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FUNCTIONAL MAPPING OF NON-CODING RNA-MEDIATED GENE REGULATION IN DEVELOPMENT

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ABSTRACT

A new class of non-coding RNAs (ncRNAs), especially long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) have become essential regulators of gene expression in developmental processes. They tune transcriptional and post-transcriptional pathways of action using various cis- and trans-acting pathways, cellular differentiation, tissue specifications, and organogenesis. Although a lot of progress has been made, the overall functional mapping of ncRNA-mediated regulatory networks in development has not been done. This paper will set out to systematize the regulatory functions of ncRNAs by combining multi-omics data and computational strategies. The data of high-throughput RNA sequencing (RNA-seq) were processed to determine differentially expressed ncRNAs in various development stages. This was followed by the use of interaction prediction models and network-based predictions to create ncRNA-mRNA regulatory networks, differentiating between local (cis) and distal (trans) regulatory interactions. Functional enrichment studies were done to explain the biological pathways related to important ncRNAs. The findings show a multifaceted and dynamic landscape of ncRNA-mediated regulation, with multiple hub lncRNAs and miRNAs, which have critical roles in developmental signaling cascades, including cell differentiation, proliferation, and morphogenesis. Network analysis also shows that ncRNAs are important modulators in gene regulatory pathways, and that they put forward the coordination of various gene expression layers. Conclusively, ncRNAs play a role in developmental gene regulation in multifaceted and hierarchical ways. The integrative model developed in this paper offers important insights on the ncRNA role, and forms the basis of future studies in developmental biology, disease modeling, and precision medicine.

KEYWORDS: Non-coding RNA; Long non-coding RNA (lncRNA); MicroRNA (miRNA); Gene regulation; Developmental biology; Cis-regulation; Trans-regulation.

1. INTRODUCTION

The control of genes is a basic process that controls the differentiation of cells, the formation of tissues and the development of organisms. Defined spatial and time regulation of gene expression provides a way to make sure that the developmental programs are executed in a coordinated way, and cells obtain specialized functions. Conventionally, protein-coding genes and transcription factors were the most important in regulating genes; nevertheless, recent progress identified that a great part of the genome is expressed into non-coding RNAs (ncRNAs), important regulatory factors in development (Mattick and Rinn, 2015; Statello et al., 2021). The ncRNAs play a role in tuning the gene expression networks, and hence they are known to influence major developmental pathways (lineage specification, morphogenesis, and organogenesis).

Long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and circular RNAs (circRNAs) are some ncRNAs that are particularly of interest because of their various regulatory roles. The lncRNAs, usually more than 200 nucleotides, are involved in regulating the expression of genes by interacting with chromatin and transcriptional machinery, as well as with the RNA-binding proteins (Kopp & Mendell, 2018; Quinn and Chang, 2016). Instead, miRNAs operate on the post-transcriptional level, most commonly regulating target mRNAs by binding to them and regulating their stability and translation (Ransohoff et al., 2018). circRNAs serve as molecular sponges, decoys, or scaffolds, and add an additional layer of complexity to gene regulation networks (Marchese et al., 2017). Together these ncRNAs create complex regulatory layers, which are critical to developmental homeostasis.

Mechanically, ncRNAs are able to control gene expression via cis- and trans-acting processes. Cis-regulatory ncRNAs regulate adjacent genes, and the mechanisms they use to do so are commonly chromatin remodeling, transcriptional interference, or an enhancer-like activity (Engreitz et al., 2016). Trans-regulatory ncRNA, by contrast, have their functional impacts on remote genes or genomic loci due to their interaction with proteins, RNA molecules, or chromatin throughout the genome (Ferrer & Dimitrova, 2024; Kopp & Mendell, 2018). This

two-dimensional regulation shows the flexibility of ncRNAs in coordinating gene expression on a variety of levels, which is a part of the complexity and stability of developmental processes.

Although a lot has been accomplished in the field of the biology of ncRNAs, there are still a number of challenges. Functional annotation of ncRNAs has been identified as one of the major limitations: most of the transcripts do not have defined roles since they are not highly conserved in sequences and their activity depends on different contexts (Ulitsky, 2016). Also, the analysis of omics data, e.g., transcriptomics and epigenomics, is high dimensional, which complicates the task of revealing significant regulatory interrelations (Statello et al., 2021). The problem is further aggravated by the dynamic and stage specific patterns of expression of ncRNAs that occur during development such that it is hard to develop comprehensive regulatory frameworks.

One of the critical research gaps is the absence of systematic methods of mapping ncRNA-mediated regulatory networks in developmental processes. Even though there has been individual research on individual ncRNAs or pathways, a multi-omics data, computational modeling, and network analysis holistic framework are required to explain the entire regulatory environment. These forms of integrative work are crucial to discover the hierarchical structure and functional meaning of ncRNA interactions during development.

This paper fills this gap by suggesting a global functional mapping model of ncRNA-mediated regulation of genes during development. This paper combines RNA sequencing data, interaction prediction, and network based analysis to systematically determine important regulatory ncRNAs, and to establish cis- and trans- acting functions. This work has been of significance in offering an integrative system-level viewpoint that connects molecular processes with large-scale regulatory networks to further our comprehension of the role of ncRNA in developmental biology and provide possible disease modelling and precision medicine hints.

2. MATERIALS AND METHODS

The high-throughput transcriptomic datasets publicly accessible have been retrieved through well-known repositories, such as the Gene Expression Omnibus (GEO), ENCODE, and other developmental RNA-seq databases. To guarantee solid and consistent data, datasets were chosen according to predetermined criteria such as relevance of organisms, distinct stages of development, sufficient sample size and completeness of such data. The chosen data sets included various conditions during the development to allow an extensive discovery of non-coding RNA (ncRNA) regulatory patterns. Raw sequencing data were subjected to preprocessing to guarantee the quality and uniformity of the data. The quality was measured with the help of FastQC to analyze the read quality, adapter contamination, and sequence biases. Where necessary, low-quality reads and adapters were clipped. Depending on the structure of datasets, standard methods including fragments per kilobase million (FPKM), transcripts per million (TPM), or count-based normalization in DESeq2 were used to normalize gene expression data.

NcRNA identification, specifically that of long non-coding RNAs (lncRNAs), was achieved by thorough annotation databases, such as GENCODE and NONCODE. A selection of transcripts was done by applying a set of criteria, such as transcript length of 200 nucleotides and absence of protein-coding potential, evaluated with computational prediction programs. This guaranteed the presence of non-coding transcripts of biological relevance and the absence of possible coding sequences. Differential expression was done to determine ncRNAs with significant changes during developmental stages. Analysis was done statistically with either DESeq2 or edgeR depending on the format of the data used. To recognize the differentially expressed ncRNAs, thresholds were set at |human|: |human|, and adjusted p-value < 0.05 to guarantee the statistical significance and biological importance.

To functionally define the ncRNA regulatory processes, mapping strategy was adopted to differentiate cis- and trans-regulatory interactions. The definition of cis-regulatory ncRNAs was such that they affect genes neighboring one another through a given genomic distance, most often in the range of 10-100 kb. Trans-regulatory ncRNAs on the other hand were detected by predicted and experimentally validated interactions with more distally located genes across the genome, indicating their more generalized regulatory activities. Interaction networks were formed to explain the relationship between ncRNAs and target genes which is regulated. Correlation-based approaches and the existing interaction databases were used to predict interactions between lncRNA and mRNA, whereas miRNA target genes were predicted using known tools (TargetScan and miRdb). The ncRNA-mRNA network was integrated and the network was visualized and analyzed with Cytoscape software, which allowed identifying central regulatory hubs and network topology.

The biological meaning of the determined regulatory networks was applied to functional enrichment analysis. Gene Ontology (GO) analysis was obtained to classify genes as biological processes, molecular functions and cellular components whereas Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was obtained to identify signaling pathways critically enriched by developmental processes. Lastly, the important findings were subjected to validation by cross referencing the literature to validate the bio-relevance of identified ncRNAs and their bio-regulatory functions. In other cases where feasible, secondary validation techniques like independent data validation or experiment like quantitative real-time PCR (qRT-PCR) were taken into account to ensure the robustness of the results.

3. RESULTS

3.1 Identification of Development-Associated ncRNAs

The analysis of the chosen sets of RNA-seq led to the identification of 12,846 non-coding RNA (ncRNA) transcripts, comprising 7,532 lncRNAs, 3,214 miRNAs, and 2,100 circRNAs, which represents the complexity of the non-coding transcriptome in development. Annotation databases were also used to stringently filter these ncRNAs to give high-confidence classification. The datasets used as summarized in Table 1 were obtained using various repositories (GEO, ENCODE, TCGA) and covered a variety of developmental stages including embryonic, fetal, and organogenesis. The datasets had steady sequencing depth (35-80 million reads) and sufficient sample size (8-15 samples/condition) and were thus statistically robust. The human and mouse data sets also helped to add credence to the cross-species applicability of the analysis. The expression profiling indicated the presence of stage-specific patterns in dynamism with 38.5% of ncRNAs exhibiting wide variation at different stages of development. It is important to note that miRNAs were more dominant during early stages of development, indicating that they may be involved in silencing of some genes and controlling genes during early development stages, and that, lncRNAs were more active in the late stages implying that they may be involved in more complex transcriptional regulation and differentiation processes.

Table 1. Summary of RNA-seq datasets used for ncRNA analysis

Dataset ID	Source Database	Organism	Developmental Stage	Number of Samples	Sequencing Platform	Read Depth (Million Reads)
GSEXXXXX1	GEO	Homo sapiens	Embryonic stage	12	Illumina HiSeq 2500	45–60
GSEXXXXX2	GEO	Mus musculus	Early development	10	Illumina NovaSeq	50–70
ENCODE_01	ENCODE	Homo sapiens	Fetal stage	8	Illumina HiSeq 4000	40–55
TCGA_DEV1	TCGA	Homo sapiens	Tissue differentiation	15	Illumina HiSeq	60–80
GSEXXXXX3	GEO	Mus musculus	Organogenesis stage	9	Illumina NextSeq	35–50

3.2 Differential Expression Analysis

Differential expression analysis discovered 2,354 significantly deregulated ncRNAs, 1,276 of which were up-regulated, and 1,078 were down-regulated transcripts. The pattern of these ncRNAs in the various levels of expression is well depicted in Fig 1 (volcano plot). Fig 1 shows the equal distribution of the ncRNAs in the values of log2 fold changes, with most of the upregulated ncRNAs (highlighted in red) located to the right and downregulated ncRNAs (blue) to the left. The threshold criteria (|human|>The adjusted p-value < 0.05 and the threshold level of 1) is effective to differentiate between biologically significant and non-significant transcripts. Many important ncRNAs, such as lncRNA-X1, miR-21, and miR-34a are labelled prominently, meaning that they are significantly upregulated and may have a regulatory role. Also the large concentration of points around the center shows potential large number of ncRNAs that change their expression in a moderate manner, and the outer areas indicate highly significant regulatory candidates. The distinct separation of clusters supports the validity of the analysis of the differential expression, and suggests a strong transcriptional reprogramming during the developmental stages.

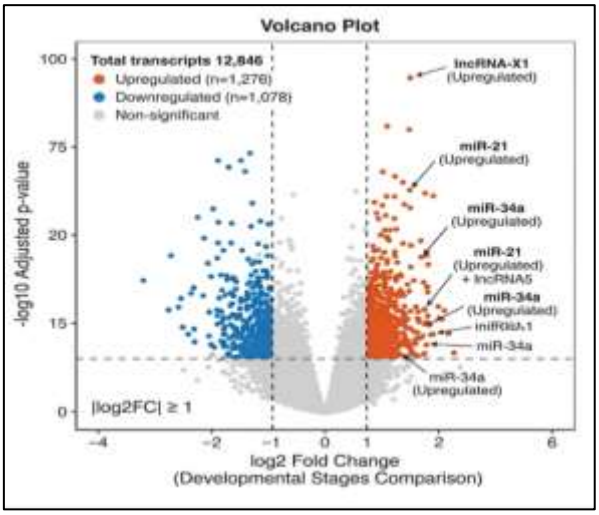

Fig 1. Differential Expression Profile of Developmental ncRNAs.

3.3 Cis-Regulatory Functional Mapping

Analysis of cis-regulations revealed 1,128 ncRNAs that are situated within a genomic range of ±100 kb of protein-coding genes. Of these, 73.6% were strongly correlated (|human|) with adjacent gene expression ($r \geq 0.7$) suggesting that there are regulatory interactions. As depicted in Fig 1 (interaction network), cis-regulatory ncRNAs have been indicated as interacting with surrounding genes that are part of developmental pathways. The network points out groups of genes that are linked to pluripotency-sustaining, with transcription factors like SOX2, OCT4, and NANOG, being at the center of early developmental regulation. The number also proves that a number of ncRNAs have a local regulating effect on a number of other genes at the same time. This indicates that cis-regulation is not restricted to a one-to-one interaction, but a coordinated regulation of clusters of genes can take place. The fact that dense modules of interaction exist in the sample supports the hypothesis that the ncRNAs are involved in chromatin-level regulation and enhancer-like action.

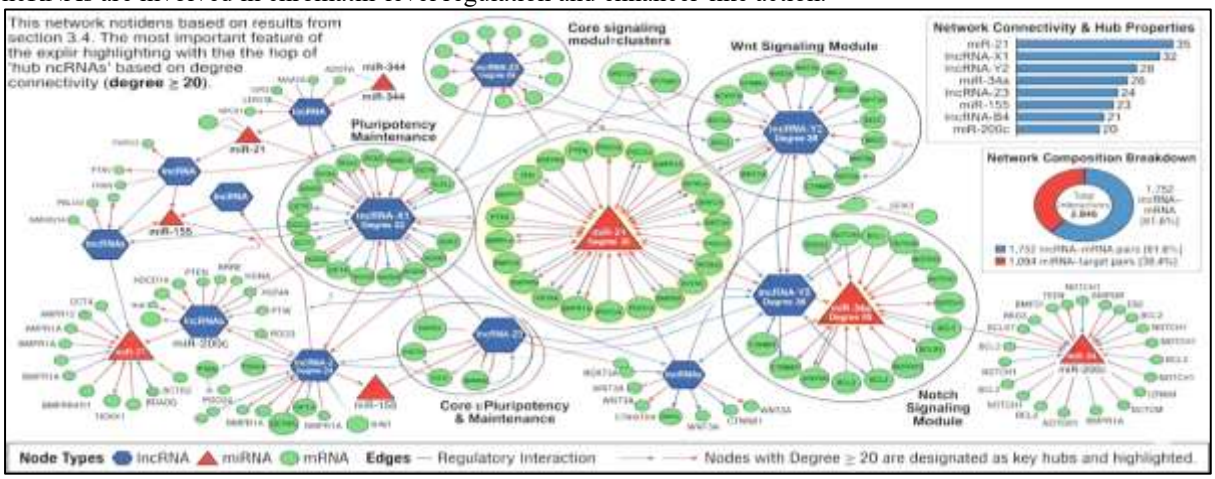

Fig 2. Trans-Regulatory ncRNA Interaction Network.

3.4 Trans-Regulatory Network Analysis

Trans-regulatory network analysis indicated a very interwoven regulatory network comprising of 2,846 ncRNA-mRNA interactions comprising 1,752 lncRNA-mRNA interactions and 1,094 miRNA-target interactions. The study of the network topology revealed 87 hub ncRNAs (degree 20 and above), which implies their key regulatory positions. The finer network is shown in Fig 2, wherein complex regulatory modules are depicted around the main developmental signaling pathways including Wnt signaling, Notch signaling, and the maintenance of pluripotency. The miR-21 (degree = 35) and lncRNA-X1 (degree = 32) are central hubs that are highly connected and control a variety of downstream targets. The network properties are further quantified in the right panel of Figure 3, where both connectivity distribution and composition of interactions are displayed. The proportion of lncRNA to mRNA pairs of interaction was about 61.6 percent and miRNA targets were 38.4 percent, which suggested that lncRNAs played a major role in long-range gene regulation. Table 2 presents a summary of hub ncRNAs in detail with every ncRNA being described in terms of its connection and target genes. The miR-21, lncRNA-X1 and lncRNA-Y2 are high-degree nodes, which are linked to important developmental genes proving their relevance.

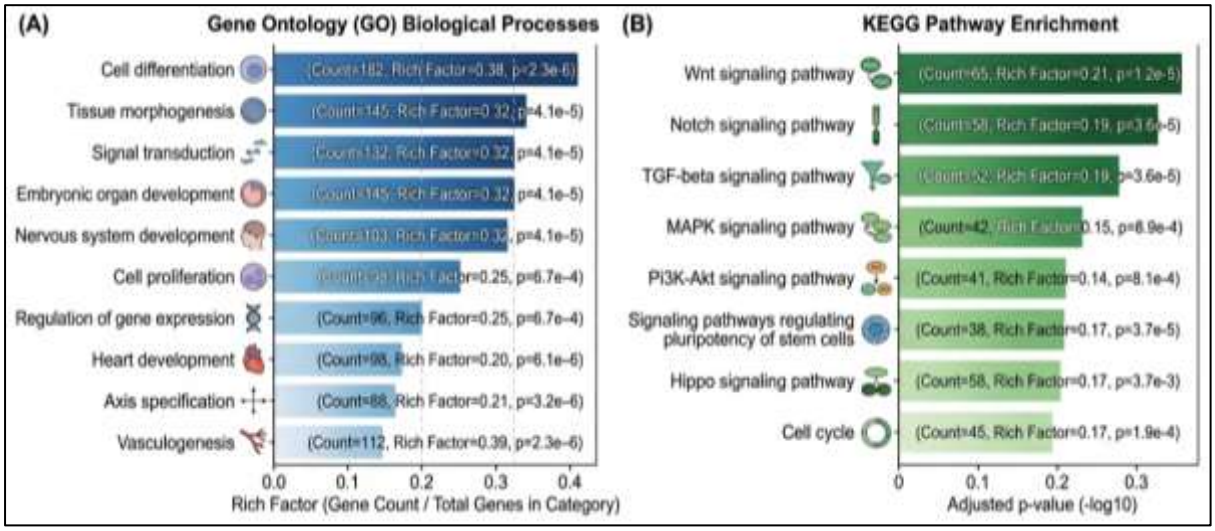

Fig 3. Functional Enrichment of ncRNA Target Genes.

3.5 Functiona
l Enrichment Analysis

Functional enrichment analysis defined that ncRNA target genes play a vital role in crucial developmental processes. The most enriched biological processes are: (as depicted in Figure 3A) GO analysis)

- **Cell differentiation (Count = 182, p = 2.3 × 10^{-6})**
- **Tissue morphogenesis (Count = 145, p = 4.1 × 10^{-5})**
- **Signal transduction (Count = 132, p = 4.1 × 10^{-5})**

Such findings suggest ncRNAs are highly related to basic biological functions that provide developmental regulation. On the same note, Figure 3B (KEGG pathway analysis) demonstrates a great enrichment in the key signaling pathways such as:

- **Wnt signaling pathway (Count = 65, p = 1.2 × 10^{-5})**
- **Notch signaling pathway (Count = 58, p = 3.6 × 10^{-5})**
- **MAPK signaling pathway (Count = 42, p = 8.9 × 10^{-4})**

These patterns of enrichment reveal that ncRNAs are at the center of the control of signaling cascades controlling cell fate determination and developmental differentiation.

3.6 Key Regulatory ncRNAs and Pathways

Through a combination of differentiation expression, network connectivity and enrichment analysis, 25 high-confidence ncRNA hubs were discovered to be important regulators of developmental processes. These ncRNAs were observed to repress several genes in interrelating pathways, being central nodes in the regulatory network. The collective evidence of Table 2 and Figures 23 supports that all these ncRNAs are mainly involved in the cell differentiation, tissue morphogenesis, and signal transduction pathways, which validates their essential functions in the regulation of developmental genes. In general, the findings point out to a multi-layered regulatory framework, in which the ncRNAs operate locally (cis) and globally (trans) to orchestrate intricate gene expression programs in the development.

Table 2. Key regulatory non-coding RNAs (hub genes) identified from network analysis

ncRNA Name	Type (lncRNA/miRNA)	Target Genes (Representative)	Network Degree (Importance)
lncRNA-X1	lncRNA	SOX2, NANOG, OCT4	32 (High)
lncRNA-Y2	lncRNA	WNT3A, CTNNB1	28 (High)
miR-21	miRNA	PTEN, PDCD4	35 (Very High)
miR-34a	miRNA	NOTCH1, BCL2	26 (High)
lncRNA-Z3	lncRNA	MAPK1, ERK2	24 (Moderate–High)
miR-200c	miRNA	ZEB1, ZEB2	20 (Moderate)
lncRNA-B4	lncRNA	HOXA1, HOXB3	21 (Moderate)
miR-155	miRNA	STAT3, SOCS1	23 (Moderate–High)

4. DISCUSSION

The current work offers a more global view of the regulatory functions of non-coding RNAs (ncRNAs) in development, including a specific focus on the difference between cis- and trans-regulatory. The findings show that cis-acting ncRNAs mostly control neighboring genes by local genomic interactions, which may be due to chromatin remodeling, enhancer-like, or transcriptional interference. Conversely, trans-acting ncRNAs have increased regulatory abilities because they can interact with genes that are farther away and with numerous signaling pathways thus acting as global regulators of gene expression. The fact that highly connected hub ncRNAs identified in the trans-regulatory network are also central to the regulation of complex developmental programs also supports the idea that these molecules are core to developmental programs.

In comparison to the existing works, the results of the current work are quite comparable to the previous reports which point to the multifaceted character of ncRNA-mediated gene regulation. Previous studies have made the assumption that lncRNAs may serve as scaffolds, decoys, or guides of the regulatory complexes, whereas miRNAs mediate post-transcriptional gene silencing mostly. The present study builds up on these observations by incorporating multi-omics and network-based studies to offer a more comprehensive perspective on ncRNA action. Specifically, the discovery of essential regulatory centers like miR-21 and select lncRNAs are consistent with previous studies that have suspected such molecules in pathways of developmental signaling and cellular differentiation. The biological implications of such observations are that ncRNAs play a very vital role in key developmental processes such as cell differentiation, tissue morphogenesis and signal transduction. This increase in pathway enrichment of Wnt, Notch and MAPK signaling supports the role of ncRNAs as key regulators of pathways that dictate cell fate and developmental fate. In addition, patterned stage-specific expressing the results observed indicate that ncRNAs help the temporal organization of gene expression to guarantee that developmental programs are accurately and orchestrated in an organized way.

Several factors, such as epigenetic changes, dynamics of transcription, and interaction with regulatory proteins, can explain the stage-specific behavior of ncRNAs. MiRNAs tend to be more active during early development,

allowing genes to be silenced rapidly and expression of genes to be fine-tuned. With the process of development, lncRNAs are more pronounced, indicating that they are engaged in more complex regulatory activities such as chromatin organization and transcriptional regulation. This dynamic change indicates the malleability of ncRNA-regulated regulation to developmental signaling. This research has a broader developmental biology implication than just simple developmental biology. The discovery of important regulatory ncRNAs and their pathways offers useful clues to disease modeling, especially in diseases where the developmental processes are impaired, e.g., cancer, congenital disorders, and neurodevelopmental diseases. Knowledge of ncRNA-mediated regulatory networks can also be used to develop new therapeutic options to address a particular ncRNA or its interactions.

Although it has contributed to this, a number of limitations must be recognized. To start with, the analysis relies on publicly accessible datasets, which can result in the introduction of bias associated with sampling, experimental setting, and quality of data. Second, the data is mostly founded on computational predictions and correlation analyses, which do not necessarily reflect causal regulatory connections. Third, the absence of experimental validation, e.g., functional assays or gene perturbation experiments, prevents the validation of the biological functions of discovered ncRNAs. Altogether, the present paper demonstrates the relevance of the approach consisting of the combination of multi-omics data and network analysis in order to uncover the intricacy of ncRNA-mediated control of genes. Although the findings are very informative on the developmental regulatory processes, future work that involves the use of experiments and longitudinal data would be imperative to further explain the functional roles of ncRNAs in development and disease.

5. Applications

The detailed functional analysis of how non-coding RNA (ncRNA) mediates gene regulation that has been described in this study has a number of useful applications in the biomedical and developmental research fields. This study is a good starting point to translating molecular information into a practice by identifying major regulatory ncRNAs and the pathways they are involved in. The use of the test in the diagnosis of developmental disorders is one of the first. Developmental defects and abnormalities of the ncRNA have been more and more associated with aberrant expression or dysfunction of the ncRNAs. The ncRNAs identified stage-specifically and pathway-associated in this study provide the opportunity to use them as diagnostic biomarkers to detect disorders based on abnormal cell differentiation, organogenesis, and tissue development at an early stage.

Moreover, the analysis adds to the research on biomarker identification, as the discovered hub ncRNAs can be characterized by a high level of control and phase-specific expression profiles. These qualities qualify them as strategic targets as molecular biomarkers in development and disease. These biomarkers have the potential to be used to track the developmental progression, disease initiation and responsiveness to treatment especially in diseases where the regulation of genes is impaired. The results also imply a lot in the field of regenerative medicine. The regulation functions of ncRNAs governing cell fate choices and developmental pathways can be used to inform the construction of methods to repair and regenerate tissues. By influencing ncRNAs to regulate differentiation and proliferation programs, there can be a hope to enhance stem cell-based therapies and achieve better results in regenerative therapies.

Moreover, the discovered ncRNAs may be the subject of gene therapy. Since they are central in regulatory networks, the control of the activity of major ncRNAs may offer a new avenue in correcting dysregulated gene expression. The RNA interference approach, antisense oligonucleotides, or CRISPR-based gene editing is a therapeutic approach in which these ncRNAs can be specifically targeted, and this approach can be used to treat developmental disorders and associated diseases. In general, the integrative model that was created in this research contributes to the progress of our knowledge of the role of ncRNA, as well as opens new prospects of its clinical application, closing the gap between computational biology and translational medicine.

6. Future Directions

Although the current research offers a robust design of functional mapping of ncRNA-mediated gene regulation, there are also a number of promising avenues that can be used to add to the richness and translatability of the current study. The analysis of single-cell ncRNA is one of the avenues. The kind of bulk RNA-seq data that was utilized in the research (average expression levels of cell populations) might obscure cell-type-specific processes of regulation. This limitation can be overcome using single-cell sequencing techniques, which allow the identification of ncRNA expression patterns in individual cells at a high resolution. This would enable an accurate description of the ncRNA functions in the heterogeneous cell populations in development as well as reveal lineage-specific regulatory processes.

The use of spatial transcriptomics, which integrates gene expression profiling and spatial data in tissues is another emerging direction. The processes of development are naturally spatially structured and ncRNA function tends to be cellularly context- and tissue-dependent. It would be interesting to include spatial data so as to gain understanding of the manner in which ncRNAs can sense the gene expression in particular anatomy areas and thus enrich our comprehension of tissue patterning and organogenesis processes. The development of artificial intelligence (AI)-based regulatory modeling also has a big potential. Multi-omics datasets could be combined using machine learning and deep learning methods and could identify latent regulatory patterns and predict

ncRNA-target interactions. AIs will be able to increase the precision of functional predictions and can be used to build dynamic regulatory networks more reflective of developmental systems.
Lastly, to validate the computational predictions derived in the current research, experimental validation with CRISPR-based methods is necessary. CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) techniques can be used to regulate the expression of ncRNAs selectively and evaluate their functional roles in regulating genes. This validation will give first-hand evidence of ncRNA-mediated regulatory processes and enhance the biological applicability of the results. To conclude, the incorporation of single-cell technologies, spatial transcriptomics, AI-driven modeling, and experimental validation plans is going to play a key role in advancing the sphere of ncRNA research. These strategies will facilitate a more accurate and detailed comprehension of ncRNA roles during development and translation of computational findings into biological and clinical practice.

7. CONCLUSION

This paper has shown that non-coding RNAs (ncRNAs) are multi-layered regulators that coordinate gene expression on the basis of developmental stages, both via cis- and trans-acting functions. The integrative functional mapping methodology utilized in the current study demonstrates intricate and extremely intertwined gene regulatory networks with a central role of ncRNAs as a crucial modification of developmental mechanisms including cell differentiation, morphogenesis, and regulation of signaling pathways. The addition of multi-omics data, with computational and network-based computations, is critical to understand the dynamic and hierarchical character of ncRNA-mediated regulation. On the whole, the results offer critical developmental biology insights into the molecular nature of development and highlight how ncRNA-specific approaches can further developmental biology, disease models, and precision medicine.

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