In recent years, the scientific community has increasingly focused on understanding plant responses to heavy metals such as copper, aluminum, and mercury. However, the precise molecular mechanisms underlying these responses remain largely unidentified. Chen et al. provided ground-breaking insights into the role of long non-coding RNAs (lncRNAs) in heavy metal uptake, specifically examining lead accumulation in poplar (*Populus tomentosa*) [122]. Using RNA sequencing (RNA-seq) analysis, they identified 226 lncRNAs associated with lead uptake, exhibiting differential expression linked to crucial pathways, including signal transduction, primary metabolism, energy biosynthesis, and secondary metabolism. Among these, PtoMYB46 was found to positively influence plant growth by downregulating genes such as Auxin Response Factor 2 (ARF2), thereby enhancing transketolase activity and photosynthetic efficiency. Additionally, PtoMYB46 suppressed the expression of PtoMATE, a gene involved in citric acid (CA) secretion, effectively increasing lead uptake efficiency.

Similarly, research on *Medicago truncatula* has revealed molecular responses to aluminum stress mediated by lncRNAs. A study identified 515 differentially expressed lncRNAs from a total of 3284, highlighting their involvement in pathways such as signal transduction, the tricarboxylic acid (TCA) cycle, and hormonal regulation. Functional annotation predicted that these lncRNAs influenced 1254 genes, emphasizing their critical role in modulating molecular and biological pathways to counteract aluminum stress [123].

7.1.3 lncRNAs in drought

For functioning of development process in plant properly water plays a vital role for growth and crop production. In current scenario, water scarcity and quality is a major issue affecting crop yield especially in regions where agriculture heavily relies on irrigation [124]. Stress caused by the decrease in water leads to alterations in the molecular, physiological, ecological, morphological, and biochemical alterations in plant [125].

In plant, water shortage is caused due to extremely high or temperatures, low rainfall, salinity, and high light intensity. Under condition of drought stress, plants undergo profound physiological alterations, including reduced respiration rates, osmotic disequilibrium, disrupted metabolic homeostasis, and retardation of the photosynthetic process by decrease in the activity of enzymes such as Rubisco and phosphoenolpyruvate [126]. These impairments compromise metabolism, and productivity across all developmental stages, ultimately diminishing crop quality and yield [127].

Under stressors such as drought stress, plants invoke a sophisticated cascade of protein phosphorylation pathways, driven by the synthesis of reactive oxygen species, phosphatidylinositol, and secondary messengers analogous to Ca^{2+}, orchestrating precise adaptive defense. In these pathways, transcription factors involved to avoid damage of cell by modulating the expression of genes that provide protection for resilience against stress, thereby bolstering the plant's resilience against stress-induced damage [128]. To confer defense against drought stress, plants have evolved multifaceted mechanisms, including intricate molecular signalling cascades, regulation of extensive gene networks, and activation of biochemical pathways involving protein dynamics, hormone synthesis, and the accumulation of proline and carbohydrates. Additionally, the identification of quantitative trait loci (QTLs) enhances understanding of stress resilience [129]. Recent reports elucidate that among the various strategies conferring tolerance to drought stress, the upregulation of genes involved in the biosynthesis of stress hormones such as jasmonic acid (JA) and abscisic acid (ABA) plays a crucial role in enhancing resilience [130, 131].

The drought stress response is intricately governed by long non-coding RNAs (lncRNAs), which orchestrates the regulation of drought-responsive gene transcription, mediates chromatin remodeling, modulates antisense transcription, and fine-tunes eTM activity, exemplifying their critical role in sophisticated regulatory networks [14, 132]. Plants exhibit a complex regulatory balance under drought stress and produce several proteins involved in inhibition or regulation of a wide range of non-coding RNAs (ncRNAs), improving cellular resilience or affecting the expression of genes to aid in adaptation [133]. To provide tolerance against drought stress lncRNAs cause several changes occur in plants such as secondary metabolites biosynthesis, metabolism of sucrose, and phyto-hormone signal transduction [134, 135]. Many studied evolved the role of drought tolerance lncRNAs in different plant species such as rapeseed [136], cassava [129], *Arabidopsis* [137, 138], rice [138], and tomato [139].

A recent study by Yang et al. aimed to identify drought-responsive RNAs, including miRNAs, lncRNAs, and mRNAs in rice. The analysis revealed a total of 32 miRNAs and 191 lncRNAs, highlighting their significant roles in the plant's adaptive response to drought stress. Notably, Os05g0586700, a drought-responsive gene implicated in plant membrane repair under drought conditions, was identified as a predicted target for as many as 16 drought-specific lncRNAs [140].

The role of lncRNAs to provide tolerance against drought stress in sugar beet by high-throughout RNA-sequencing. Under condition of drought stress, study identified 386 lncRNAs differentially expressed in sugar beet. Among the differential expressed lncRNAs, two lncRNAs *viz*., TCONS_00038334 and TCONS_00055787, identified major changes in expression approximately 1800-fold downregulation and 6000-fold upregulation, respectively [126].

7.1.4 lncRNAs in salinity

Among the various abiotic and biotic stressors, stress caused by excessive salinity significantly hampers plant productivity. Salt stress not only impairs crop growth but also degrades soil fertility, posing a significant challenge to sustainable agriculture. It is estimated that approximately 6% of the world's agricultural land contains a high concentration of salt elements [141]. The primary cause of salt accumulation in soils is the prolonged use of irrigation water, which deposits dissolved salts as it

evaporates [142]. Reports indicate that salinity affects 6% of drylands and 20% of irrigated soils globally, further exacerbating the issue and threatening agricultural productivity. Recent reports indicated that soil salinization decomposing income of people depends on agricultural for survival and recorded approximately US$31 million economic loss [143].

Plants grown in saline soils experience a significant decline in productivity due to heightened oxidative and osmotic stress, nutrient deficiencies, and disruptions in intracellular ion homeostasis [144]. In plants under salt stress condition, there is chance to change metabolic activity of plant and in cell solute ratio of Na^+/K^+ enhanced, which leads to develop several metabolic disorders [145, 146].

To withstand such challenging conditions, plants have developed intricate adaptive mechanisms, including epigenetic modifications and the regulation of critical processes such as transcription and translation. Enhancing salt stress resistance in plant species necessitates a deeper understanding of the underlying molecular mechanisms, a task that calls for focused efforts by agricultural scientists. Recent studies have highlighted the pivotal role of long non-coding RNAs (lncRNAs) in conferring tolerance to salt stresses, offering promising avenues for advancing crop resilience in many plant species such as cotton [147], sorghum [148], rice [149], tomato [146], and chickpea [150]. lncRNAs act as activate change response of several genes such as hormones *viz*., ABA and GA as well as upregulate the activity of Na^+ transporters to provide tolerance against salt stress.

The expression of various lncRNAs under saline stress in grapevine identified novel lncRNAs (3952) in grapevine. In study after supplementation of salt 12 h under condition of salt stress reports identified differentially expressed lncRNAs (1661) modulated either indirectly or directly several mRNAs with the use of miRNA-mediated, *trans*-, and *cis patterns* [151].

7.2 lncRNAs in biotic stress

In agriculture, apart from abiotic stress crop production is decreased due to microorganism's attack on plants such as fungi, bacteria, insects, and nematodes. Worldwide, approximately $20 billion of agricultural economic loss due to the loss of food production 10–30% by pathogen attack in plants is estimated (https://www.ars.usda.gov, accessed on 26 December 2024). To defense against pathogen attack, several biochemical and cellular changes occur in plant system such as metabolic and protein structural and functional changes in favor to response by activation of innate immune system of plant [152].

PAMP-triggered immunity a first layer of defense, plants' multilayered immune systems are based on which pathogen identified by cell-surface receptors identifies microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) and triggers PAMP of immune responses (PTI) [153]. In this intricate defense mechanism, plants synthesize phytohormones such as salicylic acid and jasmonic acid, and enhance the activity of protein kinases and reactive oxygen species (ROS) production, and calcium ion (Ca^{2+}) signalling cascades, effectively mitigating the proliferation of pathogenic bacteria [154]. Additionally, the effector-triggered immunity (ETI) pathway orchestrates a formidable immune response through highly specialized resistance (R) proteins [155]. These R-proteins possess exceptional specificity in recognizing pathogen-derived effectors at the infection site, thereby

mobilizing precise and robust defensive countermeasures. This R protein developed by the PTI and ETI cascade typically controls downstream signaling networks.

To provide immune response to plant towards biotic stress lncRNAs plays a vital role identified in many plant species by regulating their downstream immune signaling pathways. Late blight is a serious disease in tomato caused by *Phytophthora infestans* and the study by Zhang et al. identified interactions of lncRNA-miRNA providing immune response. The result identified that as a competitive endogenous RNA (ceRNA) for miR394, lncRNA40787 dramatically lowers miR394 levels and prevents it from cleaving the JA biosynthesis gene LCR. This regulatory mechanism fortifies tomato plants against *P. infestans*, thereby augmenting their stress tolerance through elevated JA-mediated defense pathways [156].

8. Conclusions and future prospectives

Long non-coding RNAs (lncRNAs) have emerged as indispensable regulators in plant biology, offering significant insights into how plants adapt to environmental stresses and developmental challenges. Their roles in modulating transcriptional, post-transcriptional, and epigenetic networks highlight their potential as powerful tools for improving plant resilience to abiotic and biotic stressors. Mechanisms such as chromatin remodeling, histone modification, and DNA methylation further underscore their critical involvement in plant stress responses and adaptation. Looking ahead, the integration of advanced sequencing technologies, bioinformatics tools, and gene-editing systems like CRISPR/Cas provides a promising avenue to unravel the precise regulatory mechanisms of lncRNAs. Future research should focus on harnessing lncRNAs for breeding stress-tolerant crops, improving nutrient use efficiency, and addressing the global challenges of food security and climate change. Translating these findings into practical applications can revolutionize sustainable agriculture by enabling the development of climate-resilient crops, ensuring productivity under diverse environmental conditions.

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Conflict of interest

The authors declare no conflict of interest.

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