NON-CODING RNA MEDIATED POST-TRANSCRIPTIONAL REGULATION IN HUMAN DISEASES

EDITED BY: Shaveta Kanoria, Santosh Kumar and Florent Hubé

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NON-CODING RNA MEDIATED POST-TRANSCRIPTIONAL REGULATION IN HUMAN DISEASES

Topic Editors:
Shaveta Kanoria, Wadsworth Center, United States
Santosh Kumar, National Institute of Technology Rourkela, India
Florent Hubé, UMR7216 Epigénétique et destin cellulaire, France

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EDITORIAL
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Editorial: Non-Coding RNA Mediated Post-Transcriptional Regulation in Human Diseases

Santosh Kumar[1]* *and Shaveta Kanoria*[2]*

[1]*Life Science Department, National Institute of Technology, Rourkela, India,* [2]*Wadsworth Center, Center for Medical Science, New York State Department of Health, Albany, NY, United States*

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Editorial on the Research Topic

Non-Coding RNA Mediated Post-Transcriptional Regulation in Human Diseases

Recent advances in the technology and analysis aspect of RNA sequencing have unraveled many non-coding RNAs. These non-coding RNAs regulate various post-transcriptional processes and cellular functions under normal and diseased conditions. In this research topic, the published articles cover a vast diversity of non-coding RNA types, including long non-coding RNA (lncRNA), circular RNA (circRNA), transfer RNA (tRNA), and microRNA (miRNA). The objective of this topic is to highlight the intricate relationship of ncRNA with post-transcriptional processes and their role in human diseases. A total of five research articles and four reviews are published. These research articles and reviews focusing on the objective of this topic explore the role of ncRNAs in a range of physiological processes and human diseases, including colorectal cancer, lung function and diseases, pulmonary hypertension, cystic fibrosis, adipogenesis, and reproduction.

The article by Fei Yao et al. investigated the circular RNA profiles and colorectal cancer (CRC) chemoresistant cell lines. They studied the differential expression of circRNA and analyzed their role in the chemoresistance of CRC. In another research, Pengpeng Zhang et al. studied the expression of circular RNA using RNA sequencing in brown adipogenesis at various differentiation stages. They utilized an array of computational tools to understand and predict the potential role of circRNA in brown adipogenesis. The review article by Soni and Biswas highlighted the role of lncRNA and miRNA in the post-transcriptional regulation of several lung diseases, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, and idiopathic pulmonary fibrosis. Another review article by Chaofan He et al. explored the role of non-coding RNA in reproductive processes and reproductive diseases. They described the role of miRNA, lncRNA, and PIWI-interacting RNA in spermatogenesis and follicular development. In addition, they also discussed the role of non-coding RNA in male and female reproductive diseases.

In a pilot study of X-linked microRNA expression, McKiernan et al. studied the top seven X-linked microRNAs, namely, miR-224-5p, miR-452-5p, miR-450-5p, miR-542-3p, miR-450a-5p, miR-424-5p, and miR-545-5p, which are significantly upregulated in cystic fibrosis versus non-cystic fibrosis monocytes correlating with lung function. Further, they found miR-224-5p to be correlating with lung function in cystic fibrosis, whereas the miR-545-5p and miR-224-5p levels correlate with exacerbation rate. Zhou et al. demonstrated the potentially extensive involvement of tRNA-derived fragments (tRFs) in cancers and provided a reasonable list of cancer-associated tRFs for further investigations. Another review article by Xia et al. described the role of lncRNAs in renal fibrosis (RF). They evaluated the recent publications on lncRNAs in RF and the potential applications of lncRNAs as diagnostic and prognostic biomarkers in RF. They also proposed potential therapeutic targets for treating RF-associated diseases and subsequent CKD. The article by Liu et al. indicated that the lncRNA KCNQ1OT1 ceRNA network could be involved in

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Edited by:
William C. Cho,
QEH, Hong Kong SAR, China

Reviewed by:
Cuncong Zhong,
University of Kansas, United States
Estefania Lozano Velasco,
University of Jaén, Spain

***Correspondence:**
Santosh Kumar
kumarsantosh@nitrkl.ac.in
Shaveta Kanoria
shavetak@gmail.com

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regulating the CRC tumor microenvironment. Interestingly, they found that lncRNA KCNQ1OT1 was significantly upregulated in CRC tissues and inversely associated with the survival of patients, indicating KCNQ1OT1 as a possible functional contributor and therapeutic target for CRC. The review article by Zhang et al. elucidated the involvement of non-coding RNA networks in pulmonary hypertension. The authors constructed ncRNA networks by assembling ncRNAs and their interacting RNAs or genes, providing a better understanding of the roles of ncRNAs in pulmonary hypertension and potential clinical applications of the ncRNAs in pulmonary hypertension.

The Research Topic of articles in this topic advances our understanding of the role of ncRNA in various post-transcriptional processes and human health and diseases.

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SAK and SHK contributed equally to writing and editing the manuscript.

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REVIEW
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Long Non-coding RNA: An Emerging Contributor and Potential Therapeutic Target in Renal Fibrosis

Weiping Xia, Yao He, Yu Gan, Bo Zhang, Guoyu Dai, Feng Ru, Zexiang Jiang, Zhi Chen and Xiang Chen**

Department of Urology, Xiangya Hospital, Central South University, Changsha, China

Renal fibrosis (RF) is a pathological process that culminates in terminal renal failure in chronic kidney disease (CKD). Fibrosis contributes to progressive and irreversible decline in renal function. However, the molecular mechanisms involved in RF are complex and remain poorly understood. Long non-coding RNAs (lncRNAs) are a major type of non-coding RNAs, which significantly affect various disease processes, cellular homeostasis, and development through multiple mechanisms. Recent investigations have implicated aberrantly expressed lncRNA in RF development and progression, suggesting that lncRNAs play a crucial role in determining the clinical manifestation of RF. In this review, we comprehensively evaluated the recently published articles on lncRNAs in RF, discussed the potential application of lncRNAs as diagnostic and/or prognostic biomarkers, proposed therapeutic targets for treating RF-associated diseases and subsequent CKD transition, and highlight future research directions in the context of the role of lncRNAs in the development and treatment of RF.

Keywords: long non-coding RNA, kidney disease, renal fibrosis, fibrosis, diabetic nephropathy

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Edited by:
Shaveta Kanoria,
Wadsworth Center, United States

Reviewed by:
Gary S. Stein,
The University of Vermont, United States
Zhijie Xu,
Central South University, China

****Correspondence:***
Zhi Chen
czhi1011@126.com
Xiang Chen
cxiang1007@126.com

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INTRODUCTION

Renal fibrosis (RF) is a universal pathological process that leads to terminal renal failure in chronic kidney disease (CKD) (Sun et al., 2016); it is induced in response to diverse factors, such as external injury, inflammation, ischemia, hypoxia, myofibroblast activation and migration, and matrix deposition and remodeling (Liu et al., 2017; Humphreys, 2018; Bijkerk et al., 2019; Perry et al., 2019; Zhang S. et al., 2020). Many diseases are associated with RF development, including obstructive kidney disease, chronic glomerulonephritis, chronic pyelonephritis, systemic lupus erythematosus nephropathy, and hereditary nephropathies, such as Alport syndrome, diabetic nephropathy (DN), hypertensive nephropathy, and drug-induced nephropathy (González et al., 2008; Davidson, 2016; Seccia et al., 2017). However, the mechanism underlying RF development remains poorly understood, and existing treatments are ineffective. To understand the etiology and pathogenesis of RF and to delay or reverse disease progression, numerous researchers have investigated the mechanism underlying RF development. Wang Y. et al. (2019) demonstrated that inhibiting the expression of transient receptor potential melastatin-2 (TRPM2) might protect against RF and inflammation by preventing TGF-β1-mediated JNK activation. TGF-β can affect RF development *via* canonical or non-canonical TGF-β signaling pathways. Thus, anti-TGF-β treatment could ameliorate RF, but elucidating the exact mechanisms underlying RF development and identifying a treatment remain a challenge (Meng et al., 2016; Gu et al., 2020). Recently,

long non-coding RNAs (lncRNAs) have attracted much attention. Accumulating evidence suggests that lncRNAs influence several biological processes, such as epithelial–mesenchymal transition (EMT), alternative splicing, proliferation, autophagy, apoptosis, and protein synthesis (Lu et al., 2017; Xu et al., 2017; Catana et al., 2020; Zhao X. et al., 2020). lncRNAs also play an important role in the progression and prognosis of many diseases (Yan et al., 2019; Shen et al., 2020; Wu et al., 2020). Wu et al. (2020) showed that knockdown of lncRNA SNHG17 in prostate cancer cell lines decreased proliferation, invasion, migration, and EMT transition capability while promoting apoptosis. Overexpression of lncRNA NEAT1 promotes proliferation, migration, and invasion and increases the EMT process in HeLa and SiHa cells (Shen et al., 2020). Similarly, several studies indicate that lncRNAs play a crucial role in the initiation and pathological progression of RF.

CHARACTERISTICS AND MECHANISMS OF ACTION OF LncRNA

Long non-coding RNA are defined as a class of endogenous non-coding transcripts with a length of over 200 nucleotides (Engreitz et al., 2016) that lack a specific integrated open reading frame and do not have protein-coding capacity (Kapranov et al., 2007; Yan et al., 2017). These transcripts usually have an mRNA-like structure; after splicing, lncRNAs have their own promoters, poly-adenosine tails, 5′ cap structures, and splice variants (Okazaki et al., 2002; Quinn and Chang, 2016). However, compared with those of protein-coding genes, the promoters of lncRNA are more conserved and the expression of these RNA occurs at relatively low levels (Derrien et al., 2012). In humans, lncRNAs regulate more than 70% of gene expression, with dynamic expression and varying molecular mechanisms underlying their ontogenesis, suggesting these RNAs play different biological roles and are involved in various disease processes. Unlike miRNAs that play a role at the post-transcriptional level, lncRNAs do not have a universal mode of action. Indeed, lncRNAs can bind RNA, DNA, or proteins as part of many biological processes (Dykes and Emanueli, 2017). Furthermore, lncRNA binding can be either enhanced or inhibited within an organism (Ignarski et al., 2019). Increasing studies on lncRNAs illustrate that these molecules play crucial roles in regulating specific cellular processes, such as modulating gene expression at the transcriptional, post-transcriptional, and epigenetic levels (Kung et al., 2013; Cao, 2014). Furthermore, the subcellular localization of lncRNA has an impact on their function and mechanism of action. For example, if an lncRNA is located in the cytoplasm, it can act as competing endogenous RNA (adsorbed miRNA), thereby affecting gene expression by regulating mRNA stability, degradation, and translation (Tay et al., 2014). Nuclear lncRNAs can regulate chromosome architecture, gene transcription, and rate of transcription to repress or activate gene expression (Yang Z. et al., 2019). Thus, lncRNAs are extensively involved in cell proliferation, survival, apoptosis, migration, and other cellular activities and play a vital role in biological process and disease progression.

Long non-coding RNA can be classified into six categories based on their location in the proximal protein-coding genes and the genome, i.e., bidirectional, intergenic, exon intronic antisense, natural antisense, sense overlapping, and intron sense overlapping (Ponting et al., 2009). Although studies on the mechanism of action of lncRNAs are limited, accumulating evidence indicates that lncRNAs regulate target gene expression through four main mechanisms. The first mechanism is represented by the molecular signal model (e.g., **Figure 1A**). lncRNAs exhibit cell-specific expression and distinct responses to different stimulating factors, indicating that the expression of lncRNAs is considerably controlled at the transcriptional level (Wang and Chang, 2011). Furthermore, lncRNAs respond to intra- and extracellular signaling pathways and act as modulators of signaling pathways. Previous studies have shown that lncRNAs are specifically transcribed under different stimuli *via* distinct signaling pathways and are involved in specific signal transduction processes (Mercer and Mattick, 2013). lncRNA can perform regulatory functions by functioning as signaling molecules without any involvement of protein translation. The second lncRNA mechanism is represented by the molecular decoy model (e.g., **Figure 1B**). After transcription, lncRNA can competitively bind to some RNA or proteins, thereby freeing a specific DNA region or target protein for other interactors (e.g., transcription factor or transcriptional regulator) and leading to the degradation of the target mRNA. Similarly, lncRNAs can bind to miRNAs, thereby eliminating the effects of miRNAs under physiological conditions, which would otherwise decrease or enhance mRNA stability to regulate the normal function of downstream target genes (Yao et al., 2019). For example, lncRNA MALAT1 can adjust pre-mRNA alternative splicing by trapping splicing factors inside nuclear speckles, whereas cytoplasmic lncRNAs can bind to miRNAs and derepress mRNA translation (Tripathi et al., 2010; Cesana et al., 2011). The third method represents the molecular guide model (e.g., **Figure 1C**). After transcription, lncRNAs interact with transcription factors and transcriptional regulators to guide transcription complexes to specific sites in the genome. Chromatin modifiers induce changes in local histone modifications to impact the expression of adjacent genes (Mercer and Mattick, 2013). Previous studies suggest that lncRNA-mediated transcriptional regulation can affect transcription, mRNA stability, or translation through homeopathic or *trans*-activation patterns (Wang and Chang, 2011). The last lncRNA mechanism represents the molecular scaffold model (e.g., **Figure 1D**). This is one of the most functionally intricate mechanisms of action of lncRNA, where lncRNA serves as a central platform to bind two or more macromolecules (e.g., proteins, DNA, or other RNA species) (Ignarski et al., 2019) and brings them into close proximity to chromatin. This mechanism achieves information convergence and integration between different signaling pathways, in a spatiotemporal manner, suggesting that lncRNAs play a central role in epigenetic processes as well as chromatin modification. Once we achieve a better understanding of the mechanisms underlying the abovementioned scaffolding role of lncRNA, whereby macromolecules are packaged in close proximity and expression is regulated, strategies can be designed to leverage

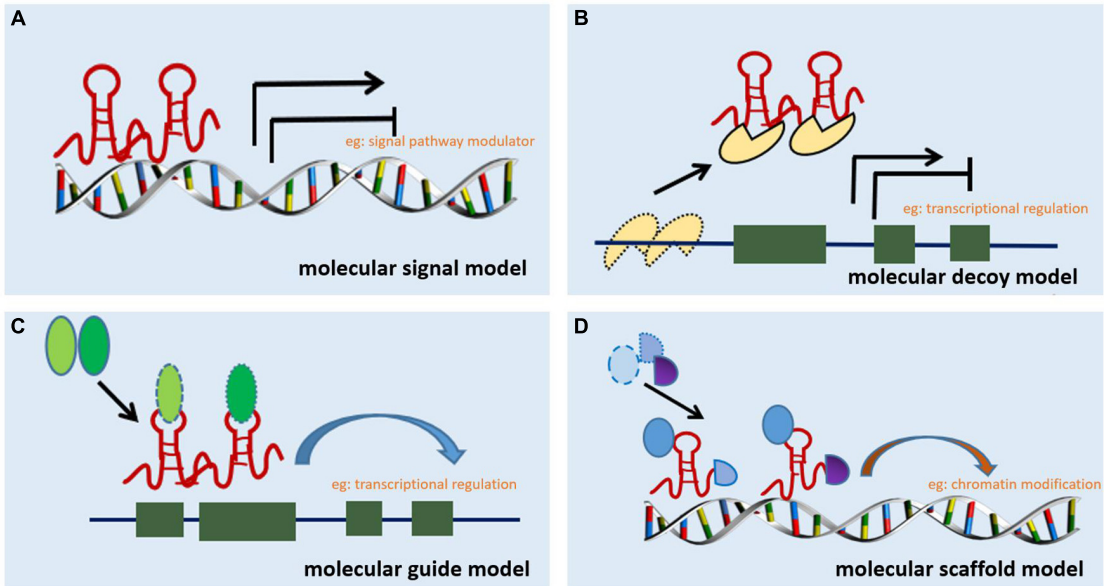

FIGURE 1 | Mechanisms of action of lncRNA. **(A)** Molecular signal model: lncRNAs act as signaling pathway modulators to influence gene regulation in response to different stimuli. **(B)** Molecular decoy model: lncRNAs competitively bind to some RNA or proteins, causing the target to free a specific DNA region or protein, and then regulate the normal function. **(C)** Molecular guide model: lncRNAs interact with transcription factors or transcriptional regulators to guide transcription complexes to specific sites in the genome. **(D)** Molecular scaffold model: lncRNAs serve as a scaffold to bind two or more macromolecules (e.g., proteins, DNA, or other RNA species) and bring them into close proximity to chromatin.

specific signaling components to redirect and reshape cell function (Wang and Chang, 2011). A number of studies indicate that lncRNAs are also involved in the pathological process of RF, but a clear relationship among these processes remains unknown.

In this paper, we summarized recent studies on the role of lncRNAs in the pathogenesis of RF (**Table 1**). Understanding the precise mechanism of action of lncRNAs in RF may aid future studies and might be helpful for developing more effective therapies aimed at preventing RF.

SEVERAL LncRNAs PLAY A PROMOTING ROLE IN RF

LncRNA Erbb4-IR

Long non-coding RNA Erbb4-IR is a Smad3-related lncRNA that plays a role in kidney fibrosis (Zhou et al., 2014; Feng et al., 2018). This lncRNA is situated in the intron area of the *Erbb4* gene on chromosome 1 of mouse (Sun et al., 2018). In a unilateral ureteral occlusion (UUO) kidney model, Erbb4-IR was found to be highly upregulated in mouse tubular epithelial cells (mTECs). The level of lncRNA Erbb4-IR affected the transcription course of collagen I and α-SMA *via* a Smad3-related mechanism in TGF-β1-treated mTECs. Research has also revealed that *Smad7* is a target gene of Erbb4-IR and that specifically silencing the expression of Erbb4-IR resulted in the upregulation of renal Smad7, thereby blunting the TGF-β1/Smad3-induced RF *in vivo* and *in vitro* (Feng et al., 2018).

Advanced glycosylation end products (AGEs) induce RF through the TGF-β/Smad pathway by binding to either Smad7 or Smad3. Thus, these molecules can be potentially exploited as novel treatment modalities for DN (Chung et al., 2010). Chung et al. (2010) revealed that Erbb4-IR is highly upregulated after stimulation of mTECs and mouse mesangial cells (MMCs) with AGEs [rather than *via* high glucose (HG) *via* a Smad 3-dependent mechanism]. Furthermore, Smad3 knockdown blunted AGE-induced Erbb4-IR expression. Bioinformatic analysis revealed the presence of an Erbb4-IR binding site in the 3′ UTR of miR29b. Previous studies confirm that miR-29b protects against the progression of RF by regulating the TGF-β/Smad-dependent pathway under diabetic conditions. Additionally, miR-29b directly binds to the 3′ UTRs of collagen I and collagen IV, thereby suppressing RF (Xiao et al., 2012). Furthermore, Erbb4-IR knockdown in db/db mice resulted in reduced expression of collagen I and IV at mRNA and protein levels, an observation that may suggest effective future therapeutics for type 2 DN (T2DN) (Sun et al., 2018).

LncRNA HOTAIR

Long non-coding RNA HOTAIR influences the progression of liver and myocardial fibrosis (Bian et al., 2017; Yu et al., 2017; Pan et al., 2018) while also being involved in the progression of renal interstitial fibrosis (RIF). Zhou et al. (2019a) showed that the overexpression of lncRNA HOTAIR downregulates miR-124 to activate the Notch1 pathway, thereby promoting EMT in TGF-β1-induced HK-2 cells and RIF in UUO rats. Paeonol reversed the effects of the HOTAIR/miR-124/Notch 1/Jagged1 axis on RIF

TABLE 1 | LncRNA dyregulated in RF diseases.

lncRNA categories	Main disease model/cell lines	Suggested function	Target gene/signaling	Function	Application	References
Erbb4-IR	UUO mouse kidney, TGF-β1 treated mTECs	Affect the transcription of collagen I and α-SMA	Smad7	Pro-RF	Therapeutic target for RF and T2DN	Feng et al., 2018
	T2DN mice (db/db), AGEs treated mTECs and mMCs	Transcriptional repression	miR-29b			Sun et al., 2018
HOTAIR	UUO rat's kidney, TGF-β1 induced HK-2 cells and NRK-49F cells	miRNA binding	miR-124, Notch1/Jagged1 signaling pathway	Pro-RF	Therapeutic target for RIF	Zhou et al., 2019a,b
LINC00667	Human CRF tissues, CRF rat models, renal tubular epithelial cells	miRNA binding	miR-19b-3p	Pro-RF	Therapeutic target and prognostic marker in CRF	Chen et al., 2019
Arid2-IR	UUO mice kidney, TGF-β1 treated HK-2 cells	Affect interleukin-1β-NF-κB signaling and inflammatory cytokine	Smad3	Pro-RF	Therapeutic target for renal Inflammation	Zhou Q. et al., 2015
	HFD and STZ-induced diabetic mice/HG-induced mMCs	Affect the expression of ECM marks	–		Therapeutic target for DKD	Yang Z. et al., 2019
Blnc1	DN patient's serum, STZ-induced DN rats, HG-induced HK2 cells	Affect inflammation, oxidative stress and RF	Nrf2/HO-1 and NF-κB pathways	Pro-RF	A potential therapeutic target in DN	Feng et al., 2019
NONHSAG053901	DN mouse model/MCs	Mediate renal inflammation	Egr-1/TGF-β pathway	Pro-RF	Diagnostic and therapeutic target in DN.	Peng and Huang, 2019
TCONS_00088786	UUO rat's kidney, TGF-β1 induced NRK52E cells	miRNA binding	miR-132	Pro-RF	therapeutic target in RIF	Zhou et al., 2018
ARAP1-AS2	HG-induced HK2 cells	Participate in cytoskeleton rearrangement and EMT processes	–	Pro-RF	A pathogenic role in DN	Li L. et al., 2020
ATB	UUO model/TGF-β1 induced HK2 cells	Participate in EMT	–	Pro-RF	Therapeutic target in RF	Zhou and Jiang, 2019
Gm4419	db/db DN mice/HG treated MCs	Affect inflammation, fibrosis and proliferation	NF-κB/NLRP3 inflammasome signaling pathway	Pro-RF	Therapeutic target for DN	Yi et al., 2017
NR_033515	DN patient's serum, HFD and STZ-induced diabetic kidney mice/HG-induced MMCs cells, BSA-stimulated HK2 cells	miRNA binding	miR-743b-5p	Pro-RF	A diagnostic and therapeutic target for DN	Gao et al., 2018
CHCHD4P4	Glyoxylate-treated mouse kidneys /COM treated HK-2 cells	Affect the EMT and cell proliferation	–	Pro-RF	A diagnostic and therapeutic target in calcium oxalate-induced kidney damage	Zhang et al., 2017
LOC105375913	Focal segmental glomerulosclerosis patient's tubulointerstitial tissues/HK-2 cells	miRNA binding	miR-27b	Pro-RF	Therapeutic target for tubulointerstitial fibrosis	Han et al., 2019
MALAT1	STZ-induced DN/HG-induced mouse podocytes	Expression changed	Serine/arginine splicing factor 1 (SRSF1)	Pro-RF	A diagnostic and therapeutic target in RF diseases	Hu et al., 2017
	db/db DN mouse and HG-induced HK-2 cells	miRNA binding	miR-145-ZEB2 axis			Liu D. W. et al., 2019a
	UUO mouse, TGF-β1 induced HK2 cells	miRNA binding	miR-145-FAK axis			Liu et al., 2020
NEAT1	STZ-induced diabetic mice, HG-induced MMCs cells	Expression changed	Akt/mTOR signaling	Pro-RF	A pathogenic role in DN	Huang et al., 2019
	HFD- and STZ-induced DM mice, BSA treated HK2 cells	Expression changed	ERK1/2 signaling		Therapeutic target for DKD	Yang et al., 2020
	DN patient's plasma, HG-induced MMCs cells	miRNA binding	miR-23c		Therapeutic target for DN	Li N. et al., 2020
PVT1	DN patient's serum, HG-induced Mouse podocyte clone 5 (MPC5) podocytes and primary podocytes, STZ-induced DN mice	Expression changed	FOXA1	Pro-RF	Therapeutic target in DN	Liu D. W. et al., 2019
	db/db DN mouse, HG-treated MMCs	miRNA binding	miR-93-5p			Li J. et al., 2020
	DN patient's serum, HG-treated MCs	miRNA binding	miR-23b-3p			Zhong et al., 2020

(Continued)

TABLE 1 | Continued

lncRNA categories	Main disease model/cell lines	Suggested function	Target gene/signaling	Function	Application	References
XIST	MN patient's kidney biopsy, Ang II treated podocytes	miRNA binding	miR-217	Pro-RF	Diagnosis biomarker and therapeutic target for MM	Jin et al., 2019
	DN patient's kidney, STZ-induced DN mice, HG treated HK-2 cells	miRNA binding	miR-93-5p		Therapeutic target for DN	Yang J. et al., 2019
1700020I14Rik	db/db DN mice/HG-treated MCs cells	miRNA binding	miR-34a-5p	Anti-RF	Therapeutic target in DN	Li et al., 2018
CYP4B1-PS1-001	db/db DN mice, HG-induced MMCs cells	Affect proliferation and fibrosis	–	Anti-RF	A biomarker for prognosis and a therapeutic target in DN	Wang et al., 2016a
	db/db DN mice, HG-induced MMCs cells	Protein binding	NCL			Wang S. et al., 2018
ENSMUST000 00147869	db/db DN mice/HG-induced MMCs cells	Affect proliferation and fibrosis	–	Anti-RF	Therapeutic target for DN	Wang et al., 2016b
MEG3	TGF-β1 induced HK2 cells	miRNA binding	miR-185	Anti-RF	Therapeutic target for RF	Xue et al., 2019
ZEB1-AS1	DN patient kidney biopsy, HG-induced HK2 cells	miRNA binding	miR-216a-5p	Anti-RF	Therapeutic target in DN	Meng and Zhai, 2020
	DN patient kidney biopsy, STZ-Induced DN mice, HG-induced HK2 cells	Expression changed	P53			Wang J. et al., 2018
ENST00000453774.1	Clinical RF specimens, UUO mice, TGF-β-induced HK-2 cells	Expression changed	Nrf2-keap1/HO-1/NQO-1 signaling	Anti-RF	Therapeutic target in RF	Xiao et al., 2019
NR03832	STZ-Induced DN Rats/HG treated HK-2 cells	miRNA binding	miR-324-3p	Anti-RF	Therapeutic target in DN	Ge Y. et al., 2019
MIAT	UUO, IRI kidneys	Affect myofibroblast formation	–	Pro-RF	Therapeutic target in RF	Bijkerk et al., 2019
	Human renal fibrotic tissues, UUO mice/TGF-β1 induced HK-2 cells	miRNA binding	miRNA-145	Pro-RF	Therapeutic target in RIF	Wang Z. et al., 2020
	STZ-induced diabetic rats/HG treated HK2 cells	Expression changed	Nrf2	Anti-RF	Therapeutic target	Zhou L. et al., 2015
GAS5	HG treated HK-2 cells	miRNA binding	miR-27a	Pro-RF	Diagnostic and therapeutic targets for DN	Lv et al., 2019
	TGF-β1 treated HK-2 cells and the kidneys of HDF/STZ mice	miRNA binding	miR-96-5p	Pro-RF	Therapeutic targeting in DKD	Wang W. et al., 2020
	HG-treated HK-2 cells	miRNA binding	miR-452-5p	Anti-RF	Therapeutic targeting in DN	Xie et al., 2019
	DN patients and HG treated MCs	miRNA binding	miR-221	Anti-RF	Therapeutic targeting for DN	Ge X. et al., 2019
	STZ treated DN rats	Recruiting EZH2 to MMP9 promoter region	MMP9 promoter region	Anti-RF	Therapeutic target in RF	Ge X. et al., 2019
TUG1	LPS treated HK2 cells	miRNA binding	miR-223	Anti-RF	Therapeutic target for lupus nephritis	Xu et al., 2018
	db/db DN mice, HG-treated NRK-52E cells	miRNA binding	miR-21		Therapeutic target for DN	Wang F. et al., 2019
	STZ-treated DN Rats, HG treated MMCs	Affect mesangial cells' proliferation and ECM accumulation	PI3K/AKT pathway		Therapeutic target for DN	Zang et al., 2019
	HG treated human podocyte cell line (CIHP) cells	Affect podocytes apoptosis	ERS–CHOP–PGC-1α signaling	Pro-RF	Therapeutic target for DN	Shen et al., 2019
	Renal I/R rat, I/R injured HK2 cells	miRNA binding	miR-449b-5p		Diagnosis biomarker and therapeutic target for AKI	Xu et al., 2020

Re = references; RF = renal fibrosis; Pro-RF = promoting renal fibrosis; anti-RF = anti-renal fibrosis.

and inhibited the effects of HOTAIR on EMT and migration of NRK-49F cells (Zhou et al., 2019b).

LncRNA LINC00667

GSE37171 chip analysis revealed that lncRNA LINC00667 is upregulated in human chronic renal failure (CRF) tissues. The expression of TGF-β1, α-SMA, connective tissue growth factor (CTGF), and tissue inhibitor of metalloproteinase 1 at the mRNA and protein levels was also upregulated. A follow-up study showed that miR-19b-3p directly binds to lncRNA LINC00667 and CTGF. CTGF was upregulated and miR-19b-3p was downregulated in human CRF tissues. Additionally, miR-19b-3p overexpression offsets the positive profibrosis effect on lncRNA LINC00667 and the expression of fibrogenic factors in a CRF rat model (Chen et al., 2019). Thus, lncRNA LINC00667 is a potential therapeutic target and a novel prognostic marker in CRF.

LncRNA Arid2-IR

Using high-throughput RNA-sequencing (RNA-Seq), 151 differently expressed Smad3-associated lncRNAs were found between the UUO kidney and Smad3 knockout mice (Zhou Q. et al., 2015). Smad3 plays an important role in renal inflammation and fibrosis, and a Smad3 binding site was located in a highly conserved region 1.6 kb upstream of Arid2-IR. Smad3 knockdown counteracted Arid2-IR upregulation in UUO kidneys. Thus, lncRNA Arid2-IR is a potential transcriptional target of Smad3. Further investigation clearly demonstrated that Arid2-IR knockdown in mTECs did not affect the TGFβ1-induced fibrotic process, including ECM marker expression,

but inhibited NF-κB/p65 phosphorylation and interleukin-1β-mediated DNA binding, thereby inhibiting proinflammatory cytokine and chemotactic factor secretion. These results are consistent with the finding that *in vitro* deletion of Arid2-IR from UUO kidney cells has no effect on RF and the TGF-β/Smad3 pathway other than the inactivation of NF-κB signaling.

Transcription of lncRNA could be directly regulated by transcription factors. Yang Y. L. et al. (2019) confirmed that early growth response protein-1 (Egr1) is highly expressed in the kidneys of mice with diabetic kidney disease (DKD). Egr1, a transcription factor, enhances the proliferation rate and ECM production of mesangial cells (MCs) in DKD. Arid2-IR expression was significantly decreased after Egr1 knockdown, and Arid2-IR overexpression in HG-cultured MMCs was offset by Egr1 knockdown, thereby downregulating collagen 1 and α-SMA expression. Yang Y. L. et al. (2019) proved that Arid2-IR was also regulated by Egr1 in high-fat diet (HFD)-fed mice and in mice with streptozotocin (STZ)-induced diabetes. Nevertheless, more precise cross talks between Egr1 and Arid2-IR should be investigated.

LncRNA Blnc1

Recent studies have demonstrated that the level of Blnc1 was increased in the serum of patients with DN and in rats with STZ-induced DN (Feng et al., 2019). Additionally, tissue damage and fibrosis were increased compared with those in the control group. Furthermore, Feng et al. (2019) showed that Blnc1 inhibition significantly reduced the level of fibrosis, inflammation, and oxidative factors *in vitro*. Additionally, HG injury in HK-2 cells significantly reduced the level of NRF2/HO-1 protein and activated the NF-κB pathway. These effects were reversed upon Blnc1 inhibition. Thus, Blnc1 serves as a novel regulator of inflammation, oxidative stress, and RF *via* the NRF2/HO-1 and NF-κB pathways in DN.

LncRNA NONHSAG053901

Ho et al. (2016) confirmed that renal failure activates Egr-1, and Egr-1 deficiency alleviates TGF-β-induced renal inflammation and fibrosis. Concordantly, further investigations showed that lncRNA NONHSAG053901 binds directly to Egr-1. lncRNA NONHSAG053901 overexpression increased the expression of proinflammatory cytokines and RF biomarkers *via* the Egr-1/TGF-β pathway in MMCs. These effects were partially or fully restored upon co-transfection of siRNA against Egr1 (Peng and Huang, 2019). Thus, lncRNA NONHSAG053901 may serve as a promising target for antifibrotic therapies.

LncRNA TCONS_00088786

To identify new potential molecular targets and biomarkers for RF, the strategy of high-throughput RNA-Seq followed by qRT-PCR was employed. This enabled the identification of differentially expressed RNA in urine and kidney tissue from rats after a 2-week UUO (Sun et al., 2017). The expression of 24 lncRNAs was upregulated and that of 79 lncRNAs was downregulated in the kidneys of UUO rats; the expression of 625 lncRNAs was upregulated and that of 177 lncRNAs was downregulated in the urine of UUO rats. In this study, TCONS_00088786—harboring a putative promoter containing a few conserved Smad3 binding motifs—was identified. Additionally, TCONS_00088786 was found to be dose- and time-dependently expressed in response to TGF-β induction and influence the expression of some fibrosis-related genes *via* a negative feedback loop in NRK52E cells.

Zhou et al. (2018) showed that the expression of lncRNA TCONS_00088786 was significantly increased in the UUO kidney *in vivo* and in TGF-β-treated NRK52E cells. Increased TCONS_00088786 levels upregulate the expression of miR-132, collagen I, and collagen III. Silencing of lncRNA TCONS_00088786 results in decreased expression of miR-132, collagen I, and collagen III. Therefore, this study demonstrated that lncRNA TCONS_00088786 contributes to the progression of interstitial fibrosis by upregulating the expression of miR-132. These findings indicate that TCONS_00088786 could definitely serve as a new therapeutic target for RIF.

LncRNA ARAP1-AS2

Li L. et al. (2020) observed enhanced ARAP1-AS2 and ARAP1 expression in HG-stimulated HK-2 cells. Cdc42-GTP, cytoskeletal remodeling, cell viability, and migration were also increased in HG-treated HK-2 cells. Inhibition of ARAP1 expression counteracts the effects of HG and ameliorates RF. Furthermore, the overexpression of ARAP1-AS2 significantly increased EMT by positively regulating the expression of ARAP1. However, the mechanism by which ARAP1-AS2 regulates ARAP1 expression remains unclear. These results suggested that ARAP1-AS2/ARAP1 may affect RF *via* increased Cdc42-GTP levels in HG-treated HK-2 cells, a phenomenon that suggests new strategies to minimize DN progression (Li L. et al., 2020).

LncRNA-ATB

Livin, a member of the anti-apoptotic protein family, is associated with the development, progression, and drug resistance of many human tumors *via* the stimulation of EMT (Li et al., 2013; Ge et al., 2016). Zhou and Jiang (2019) investigated the expression of livin in UUO models and TGF-β1-treated HK-2 cells and found that UUO elicits a high expression of livin and lncRNA-ATB. When livin was knocked-out using siRNA, the expression of lncRNA-ATB was significantly downregulated, thereby inhibiting TGF-β1-induced EMT in HK-2 cells. Hence, lncRNA-ATB and livin could serve as prominent therapeutic targets for RF.

LncRNA Gm4419

Several studies demonstrate that the activation of NF-κB and NLRP3 is a critical link between inflammation and DN progression. Using RNA-Seq in the kidney tissues of db/db DN mice, Yi et al. (2017) identified 14 abnormally expressed lncRNAs, including lncRNA-Gm4419. Additionally, the expression of lncRNA Gm4419, p50, and NLRP3 inflammasomes was upregulated in MCs cultured in the presence of HG, and Gm4419 knockdown significantly downregulated the expression of proinflammatory cytokines and RF biomarkers in MCs. Furthermore, Gm4419 directly interacted with the p50 subunit of NF-κB to activate the NF-κB pathway. Additionally, the promoter region of the gene coding for the

NLRP3 inflammasomes contains a p50 binding site in MCs. Overexpression of p50 or Gm4419 might increase the expression of NLRP3 inflammasomes, but Gm4419 overexpression did not alter the expression of proinflammatory cytokines and NLRP3 inflammasomes in MCs after transfection with SN50 (a p50-specific inhibitor) (Yi et al., 2017). Taken together, this study indicates that Gm4419 is a novel NF-κB-associated lncRNA, which activates the NF-κB/NLRP3 inflammasome signal *via* the interactions of p50 with Gm4419 and NLRP3 inflammasomes in MCs.

LncRNA NR_033515

Gao et al. (2018) proposed that lncRNA NR_033515—whose expression is dramatically upregulated in the serum of patients with DN and is closely correlated with the different stages of DN—could serve as a crucial diagnostic and therapeutic target. lncRNA NR_033515 also plays a prominent role as a diagnostic marker of DN. *In vitro* studies demonstrated that increased NR_033515 expression in MMCs promotes the expression of PCNA and cyclin D1; upregulates ASK1, FN, α-SMA, and P38 expression; and is positively associated with the expression of EMT biomarkers (E-cadherin and vimentin) *via* miR-743b-5p (Gao et al., 2018). Further investigations are needed to validate the potential interactions between NR_033515 and miR-743b-5p in DN.

LncRNA CHCHD4P4

When renal tubules become injured due to calcium oxalate crystal deposition, EMT occurs in the epithelial cells of renal tubules, thereby initiating RF. In total, 376 lncRNAs were differentially expressed between the glyoxylate-exposed and healthy mice kidney groups (Zhang et al., 2017). Further analysis of the human and mouse lncRNAs, i.e., CHCHD4P4 homologs—which were identified in mice and humans using BLAST—revealed a 425-bp-long lncRNA located on chromosome 3 (Zhang et al., 2017). Calcium oxalate monohydrate (COM) induced the overexpression of CHCHD4P4 in HK-2 cells. Silencing of CHCHD4P4 resulted in the inhibition of mesenchymal-like morphological features and decreased the transcription of vimentin, zinc finger E-box binding homeobox1 (ZEB1), and Snail. More importantly, CHCHD4P4 overexpression inhibited cell proliferation by promoting the apoptosis of HK-2 cells treated with COM, suggesting that CHCHD4P4 might aid the early diagnosis and treatment of kidney disease. Additional research is necessary to explore the mechanism by which CHCHD4P4 regulates the expression of EMT-related genes.

LncRNA LOC105375913

In the tubulointerstitial tissue of patients with focal segmental glomerulosclerosis (FSGS), the level of LOC105375913 was significantly increased and positively correlated with the tubulointerstitial fibrosis score (Han et al., 2019). Overexpression of LOC105375913 in HK-2 cells increased the expression of FN, collagen I, and Snail at mRNA and protein levels in HK-2 cells and in the tubular cells of patients with FSGS. Bioinformatic analysis and RNA pull-down revealed that LOC105375913 functions as ceRNA and competitively binds to miR-27b, thus regulating Snail expression and causing tubulointerstitial fibrosis in mice and in C3a-stimulated HK-2 cells. p38 and the transcription factor XBP-1s regulate LOC105375913 expression in HK-2 cells. Overexpression of XBP-1s or p38 also increases the level of endogenous LOC105375913, promotes the binding of miR-27b to LOC105375913, and increases the expression of fibrosis markers in HK-2 cells. Conversely, this binding between LOC105375913 and miR-27b was significantly inhibited upon XBP-1s knockdown or p38 inhibition, and this resulted in decreased expression of fibrosis markers in HK-2 cells (Han et al., 2019).

LncRNA MALAT1

MALAT1 is also known as mascRNA because it is located in the cell nucleus with a cytoplasmic tRNA-like small RNA (Zong et al., 2016). Hu et al. (2017) showed that the expression of lncRNA MALAT1 is dramatically increased in the background of STZ-induced DN when proteinuria was present and is correlated with by HG-induced podocyte damage. MALAT1 knockdown enhances the integrity of podocyte architecture and function by downregulating SRSF1 expression and reduces the nuclear accumulation of β-catenin triggered by HG. Furthermore, β-catenin also regulates MALAT1 expression due to its ability to bind to the MALAT1 promoter region. Downregulated expression of the β-catenin gene decreases MALAT1 expression, while MALAT1 regulates the pattern of post-transcriptional β-catenin splicing. These results demonstrate the feedback loop mechanism that exists between β-catenin and MALAT1 during podocyte damage (Hu et al., 2017). Furthermore, the expression of MALAT1 is increased in the plasma and kidney tissues of patients with acute kidney injury, in hypoxic kidney biopsies of mice, and in cultured and *ex vivo*-sorted hypoxic endothelial and HK-2 cells (Kölling et al., 2018). Liu B. et al. (2019) provided more evidence for the molecular mechanisms underlying EMT and RF in DN. In their study, MALAT1 was significantly upregulated, while miR-145 was downregulated in the renal tissues of DN mice. They proposed that MALAT1 functions as a "sponge" for miR-145 and subsequently upregulates the expression of its target gene *ZEB2* to promote EMT and fibrosis in HK-2 cells cultured in HG medium. Furthermore, Liu et al. (2020) found that m6A is the primary methyltransferase, which induces lncRNA MALAT1-exacerbated renal fibrogenesis in obstructive nephropathy *via* the miR-145/FAK signaling pathway. Taken together, lncRNA MALAT1 may be a potential biomarker for the diagnosis and treatment of RF in CKD.

LncRNA NEAT1

Long non-coding RNA NEAT1 is a pivotal regulator of the mitogen-activated protein kinase (MAPK) pathway in human lupus disease (Zhang et al., 2016). In STZ-induced diabetes mellitus (DM) rats and glucose-treated MMCs, NEAT1 is significantly upregulated. Interestingly, the proliferation of MMCs and fibrosis in DN are inhibited by NEAT1 siRNA *via* activation of Akt/mTOR signaling (Huang et al., 2019). As an anti-aging protein, Klotho markedly alleviates renal tubular EMT and inhibits NEAT1 expression during DN development. Silencing NEAT1 in BSA-induced HK-2 cells can reverse the

protective effect caused by Klotho *via* ERK1/2 signaling (Yang et al., 2020). These results are consistent with those obtained for STZ- and HFD-treated DN mice. To further investigate the pivotal role of NEAT1 in DN, Li N. et al. (2020) demonstrated that lncRNA NEAT1 expression was dramatically elevated in the serum of patients with DN. As described above, these findings suggest a new regulatory pathway involving NEAT1, which might be a potential therapeutic target for DKD.

LncRNA PVT1

Plasmacytoma variant translocation 1 (PVT1) is a famous lncRNA regulator in DM (Hanson et al., 2007; Alwohhaib et al., 2014). PVT1, a 1.9-kb-long lncRNA, mediates the overexpression of ECM proteins in DN (Alvarez and DiStefano, 2011) and was the first lncRNA reported to be related to kidney disease (Alvarez et al., 2016). Accordingly, PVT1 is upregulated in the serum of patients with DN (Liu D. W. et al., 2019; Zhong et al., 2020). Similarly, PVT1 expression significantly increased in mouse podocyte clone-5 and in primary podocytes in an HG environment (Liu D. W. et al., 2019). Moreover, PVT1 recruits the enhancer of Zeste homolog 2 (EZH2) to facilitate the recruitment of H3K27me3 to the FOXA1 promoter area, thus downregulating FOXA1 expression to promote apoptosis and podocyte damage in DN (Liu D. W. et al., 2019).

PVT1 is also overexpressed in the serum of patients with DN, kidneys of mice with DN, and HG-induced MMCs or human MCs (hMCs). PVT1 knockdown inhibits cellular migration, invasion, proliferation, and fibrosis. Furthermore, PVT1 knockdown blocks the PI3K/Akt/mTOR signal and promotes the apoptosis of MMCs under HG conditions by upregulating miR-93-5p (Li J. et al., 2020). Furthermore, Zhong et al. (2020) validated that silencing PVT1 might relieve HG-induced FN and α-SMA expression and proliferation in hMCs by inhibiting the NF-κB pathway *via* the miR-23b-3p/WT1 axis.

LncRNA XIST

Aberrant expression of lncRNA XIST is closely related to the inactivation of the X chromosome. This lncRNA is a well-known tumor suppressor gene or oncogene in many tumors, but little research has been conducted regarding its role in RF. Huang et al. (2014) were the first to report significantly increased lncRNA XIST levels in the urine of patients with membranous nephropathy (MN); this increase in lncRNA XIST levels in the urine is positively related to disease severity. They also found that XIST is regulated by H3K27me3 levels in the kidney of mice with MN. These results provide new insights into the diagnosis and treatment of MN. Furthermore, lncRNA XIST functions as a ceRNA for miR-217 to facilitate Toll-like receptor 4 (TLR4) expression, thus inducing the apoptosis of podocytes (Jin et al., 2019). Furthermore, lncRNA XIST levels are increased in the kidneys of patients with DN, in HG-treated HK-2 cells, and in mice with DN. XIST knockdown inhibits RIF in DN by repressing cyclin-dependent kinase inhibitor 1A and increasing miR-93-5p expression (Yang J. et al., 2019). Further studies based on the results of the abovementioned studies are warranted before lncRNA XIST can be used as a novel clinic biomarker for patients with DN.

SEVERAL LncRNAs PLAY AN ANTIFIBROSIS ROLE IN RF

LncRNA 1700020I14Rik

Long non-coding RNA 1700020I14Rik (ENSMUST00000147425) is located in chromosome 2 (Chr2: 119594296–119600744). Li et al. (2018) demonstrated that this lncRNA 1700020I14Rik functions as a miRNA sponge and competitively binds to miR-34a-5p. While other lncRNAs were significantly downregulated in HG-cultured MCs and in db/db DN mice, lncRNA 1700020I14Rik displayed the highest sequence conservation in the cells and mice compared with their homologous sequence in humans. Furthermore, overexpression of lncRNA 1700020I14Rik reduces the proliferation and expression of fibrosis markers (Col-4, FN, TGFβ1) of MCs in an HG environment by directly interacting with miR-34a-5p, which inhibits the Sirt1/HIF-1α signaling pathway. These changes were reversed by miR-34a-5p mimics. Thus, lncRNA 1700020I14Rik probably serves as an important therapeutic target for DN.

LncRNA CYP4B1-PS1-001

Long non-coding RNA CYP4B1-PS1-001 (transcript ID: ENSMUST00000118753) is located on the reverse strand of chromosome 10 and is significantly downregulated in db/db mouse models (Wang et al., 2016a). Moreover, dose-dependently decreased CYP4B1-PS1-001 expression was further confirmed in MMCs under different glucose levels. Overexpression of CYP4B1-PS1-001 altered the expression of proliferation indexes and fibrosis markers in MMCs during the progression of DN (Wang et al., 2016a). Further studies demonstrated that CYP4B1-PS1-001 promoted nucleolin (NCL) ubiquitination and degradation, thereby inhibiting the fibrosis process of MMCs and indicating that the CYP4B1-PS1-001/NCL axis might be a prognostic biomarker and effective therapeutic target for the treatment of DN (Wang S. et al., 2018).

LncRNA ENSMUST00000147869

ENSMUST00000147869, which is 629 nt long and located on chromosome 4, is downregulated in the renal tissues of DN mice (Wang et al., 2016b). Overexpression of ENSMUST00000147869 reduces the expression of fibrosis markers and proliferation indexes in MMCs under HG conditions. Wang et al. (2016b) provided new insights into the pathogenesis and development of DN.

LncRNA MEG3

Long non-coding RNA MEG3 is situated on the human chromosome 14q32 (Wylie et al., 2000). Xue et al. (2019) found that MEG3 is significantly downregulated in TGF-β1-treated HK-2 cells. MEG3 overexpression significantly decreases the expression of mesenchymal markers and increases the expression of epithelial markers. Moreover, miR-185 regulates the expression of DNA methyltransferase 1 (DNMT1), which thereby regulates the expression of MEG3 *via* modulation of CpG methylation in the MEG3 promoter in TGF-β1-induced HK-2 cells (Xue et al., 2019). Thus, the miR-185/DNMT1/MEG3 pathway is a

new mode of regulation for RF and further verification of its role in RF is needed.

LncRNA ZEB1-AS1

Long non-coding RNA ZEB1-AS1, which is transcribed from a shared bidirectional promoter with ZEB1 (Gao et al., 2019), is a cancer-related and antifibrotic lncRNA. When expressed, this gene acts as an oncogenic regulator in many human tumors (Ni et al., 2020). Meng and Zhai (2020) demonstrated that ZEB1-AS1 overexpression inhibits HG-induced RF by suppressing EMT and fibrogenesis. Mechanistically, they identified that ZEB1-AS1 impedes RF by regulating BMP7 expression and inhibits EMT of HK-2 cells by competitively binding to miR-216a-5p (Meng and Zhai, 2020). Several studies reported that BMP7, which plays a key role in many renal diseases, is a direct target of miR-216a-5p. Importantly, as predicted by bioinformatics analysis and confirmed by the luciferase reporter assay, the expression levels of fibrosis-related proteins and EMT-related markers reduced significantly with the overexpression of ZEB1-AS1 in the HG-treated HK-2 cells. The expression of these markers is restored by targeting miR-216a-5p and downregulating BMP7 in HK-2 cells (Meng and Zhai, 2020).

Previous findings showed that p53 is significantly upregulated in STZ-induced mice with DN (Peng et al., 2015). Inhibition of p53 expression may ameliorate TGF-β-induced RF induced in UUO (Overstreet et al., 2014; Peng et al., 2015). Wang J. et al. (2018) found that p53 is mainly expressed in the renal tubular cells of db/db mice at 16 weeks of age. Furthermore, they reported that p53 inhibitors and the deletion of p53 in the proximal tubule ameliorate interstitial fibrosis in db/db mice and in STZ-induced DN mice. Furthermore, p53 physically interacts with the promoter region of lncRNA ZEB1-AS1. They also verified that lncRNA ZEB1-AS1 binds to the H3K4 methyltransferase MLL1 and promotes H3K4me3 histone modification in the ZEB1 promoter, which negatively regulates ZEB1 expression in HK-2 cells and causes ECM accumulation (Wang J. et al., 2018). Taken together, these findings provide evidence that p53-lncRNA ZEB1-AS1 and the ZEB1 axis may be new potential therapeutic targets for RF in DN.

LncRNA ENST00000453774.1

Long non-coding RNA microarray profiling was used to detect lncRNA dysregulation in TGF-β-treated HK-2 cells. The results show that lncRNA ENST00000453774.1 (lncRNA 74.1) is dramatically downregulated, consistent with the results obtained from clinical RF specimens (Xiao et al., 2019). Autophagy and oxidative stress are closely related in RF. Interestingly, overexpression of lncRNA 74.1 promotes ROS defense mechanisms *via* the Nrf2-keap1/HO-1/NQO-1 pathway by accelerating pro-survival autophagy and decreasing the expression of ECM markers, collagen I, and FN. These effects were found to alleviate RF and therefore represent a potential treatment for renal diseases (Xiao et al., 2019).

LncRNA NR_038323

LncRNA_NR038323 is located on chromosome 8 (Chr8: 23336208–23366125). Ge Y. et al. (2019) demonstrated that lncRNA NR_038323—which is localized in the cytoplasm—plays an antifibrotic role in HG-treated HK-2 cells and is induced in response to a 24–72-h HG treatment. However, HG-triggered increase in endogenous lncRNA NR_038323 expression was unable to limit HG-induced expression of collagen I, collagen IV, and FN. Interestingly, this effect was almost completely reversed upon overexpression of lncRNA NR_038323 in HG-treated HK-2 cells. lncRNA NR_038323 contains the binding sites for miR-324-3p and DUSP1, suggesting that this lncRNA is a direct target of miR-324-3p. Increased expression of lncRNA NR_038323 suppressed miR-324-3p expression, which resulted in increased expression of DUSP1, a phenomenon that resulted in inhibition of the ERK1/2 and p38MAPK signal in HG-induced RF (Ge Y. et al., 2019). Furthermore, *in vivo* studies showed that lncRNA NR_038323 overexpression mediates antifibrotic effects by regulating the miR-324-3p/DUSP1 pathway in patients with DN and in rats with STZ-induced DN (Ge Y. et al., 2019). These findings suggest lncRNA_NR03832 can serve as a potential therapeutic target for DN.

LncRNAs WITH A DOUBLE-EDGED SWORD ROLE IN RF

MIAT

MIAT, also known as Gomafu, was first detected in mitotic progenitors and post-mitotic retinal precursor cells (Rapicavoli et al., 2010). This lncRNA decreases myofibroblast formation and alleviates the progression of kidney fibrosis. Bijkerk et al. (2019) observed marked expansion of pericyte-derived myofibroblasts in the interstitium of UUO 10 days after UUO. However, 2 days after unilateral ischemia–reperfusion injury (IRI), no clear expansion was observed. MIAT, which critically influences myofibroblast formation, is highly increased in myofibroblasts isolated from IRI and UUO kidneys (Bijkerk et al., 2019). Moreover, knockdown of MIAT suppresses myofibroblast formation, as evidenced by the decreased expression of α-SMA, collagen 1, Smad2, and Smad3. MIAT is also upregulated in UUO mice and human clinical kidney specimens. In TGF-β1-induced HK-2 cells, MIAT knockdown counteracts the effect of TGF-β1 on the EMT process in cells by interacting with the miRNA-145/EIF5A2 axis (Wang Z. et al., 2020).

However, in STZ-induced diabetic rats, MIAT expression is decreased and negatively correlated with the expression of serum creatinine and blood urea nitrogen (Zhou L. et al., 2015). Besides, *in vitro* studies show that the increased expression of MIAT caused by pcDNA-MIAT plasmid transfection reverses the inhibitory action of Nrf2 expression and improves renal tubule cell viability (Campo and Mylotte, 1988; Zhou L. et al., 2015). Hence, elucidating the role of these cellular pathways in the pathophysiology and modulation in different types of RF could help reverse the pathological process of RF.

LncRNA GAS5

GAS5 expression is increased in HK-2 cells in an HG environment. Moreover, Lv et al. (2019) revealed that silencing GAS5 alleviates the HG-mediated reduction in HK-2 cell viability

and apoptosis by downregulating miR-27a and BNIP3 and inactivating the JNK pathway. In STZ/HDF mouse kidneys and TGF-β1-treated HK-2 cells, GAS5 is highly expressed. GAS5 knockdown relieves renal tubular epithelial fibrosis by regulating the antifibrotic miR-96-5p, which inhibits FN1 expression (Wang W. et al., 2020).

In contrast, Xie et al. (2019) demonstrated that GAS5 expression is decreased in HK-2 cells treated with HG and that GAS5 overexpression suppresses oxidative stress, inflammation marker expression, and pyroptosis by directly targeting miR-452-5p in HG-induced HK-2 cells. Furthermore, GAS5 is downregulated in patients with DN and MCs (Ge X. et al., 2019). Overexpression of GAS5 inhibits proliferation, causes G0/1 phase arrest, and alleviates the expression of FN, collagen 4, and TGF-β1 in MCs. In addition, GAS5 upregulates SIRT1 expression and inhibits cell proliferation and fibrosis by acting as a ceRNA for miR-221. In another study, GAS5 was shown to recruit EZH2 to the matrix metalloproteinase 9 (MMP9) promoter regions, downregulating MMP9 and alleviating RIF and inflammatory reactions in STZ-induced DN rats (Zhang L. et al., 2020).

LncRNA TUG1

Long non-coding RNA TUG1, which is situated at chromosome 22q12, was first described as an important component of retinal development and photoreceptor function in mouse retinal cells. TUG1 was significantly decreased in HK-2 cells after lipopolysaccharide treatment. TUG1 overexpression was shown to protect renal tubular epithelial cells from inflammatory injury by downregulating miR-223 and upregulating Sirt1 expression, resulting in PI3K/AKT activation and NF-κB inactivation. The study further describes the protective anti-inflammatory effects of TUG1 in lupus nephritis (Xu et al., 2018). TUG1 overexpression promotes the expression of TIMP3 *via* the regulation of miR-21, ultimately inhibiting HG-stimulated NRK-52E cell fibrosis and RF in DN mice. These findings provide evidence for a new approach for DN fibrosis treatment (Wang F. et al., 2019). Furthermore, TUG1 is downregulated in DM rats and in HG-induced MCs. Finally, overexpression of TUG1 suppresses the proliferation and ECM deposition of MCs, which is caused by high-level glucose induction *via* PI3K/AKT pathway inhibition (Zang et al., 2019).

Podocytes have been observed to be very prone to damage in diabetes. Shen et al. (2019) observed that TUG1 is upregulated in HG-treated human podocyte cells. Moreover, TUG1 improves endoplasmic reticulum stress, thereby influencing podocyte apoptosis by modulating the C/EBP homologous protein (CHOP) and peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α) signaling pathways in HG-induced developing DNs. Accumulating evidence shows that overexpression of high mobility group box 1 (HMGB1) aggravates renal ischemia/reperfusion injury by promoting inflammatory responses in mice (Chen et al., 2017). TUG1 is also upregulated in renal ischemia/reperfusion (I/R) injury models. Silencing TUG1 reduces I/R-induced inflammation and apoptosis by directly regulating miR-449b-5p, HMGB1, and matrix metalloproteinase 2 expression (Xu et al., 2020).

THE FUNCTION OF LncRNAs IN RF

Long non-coding RNAs, which influence various biological processes in RF, have been emerging as key regulators. Here, we briefly summarize some currently known regulatory mechanisms

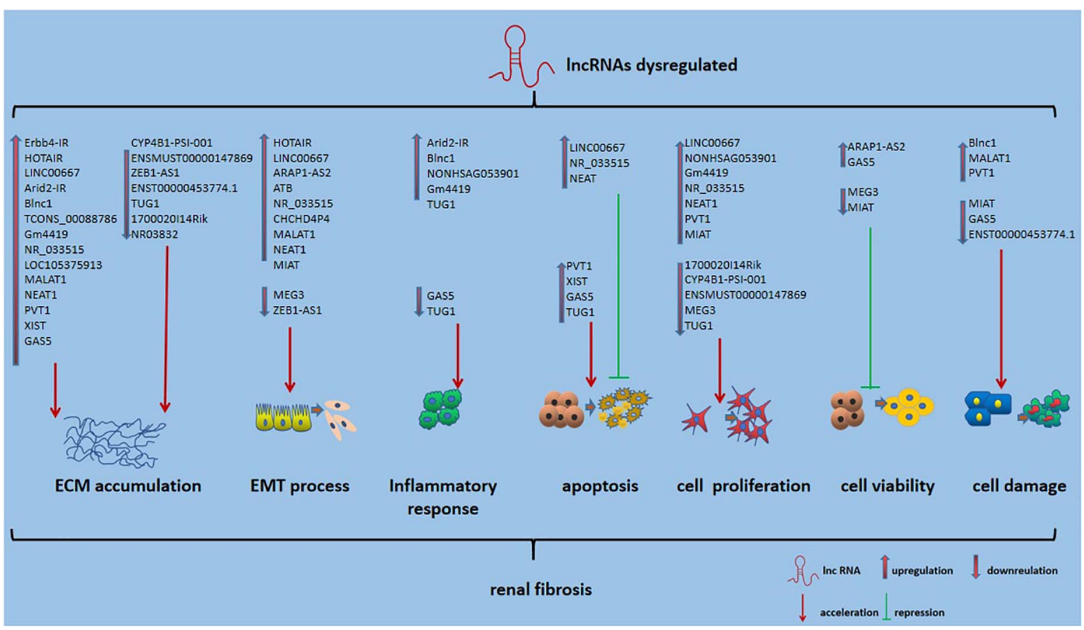

FIGURE 2 | Role of lncRNAs in RF. Also shown is that lncRNA could affect ECM accumulation, EMT process, inflammatory responses, apoptosis, cell proliferation and cell damage, and cell viability in RF.

by which lncRNAs are involved in RF, especially in DN (**Figure 2**). ECM accumulation, the EMT process, inflammatory responses, apoptosis, cell proliferation and cell damage, and cell viability have been shown to be involved in mechanisms underlying RF development.

CONCLUSIONS AND PROSPECTS

Numerous studies indicate that lncRNAs, whose expression is aberrant in pathways involved in kidney disease, are rapidly emerging as potential therapeutic targets and diagnostic markers. However, their roles in kidney fibrosis are scarcely understood, and further studies are required to elucidate the molecular mechanisms underlying their functions. For example, at least three lncRNAs, Blnc1, ENST00000453774.1, and MIAT, affected the expression of Nrf2 (Zhou L. et al., 2015; Feng et al., 2019; Xiao et al., 2019). In the DN and UUO models, lncRNA expression was negatively correlated with Nrf2 expression. Moreover, Arid2-IR and NONHSAG053901, which could directly bind to Egr-1 and positively regulate Egr-1 expression, were increased in the DN model (Peng and Huang, 2019; Yang Y. L. et al., 2019). Different lncRNAs might play similar pro/anti-RF roles by binding to the same transcription factor or protein in distinct RF diseases or in the same RF disease. However, the mechanisms of interaction between different lncRNAs and regulation of aberrant cross talks in RF require elucidation. Other confounding factors in our understanding of lncRNAs include the fact that lncRNAs can have more than one mechanism of action in RF. For example, GAS5 can not only influence EZH2 recruitment to the MMP9 promoter region and competitive binding of miR-96-5p in the DN model (Wang W. et al., 2020; Zhang L. et al., 2020) but also activate or repress their target genes. This phenomenon is extremely common, which suggests that novel mechanisms are yet to be elucidated.

Furthermore, lncRNA/miRNA interactions are a common regulatory strategy in RF, but the gene regulatory network is complex and is yet to be fully elucidated. For example, PVT1 and XIST could bind to miR-93-5p at the predicted site; silencing of PVT1 or XIST inhibits the fibrosis process in HG-treated MMCs or HK-2 (Yang J. et al., 2019; Li J. et al., 2020). Similarly, MALAT1 and MIAT could influence cell proliferation, viability, migration, and the EMT process by binding to miR-145 in TGF-β1-stimulated HK-2 cells (Liu B. et al., 2019; Liu et al., 2020; Wang Z. et al., 2020). Clinical trials on lncRNA/miRNA are still underway. Researchers believe that the therapeutic targeting of lncRNA/miRNA might elicit fewer off-target effects due to the precision of the combination (Zhao Z. et al., 2020). However, whether the combination of two or more different lncRNAs targeting the same miRNA or protein-coding gene, or the combination of two or more different miRNA targeting the same lncRNA, will increase the risk of targeted therapy off-targets is presently unclear. Moreover, with respect to RF, no relevant research report on whether simultaneous intervention of these differentially expressed lncRNAs would have a synergistic or counteracting effect on the same downstream target gene exists. Hence, more comprehensive studies on lncRNA involving CRISPR/Cas9 DNA and CRISPR/Cas13 RNA editing techniques and alternative splicing are required to provide insights into the specific mechanisms by which dysregulated lncRNA function in RF. Furthermore, lncRNA inhibition or overexpression *in vivo* without any accompanying toxic side effects, particularly in human renal tissue, remains challenging. Therefore, urologists should cooperate with molecular biologists working in the fields of materials science, to enable detailed functional verification in multiple models of RF and to identify lncRNA with clinical application potential. Only by overcoming these issues can we identify additional RF-related lncRNAs. Finally, lncRNAs are expected to serve as promising targets for antifibrotic therapies.

AUTHOR CONTRIBUTIONS

WX, XC, and ZC developed the original content and drafted the manuscript. WX, XC, ZC, YH, YG, BZ, and GD contributed to article acquisition. FR and ZJ contributed to the preparation of the table. All authors contributed to the article and approved the submitted version.

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Identification of Circular RNAs Associated With Chemoresistance in Colorectal Cancer

Fei Yao[1†], Xiaochen Xiang[1†], Chuanren Zhou[1], Qiyou Huang[1], Xiaoying Huang[1], Zhufu Xie[1], Qiang Wang[1] and Qingming Wu[1,2]*

[1] *Institute of Infection, Immunology and Tumor Microenvironment, School of Medicine, Wuhan University of Science and Technology, Wuhan, China,* [2] *Hubei Province Key Laboratory of Occupational Hazard Identification and Control, Wuhan University of Science and Technology, Wuhan, China*

Chemoresistance is a major clinical obstacle for the treatment of colorectal cancer (CRC). Circular RNAs (circRNAs) are a new type of non-coding RNA that participated in the development of chemoresistance. However, the profiles and effects of circRNAs in 5-fluorouracil (5-Fu) and cisplatin resistance of CRC are still unclear and need to be elucidated. In the present study, the profiles of circRNAs in CRC chemoresistant (HCT8/5-Fu and HCT8/DDP) and chemosensitive (HCT8) cell lines were identified *via* RNA-sequencing. In total, 48 and 90 differentially expressed (DE)-circRNAs were detected in HCT8/5-Fu and HCT8/DDP cell lines, respectively. Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway analysis were conducted on the host genes of DE-circRNAs; the results showed that the most significant enrichment pathways in HCT8/5-Fu and HCT8/DDP cell lines were base excision repair and Hippo signaling pathway, respectively. In addition, 11 common DE-circRNAs in the two drug-resistant cell lines (two are upregulated and nine are downregulated) were screened and verified by quantitative real-time PCR; hsacirc_023607 and hsacirc_007420 were found to be the circRNAs with the highest upregulation and downregulation fold changes. However, functional studies showed hsacirc_023607 has no effect on CRC chemoresistance. Therefore, the regulatory networks of targeted miRNAs related to 5-Fu or cisplatin resistance were predicted and constructed, in which hsacirc_002482 was identified as a hub gene, and its overexpression could suppress HCT8/5-Fu and HCT8/DDP cell proliferation and promote cell apoptosis, and enhance cell chemosensitivity. Taken together, these results of the study suggested that hsacirc_002482 may play important roles in chemoresistance of CRC.

Keywords: circRNA, colorectal cancer, chemo-resistance, RNA sequencing, hsacirc_002482

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Vaibhav Shukla,
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***Correspondence:**
Qingming Wu
wuhe9224@sina.com

[†] These authors have contributed equally to this work

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant tumor of the digestive system, with high morbidity and mortality (Bray et al., 2018). Surgical resection is still the main treatment for CRC at present, but for inoperable or advanced patients, chemotherapy becomes an important therapeutic approach (Aakif et al., 2016). 5-Fluorouracil (5-Fu) combined with cisplatin (DDP) can reduce the recurrence

rates and prolong survival time of patients (Martini et al., 2017). However, the patients usually acquired chemoresistance to chemotherapeutic drugs after a period of treatment, resulting a poor prognosis (Amable, 2016; Ghosh, 2019). Thus, a deeper understanding and exploration of mechanisms of chemoresistance are crucial to improve the efficacy of chemotherapy in CRC.

Circular RNAs (circRNAs) are a category of non-coding RNA with a covalently closed loop structure, mainly formed by introns or exons through back-splicing or lariat introns, which could function as cancer biomarkers because its circular structure was more stable and not susceptible to degradation by RNA exonuclease (Kristensen et al., 2019; Patop et al., 2019). CircRNAs were regarded as error products of spliceosome-mediated and have not been further studied until recent years (Sanger et al., 1976; Li et al., 2020). The development of high-throughput technology provides the possibility for in-depth study of circRNAs. Accumulating evidence demonstrated that circRNAs were not only involved in numerous biological processes (BPs), such as cell proliferation, apoptosis, invasion, and migration but also related to the chemoresistance of various cancers (Salzman, 2016; Jeyaraman et al., 2019; Wang et al., 2019). For example, Hon et al. (2019) indicated that has_circ_0000338 was highly expressed in FOLFOX-resistant HCT116 cells compared with parental cells, and knockdown has_circ_0000338 could improve the chemosensitivity. Besides, it was found that circRNA-SORE was highly expressed in sorafenib-resistant hepatocellular carcinoma cells. Mechanically, circRNA-SORE could bind with the oncogenic protein YBX1 in the cytoplasm, preventing YBX1 degradation mediated by PRP19; sorafenib resistance was overcame when silencing circRNA-SORE (Xu et al., 2020). These studies indicated circRNAs may play important regulatory roles in cancer chemoresistance. However, the expression profiles and effects of circRNAs in 5-Fu and cisplatin resistance of CRC are still unclear and need to be elucidated.

In the present study, to further explore the relationship between circRNAs and chemoresistance of CRC, we first detected circRNA expression profiles and screened the differentially expressed (DE) circRNAs in two different drug-resistant cell lines (HCT8/5-Fu and HCT8/DDP) compared to CRC parental cells (HCT8) through RNA-sequencing, and performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. In addition, to further find circRNAs both involved in 5-Fu and cisplatin-resistance of CRC, 11 common DE-circRNAs in the two drug-resistant cell lines were screen out and further verified by quantitative real-time PCR (qRT-PCR), among which hsacirc_023607 was the circRNA with the highest upregulation. However, we found that silencing hsacirc_023607 does not affect CRC chemoresistance, indicating not all common DE-circRNAs related to drug resistance; further research is needed. Thus, the targeted miRNAs associated with 5-Fu or cisplatin resistance of the common DE-circRNAs were predicted, and the circRNA–miRNA regulatory networks were constructed. hsacirc_002482 was identified as a hub gene, which was decreased in two chemoresistance cells. Gain-of-function assays showed that hsacirc_002482 overexpression suppressed HCT8/5-Fu and HCT8/DDP cell proliferation, promoted cell apoptosis and enhanced cell chemosensitivity. Taken together, these results suggested that hsacirc_002482 may play important roles in chemoresistance of CRC.

MATERIALS AND METHODS

Cell Culture

The human CRC cell line HCT8 was purchased from China Center for Type Culture Collection (Wuhan, China). The parental cells HCT8 were exposed to increasing concentrations of 5-Fu or cisplatin for more than 7 months to obtain the chemoresistant CRC cell lines (HCT8/5-Fu and HCT8/DDP). Both cells were cultured in RPMI-1640 (Gibco, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, United States) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, United States). In addition, HCT8/5-Fu cells were cultured with 5 μg/ml 5-Fu (Sigma-Aldrich, St. Louis, MO, United States), and HCT8/DDP cells were cultured with 1 μg/ml cisplatin (Sigma-Aldrich, St. Louis, MO, United States) to maintain drug resistance at 37°C in a 5% CO_2 incubator.

MTT Assay

The cells in logarithmic growth phase were seeded in a 96-well plate with 5,000 cells per well for overnight incubation, and five duplicate wells were set for each group. Different concentration gradients of 5-Fu or cisplatin were added into wells for 48 h to assess cell viability. Same-concentration gradients of 5-Fu or cisplatin were supplemented into wells and cultured for 12, 24, and 36 h to evaluate cell proliferation ability. MTT reagent (100 μl; Sigma, United States) was added into each well and then incubated for 4 h at 37°C. After that, 150 μl of dimethyl sulfoxide (DMSO) was added, and the absorbance was detected at 490 nm with a spectrophotometer.

RNA Extraction and Quality Assessment

The parental cells and drug-resistant cells were placed at room temperature for 5 min for fully lysis after adding TRIzol reagent (Invitrogen, Grand Island, NY, United States). Chloroform was added according to 200 μl chloroform/ml TRIzol, and then shook and mixed well. All samples were centrifuged at 12,000 × *g* for 15 min at 4°C. The upper water phase was taken and transferred into another centrifuge tube, and then 0.5 ml isopropanol was added and placed at 4°C for 10 min. The RNAs were at the bottom of the tubes after centrifugation and the supernatant was discarded, then they were washed and suspended in 75% ethanol, centrifuged, and dried at room temperature for 5–10 min; finally, DEPC water was added to dissolve it. The NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL, United States) was used to perform preliminary quantification of RNA, and agarose gel electrophoresis and Agilent 2100 (Agilent Technologies, Santa Clara, CA, United States) were used to evaluate RNA integrity; the purity of the RNA was evaluated *via* the ratio of OD_{260}/OD_{280}. The RNA integrity number (RIN) value is a

quantitative value reflecting the integrity of RNA. In general, the RNA samples were used for further experiments with RIN $\geq$ 7 and OD_{260}/OD_{280} ratio between 1.8 and 2.1.

RNA-Sequencing

After extracting the total RNAs of the parental cell and the two drug-resistant cells, the Ribo-Zero Magnetic kit (Epicentre, Madison, WI, United States) was used to remove the rRNA from the total RNA, and then RNase R (Epicentre, Madison, WI, United States) was used to remove linear RNAs and enrich circRNAs. The remaining RNAs were interrupted to fragments about 300 base pairs (bp) in length. The first-stranded complementary DNA (cDNA) was synthesized with random hexamer primers, and the second-strand of cDNA was digested with USER Enzyme (NEB, Ipswich, United States) before PCR amplification. Subsequently, the quality, total concentration, and effective concentration of the library were detected by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States), and QuantiFluor dsDNA System (Promega, Madison, WI, United States) was used to quantify libraries. The libraries were sequenced based on the Illumina HiSeq 2000 platform, and 150-bp paired-end reads were generated. There were three replicates in each group, and all sequencing was completed by Majorbio Biotech Co., Ltd. (Shanghai, China).

Identification of DE-Circular RNAs

Raw data were filtered to obtain high-quality sequences; HISAT2[1] was used to map clean data to the reference genome, and then reads aligned to the genome were performed comparison regional distribution and gene coverage uniformity analysis. Find_circ tools were used to identify the circRNAs (Jeck et al., 2013). The expression levels of each transcript were quantified by the reads per kilobase of model per million base pairs sequenced (RPKM). The analysis of differences in circRNAs expression between the two groups was performed using the DESeq software (Hansen et al., 2016). The DE-circRNAs were selected with $|\log2\text{FoldChange}| > 1$ and $p < 0.05$. To further find circRNAs involved in 5-Fu and cisplatin resistance in CRC, the common DE-circRNAs were selected between the two drug resistance groups.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis

Gene Ontology[2] and KEGG[3] analyses of DE-circRNAs were performed to determine the main biological functions and significantly enriched pathways. $p < 0.05$ was considered statistically significant.

Quantitative Real-Time PCR

To verify the accuracy of the sequencing results, the expression levels of common DE-circRNAs in two drug-resistant cells were detected by qRT-PCR. Total RNA was extracted from drug-resistant cells by using TRIzol reagent (Invitrogen, Grand Island, NY, United States), and complementary DNA (cDNA) was synthesized using the ReverTra Ace qPCR RT Kit (TOYOBO, Japan) according to the instructions. Subsequently, quantitative PCR was conducted using iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States). The primer sequences of common DE-circRNAs used in the study were synthesized by Sangon Biotech (Shanghai, China) and shown in **Table 1**. GAPDH was used as the internal control, and the relative expression of circRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. Each sample was analyzed in triplicate.

Prediction of Circular RNA–miRNA Networks

miRanda and circinteractome database were used to predict the targeted miRNAs of common DE-circRNAs. The circRNA–miRNA networks were displayed, and the hub gene was identified by Cytoscape software.

Cell Transfection

hsacirc_002482 overexpression plasmids (hsacirc_002482-OE) and its negative control were constructed and purchased from GenePharma (Shanghai, China). When the cell confluence reached 50%, transfection was performed using Lipofectamine 2000 reagent (Invitrogen, CA, United States) according to the instructions of the manufacturer.

Western Blotting

Proteins were lysed by radioimmunoprecipitation assay (RIPA) lysis buffer with phosphatase inhibitor (Beyotime Biotechnology, Shanghai, China), and protein concentrations were quantified using a bicinchoninic acid (BCA) Protein Assay Kit (Biosharp, Shanghai, China). Protein samples were separated by 8.75% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, United States), and then the membrane was blocked in 5% skim milk at room temperature and incubated with primary antibodies for γH2AX (1:1,000, ABclone, AP0099) and β-actin (1:5,000, ABclone, AC026) overnight at 4°C. Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h, and then visualized with ECL reagents (Bio-Rad, United States). The relative level of γH2AX protein expression was determined by densitometric analysis using ImageJ software.

Apoptosis Assay

The chemoresistance cells transfected with overexpression plasmid were seeded into six-well plates, and then 5-Fu and cisplatin were added into HCT8/5-Fu and HCT8/DDP cells for 48 h, respectively. The cells were collected and washed with precold PBS, and then about 1–5 $\times$ 10^5 cells were resuspended in 500 μl of binding buffer with 5 μl of annexin V–fluorescein isothiocyanate (FITC) and 10 μl propidium iodide (PI) staining solution for 15 min at room temperature. Accuri C6 (BD Biosciences, Franklin Lakes, NJ, United States) flow cytometer

[1]http://ccb.jhu.edu/software/hisat2/index.shtml

[2]http://geneontology.org/

[3]http://www.kegg.jp/

TABLE 1 | Sequences of the primers in the analysis of circRNA expression by qRT-PCR.

Name	Primer sequence	Product (bp)
GAPDH	Forward: 5′-CCAGCAAGAGCACAAGAGGAAGAG-3′	109
	Reverse: 5′-GGTCTACATGGCAACTGTGAGGAG-3′	
hsacirc_030252	Forward: 5′-GGGACACATTCTGGCTCATGC-3′	144
	Reverse: 5′-CGCCACAACTTGATCCTCCTTC-3′	
hsacirc_027876	Forward: 5′-CACCCACAGCGCCTATCTCA-3′	116
	Reverse: 5′-ACTCTGGGCTTCACTGGTGC-3′	
hsacirc_023607	Forward: 5′-GGCTCTGGCGTTGGTGTTTT-3′	133
	Reverse: 5′-CGTTGGCTGCCATCACTGTC-3′	
hsacirc_018467	Forward: 5′-AGAAAAAGAGCAAGAGGCCATTTCT-3′	109
	Reverse: 5′-AGTGGTCACGGTCCAGTACA-3′	
hsacirc_016764	Forward: 5′-GCTCTCCTTGCACCTGATCAA-3′	114
	Reverse: 5′-TTGTGATGTAAAACAGGAAGCAAGG-3′	
hsacirc_016305	Forward: 5′-AGGCATCTCAAGAGACTTGCGT-3′	93
	Reverse: 5′-TGGGCATCCAGAAGTGGGTC-3′	
hsacirc_008249	Forward: 5′-TACGCCATGGAAACCGCTCT-3′	80
	Reverse: 5′-TCCGCTGGTAATCCCCATCG-3′	
hsacirc_007420	Forward: 5′-CCACCAGACGAGCACCAAGA-3′	125
	Reverse: 5′-GAGTGCAGTGAAGCGTTCGG-3′	
hsacirc_006554	Forward: 5′-TGGCTGGTTTCCTGGACAGA-3′	104
	Reverse: 5′-TGCCTTCAGGATAGCGCTCT-3′	
hsacirc_002482	Forward: 5′-AAGCTAAAACCATGGGGGCAA-3′	138
	Reverse: 5′-CCTTCTGAAGGTACCTTTGAATCTCT-3′	
hsacirc_000154	Forward: 5′-GAGATGTGGACCGTGTGAAAAGA-3′	150
	Reverse: 5′-TCAAGGACTCAGAGAGCCGT-3′	

was used to measure the cell apoptosis, and data were analyzed by FlowJo software (Tree Star, Ashland, OR, United States).

Statistical Analysis

SPSS 25.0 statistical software was used for data analysis. The experimental data were expressed as mean ± SD. The independent sample *t*-test was used for the comparison of means between the two groups, and $p < 0.05$ was considered statistically significant. The expression difference of circRNAs was considered to be statistically significant with $|\log2FoldChange| > 1$ and $p < 0.05$.

RESULTS

Identification of Colorectal Cancer Chemoresistant Cell Lines

To obtain the chemoresistant CRC cell lines (HCT8/5-Fu and HCT8/DDP), the parental cells HCT8 were exposed to increasing concentrations of 5-Fu or cisplatin for more than 7 months. MTT experiments were used to detect the sensitivity of HCT8 and HCT8/5-Fu cells to 5-Fu, as well as HCT8 and HCT8/DDP cells to cisplatin. The results showed that the cell viability of all groups decreased with the concentration of the drug increased. However, the survival rate of HCT8 cells was significantly lower than that of HCT8/5-Fu or HCT8/DDP cells when the same concentration was add (**Figures 1A,C**). Moreover, the half maximal inhibitory concentration (IC_{50}) values of HCT8/5-Fu and HCT8/DDP were higher than those of chemosensitive cell lines (**Figures 1B,D**), indicating that the two drug-resistant cell lines were more resistant to drugs than paternal cell lines, which lays the foundation for follow-up research.

Expression Profiles of Circular RNAs

The circRNA expression profiles of the parental cell lines and two drug-resistant cell lines were obtained through RNA-sequencing. DE-circRNAs were screened out in drug-resistant cells compared to parental cells with the criteria of $|\log2FoldChange| > 1$ and $p < 0.05$. The heat map analysis showed the expression of the DE-circRNAs visually; the three repeats of each group clustered together, while the chemoresistant groups and control group were clustered separately (**Figure 2A**). The sequencing results indicated that a total of 7,393 circRNAs were screened out in HCT8/5-Fu cells, among which 48 circRNAs were DE, with 16 upregulated and 32 downregulated (**Figure 2B**). The top five up and downregulated circRNAs in HCT8/5-Fu cell lines are shown in **Supplementary Table 1**. In addition, 90 DE-circRNAs (42 upregulation and 48 downregulation) were found among 7,385 circRNAs in HCT8/DDP cells (**Figure 2C**). The top five up and downregulated circRNAs in HCT8/DDP cell lines are shown in **Supplementary Table 2**. In addition, the distribution of circRNAs on different chromosomes was visualized (**Figure 2D**). As shown in **Figure 2E**, the majority of candidate circRNAs

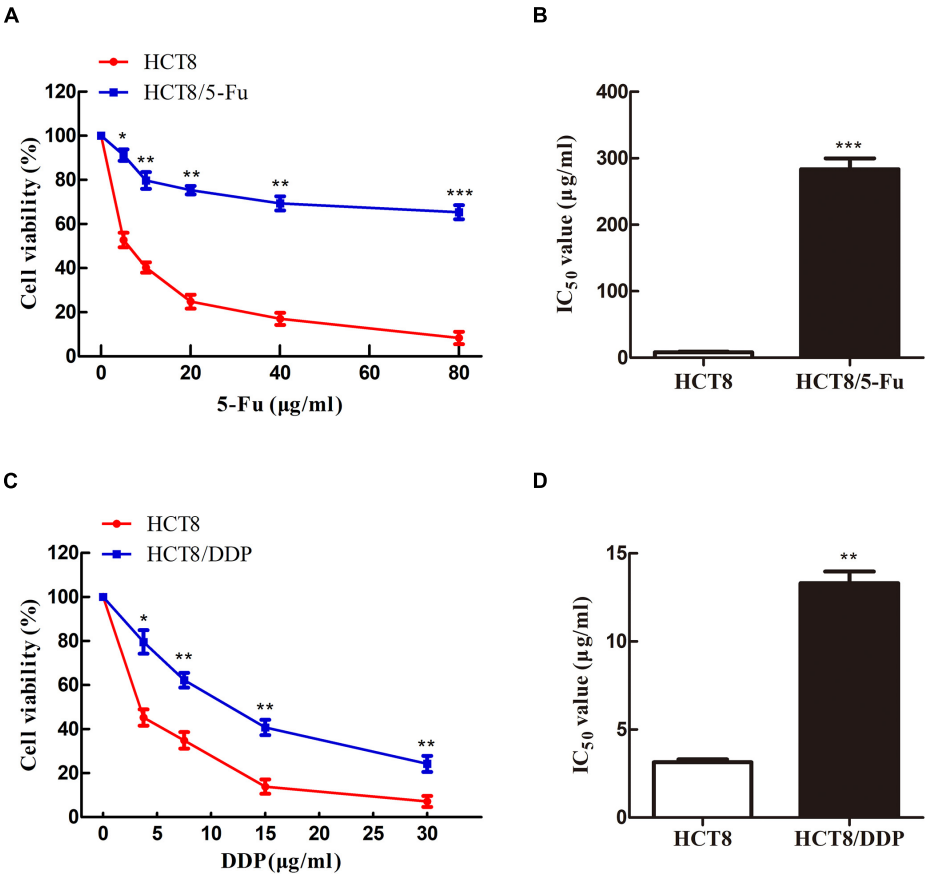

FIGURE 1 | HCT8/5-Fu and HCT8/DDP cells were more resistant to chemotherapy drugs. Cell viability and IC_{50} of HCT8 and HCT8/5-Fu cells **(A,B)**, HCT8, and HCT8/DDP **(C,D)** were assessed by MTT assay. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

originate from the exonic regions, and the rest were introns, antisense, and intergenic.

Gene Ontology Analysis of DE-Circular RNAs

Gene Ontology enrichment analysis was mainly classified to molecular function (MF), BP, and cell component (CC). The host genes of the DE-circRNAs in HCT8/5-Fu cells have a total of 1,887 GO functional annotations, among them, 1,369, 256, and 262 GO terms were significantly enriched in BP, CC, and MF, respectively (**Figure 3A**). The GO analysis of HCT8/DDP cells showed that these host genes of the DE-circRNAs were enriched to 3,208 GO entries, which were mainly related to tube development (BP), nucleoplasm and nuclear cavity (CC), and nucleic acid binding and DNA binding (MF), as shown in **Figure 3B**.

Kyoto Encyclopedia of Genes and Genomes Pathway Analysis of DE-Circular RNAs

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis showed that the host genes of the DE-circRNAs in HCT8/5-Fu cells were enriched in 15 pathways, of which base excision repair, nucleotide excision repair, and mismatch repair were highly enriched, indicating the development of 5-Fu resistance in CRC cells was possibly related to the DNA repair pathway, as shown in **Figure 4A**. γH2AX, a biomarker of DNA damage, was detected by Western blotting; the expression level of γH2AX in parental cell lines was higher than that of HCT8/5-Fu cells, suggesting HCT8/5-Fu cells have stronger DNA damage repair capacity (**Figure 4B**). In addition, the KEGG pathway enrichment analysis of the DE-circRNAs in HCT8/DDP cells showed that the Hippo signaling pathway was the most enriched pathway (**Figure 4C**). Then, the key genes of Hippo signaling were detected *via* qRT-PCR; the expression level of YAP1 and TEAD family transcription factors (except TEAD4) was significantly highly expressed in HCT8/DDP cells compared to that of parental cell lines, indicating inactivation of the Hippo signaling may play an important role in the development of cisplatin resistance (**Figure 4D**), which was consistent with previous studies on the involvement of the Hippo signaling pathway in multiple cancer chemoresistance (Wang et al., 2016, 2018).

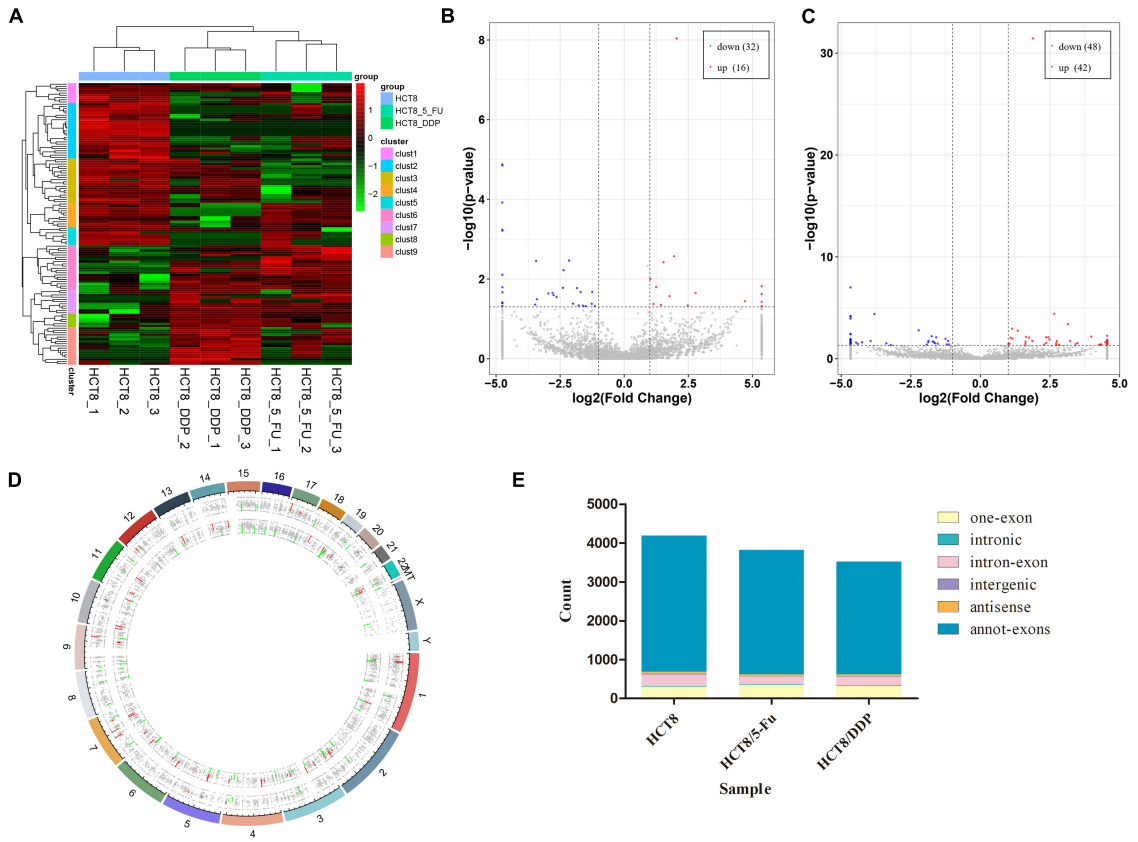

FIGURE 2 | Expression profiles of circRNAs. **(A)** Cluster analysis of DE-circRNAs. The volcano plot of DE-circRNAs in **(B)** HCT8/5-Fu cell lines and **(C)** HCT8/DDP cell lines. Red denotes increased expression, and blue indicates decreased expression. **(D)** Distribution of circRNAs on different chromosomes. **(E)** Genomic origin of the identified circRNAs.

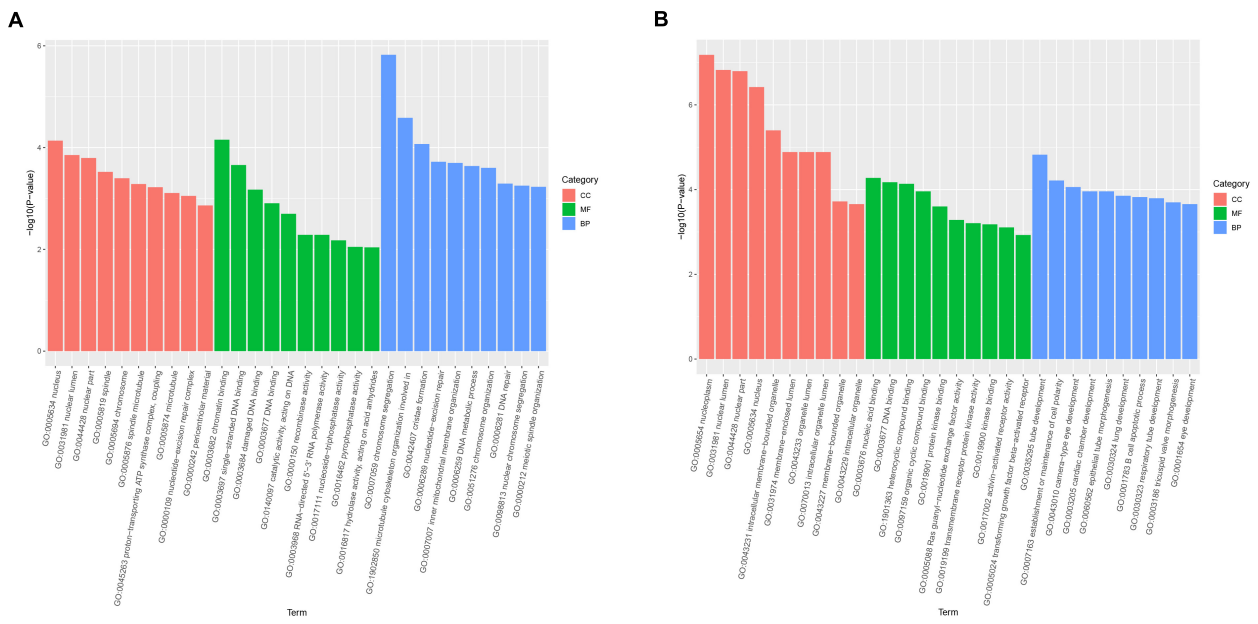

FIGURE 3 | Gene Ontology analysis of host genes of DE-circRNAs. GO analysis of the DE-circRNAs in **(A)** HCT8/5-Fu cell lines and **(B)** HCT8/DDP cell lines.

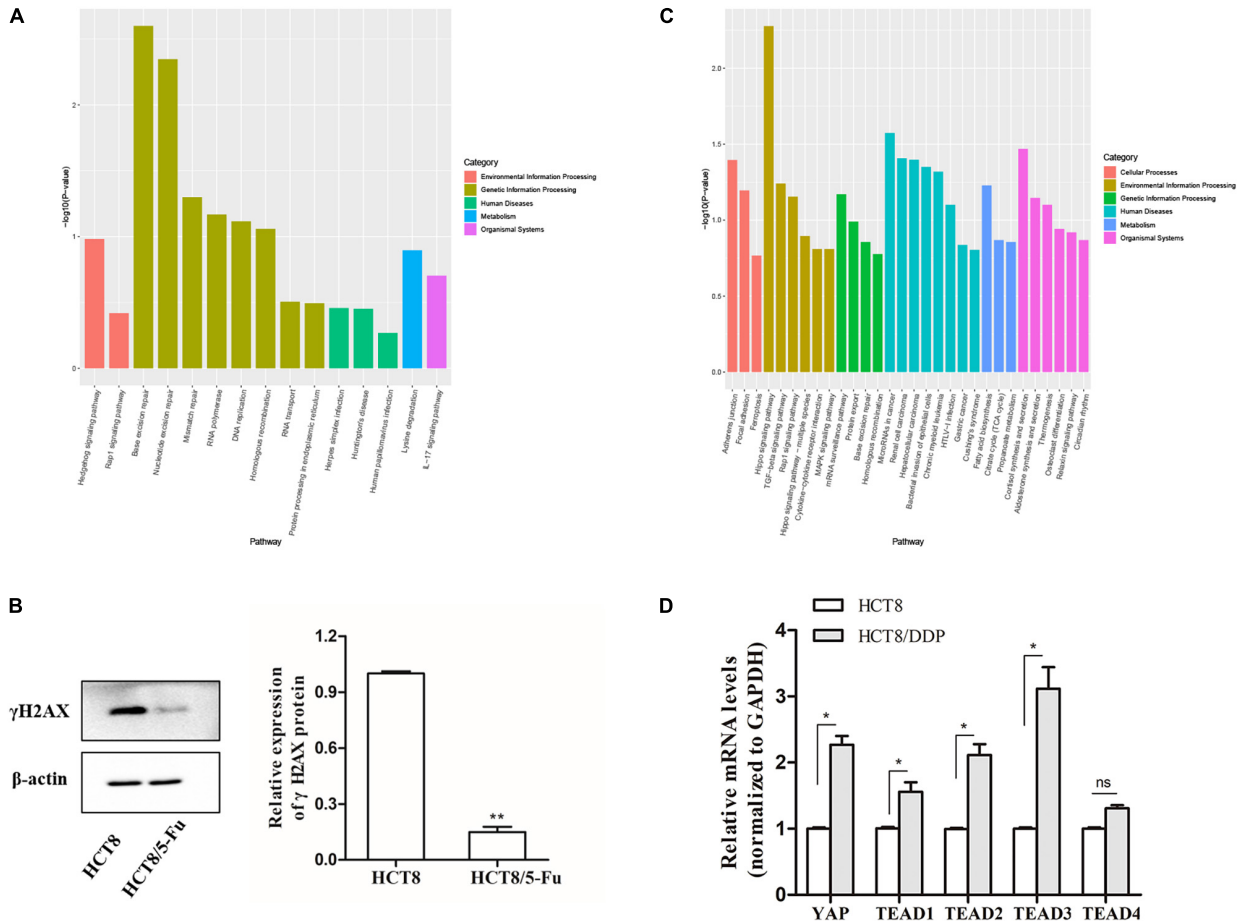

FIGURE 4 | Kyoto Encyclopedia of Genes and Genomes enrichment analysis of host genes of DE-circRNAs. **(A)** Significantly enriched signaling pathways of the DE-circRNAs in HCT8/5-Fu cell lines. **(B)** The expression of γH2AX in HCT8 and HCT8/5-Fu cells was detected *via* Western blotting. **(C)** Significantly enriched signaling pathways of the DE-circRNAs in HCT8/DDP cell lines. **(D)** The expression of key genes of Hippo signaling in HCT8 and HCT8/DDP cells was detected by qRT-PCR. $n = 3$, data are shown as mean ± SD, $^{*}p < 0.05$; $^{**}p < 0.01$ and "ns" represents no statistical significance.

Validation of Common DE-Circular RNAs by Quantitative Real-Time PCR

Venn analysis was used to screen common DE-circRNAs in two drug-resistant cells, as shown in **Figure 5A**; 11 common DE-circRNAs were found, with 2 upregulation and 9 downregulation (**Table 2**). qRT-PCR was used to detect the expression levels of common DE-circRNAs to verify the accuracy of RNA-sequencing. The verification results were consistent with the sequencing data, indicating the high accuracy of RNA-sequencing results. Among them, hsacirc_023607 was the most upregulated DE-circRNAs, about three times upregulated; and hsacirc_007420 was the most downregulated DE-circRNAs, which was expressed extremely low in CRC chemoresistant cell lines (**Figure 5B**).

Prediction of Circular RNA–miRNA Network

Circular RNAs exist as an miRNA response element (MRE) and could act as "molecular sponges" of miRNAs, inhibiting its expression. miRanda and circinteractome online tools were used to predict the targeting miRNAs of common DE-circRNA in CRC chemoresistance cell lines. A total of 951 miRNAs were predicted and displayed (**Supplementary Figure 1A**), of which the most upregulated circRNA (hsacirc_023607) and downregulated circRNA (hsacirc_007420) targeted 110 and 253 miRNAs, respectively (**Supplementary Figures 1B,C**). In order to explore whether hsacirc_023607 affects CRC chemoresistance, we knocked down the expression of hsacirc_023607 in two chemoresistant cells. As shown in **Supplementary Figure 2A**, the siRNAs significantly decreased hsacirc_023607 expression level. Unfortunately, we found silencing hsacirc_023607 has no effect on chemosensitivity, cell apoptosis, and proliferation (**Supplementary Figures 2B–D**). Therefore, we speculated that the DE-circRNAs obtained by RNA-sequencing are not all related to chemoresistance; subsequently, we further screened 133 targeting miRNAs of common DE-circRNA related to cancer resistance to 5-Fu or cisplatin (**Figure 6A**). Among which, hsacirc_002482 was identified as the hub gene through the circRNA–miRNA network analysis; hence, we selected

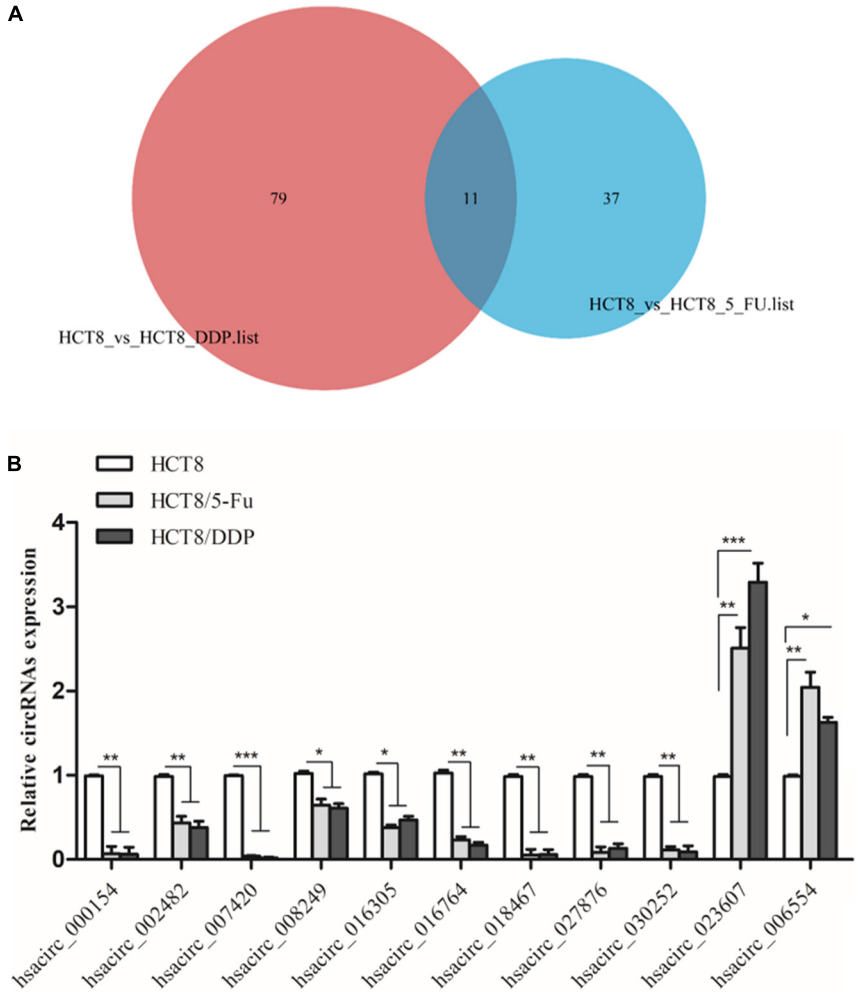

FIGURE 5 | Screening and verification of common DE-circRNAs. **(A)** Venn diagram displays DE- and overlapping circRNAs between HCT8/5-Fu and HCT8/DDP cell lines. **(B)** The relative expression levels of common DE-circRNAs in chemoresistant and chemosensitive cell lines were determined by qRT-PCR. $n = 3$, data are shown as mean ± SD, $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

TABLE 2 | Common DE-circRNAs in chemoresistant cell lines.

Gene ID	HCT8/5-Fu			HCT8/DDP		
	Regulation	log_2FC	*p*-Value	Regulation	log_2FC	*p*-Value
hsacirc_023607	Up	2.0468	9.16227E−09	Up	1.8896	3.58054E−32
hsacirc_006554	Up	1.5311	0.003791891	Up	1.0002	0.036777448
hsacirc_007420	Down	−Inf	0.000120304	Down	−Inf	7.14213E−05
hsacirc_027876	Down	−Inf	1.40889E−05	Down	−Inf	9.98353E−08
hsacirc_018467	Down	−Inf	0.000582425	Down	−Inf	6.60779E−05
hsacirc_000154	Down	−Inf	0.038921704	Down	−Inf	0.012607355
hsacirc_030252	Down	−Inf	1.40889E−05	Down	−3.8073	4.04837E−05
hsacirc_016305	Down	−3.4734	0.043758497	Down	−Inf	0.00402312
hsacirc_016764	Down	−1.6087	0.046626401	Down	−1.5865	0.008076318
hsacirc_002482	Down	−1.2670	0.041782606	Down	−1.1604	0.018296181
hsacirc_008249	Down	−1.2489	0.021168291	Down	−1.2083	0.039039397

FC, fold change; Inf, infinity.

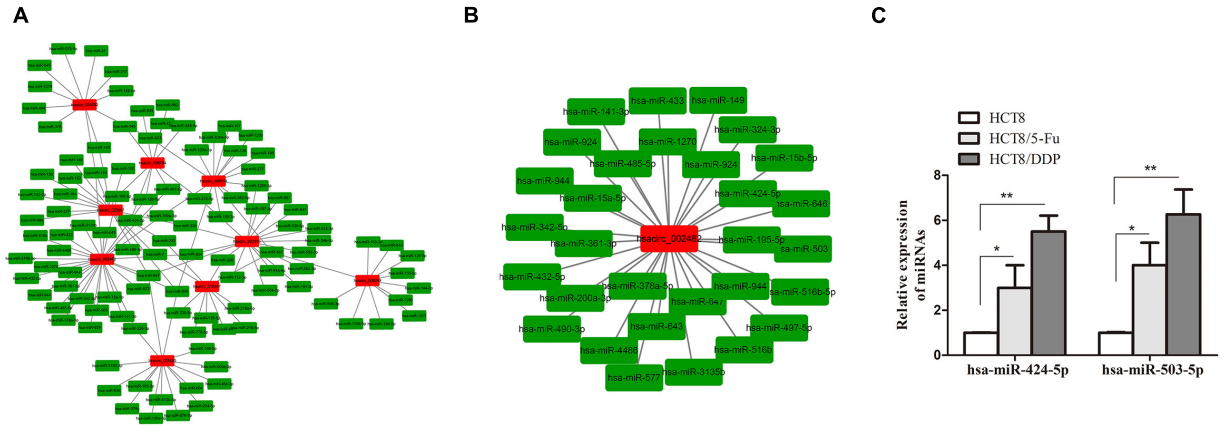

FIGURE 6 | Prediction of circRNA–miRNA regulatory networks. **(A)** The network of targeting miRNAs of common DE-circRNA related to cancer resistance to 5-Fu or cisplatin. **(B)** The regulatory network of the hub gene hsacirc_002482 targeted miRNAs related to 5-Fu or cisplatin resistance. **(C)** The expression levels of hsa-miR-424-5p and hsa-miR-503-5p in chemoresistant and chemosensitive cell lines were determined by qRT-PCR. $n = 3$, data are shown as mean ± SD, $^{*}p < 0.05$ and $^{**}p < 0.01$.

hsacirc_002482 as a candidate circRNA for further investigation. The network of hsacirc_002482 targeted 29 chemoresistance-related miRNAs was constructed (**Figure 6B**), among which hsa-miR-424-5p and hsa-miR-503-5p have been reported to regulate cisplatin and 5-Fu resistance and related to CRC chemosensitivity (Qiu et al., 2013; Xu et al., 2017; Yu et al., 2020). Thus, we detected the expression of hsa-miR-424-5p and hsa-miR-503-5p in parental and chemoresistant cell lines and found all of them were significantly highly expressed in chemoresistant cell lines (**Figure 6C**); hence, we also speculate that hsacirc_002482 might play an important role in CRC chemoresistance.

Overexpression of hsacirc_002482 Enhances Colorectal Cancer Chemosensitivity

To further explore the functional roles of hsacirc_002482 in CRC chemoresistance, we first overexpressed the hsacirc_002482 in HCT8/5-Fu and HCT8/DDP cell lines (**Figure 7A**). Then, we found elevated hsacirc_002482 partially reduced the expression of hsa-miR-503-5p, but not hsa-miR-424-5p (**Figure 7B**). Subsequently, cell proliferation, apoptosis, and viability were examined after treatment with chemotherapeutic drugs. The results showed that hsacirc_002482 upregulation could inhibit cell proliferation as well as increase apoptosis ratios of two CRC chemoresistant cells (**Figures 7C,D**). Additionally, overexpression of hsacirc_002482 could significantly enhance chemosensitivity of HCT8/5-Fu and HCT8/DDP cells compared to negative control, and the IC_{50} values were decreased (**Figure 7E**), indicating hsacirc_002482 was associated with 5-Fu and cisplatin resistance in CRC.

DISCUSSION

Chemotherapy is an important treatment method for CRC patients and widely used to prolong the survival time of patients (Ping et al., 2018; Vodenkova et al., 2020). 5-Fu and cisplatin are common chemotherapeutic drugs; however, the development of chemoresistance limited the therapeutic effect and resulted in poor prognosis (Ye et al., 2019). Therefore, exploring the mechanisms of chemoresistance in CRC is particularly important. CircRNAs are a new type non-coding RNAs, regarded as stable transcription products and potential prognosis biomarkers for cancers (Qu et al., 2015; Zhang et al., 2018; Arnaiz et al., 2019). Numerous studies have found that circRNAs participate in several biological functions, and dysregulated circRNAs are closely related to cell proliferation, migration, invasion, and chemoresistance of cancers (Li et al., 2018; Abu et al., 2019; Wang et al., 2019). For example, hsa_circ_0000745 was confirmed as a tumor promoter in cervical cancer progression; its overexpression promoted cell proliferation, migration, and invasion, which regarded as a prognosis marker of cervical cancer (Jiao et al., 2020). Jian et al. (2020) indicated hsa_circ_001680 was highly expressed in CRC tissue, which could enhance the capacity of cell proliferation and migration. Additionally, hsa_circ_001680 promoted the cancer stem cell population and induced irinotecan resistance by regulating miR-340 to affect BMI1 expression (Jian et al., 2020). Until now, the expression profiles and functions of circRNAs in CRC chemoresistance remain largely unknown; hence, the study aims to find potential circRNAs that regulate 5-Fu and cisplatin resistance in CRC.

The development of high-throughput sequencing technology provides the possibility to study circRNAs. In the present study, the expression profiles of circRNAs were compared between parental cells (HCT8) and two drug-resistant cell lines (HCT8/5-Fu and HCT8/DDP) through RNA-sequencing. The results demonstrated 48 circRNAs were aberrantly regulated in HCT8/5-Fu cells, and 90 circRNAs with significant differences were detected in HCT8/DDP cells. Subsequently, a total of 11 common DE-circRNAs in HCT8/5-Fu and HCT8/DDP cells were identified.

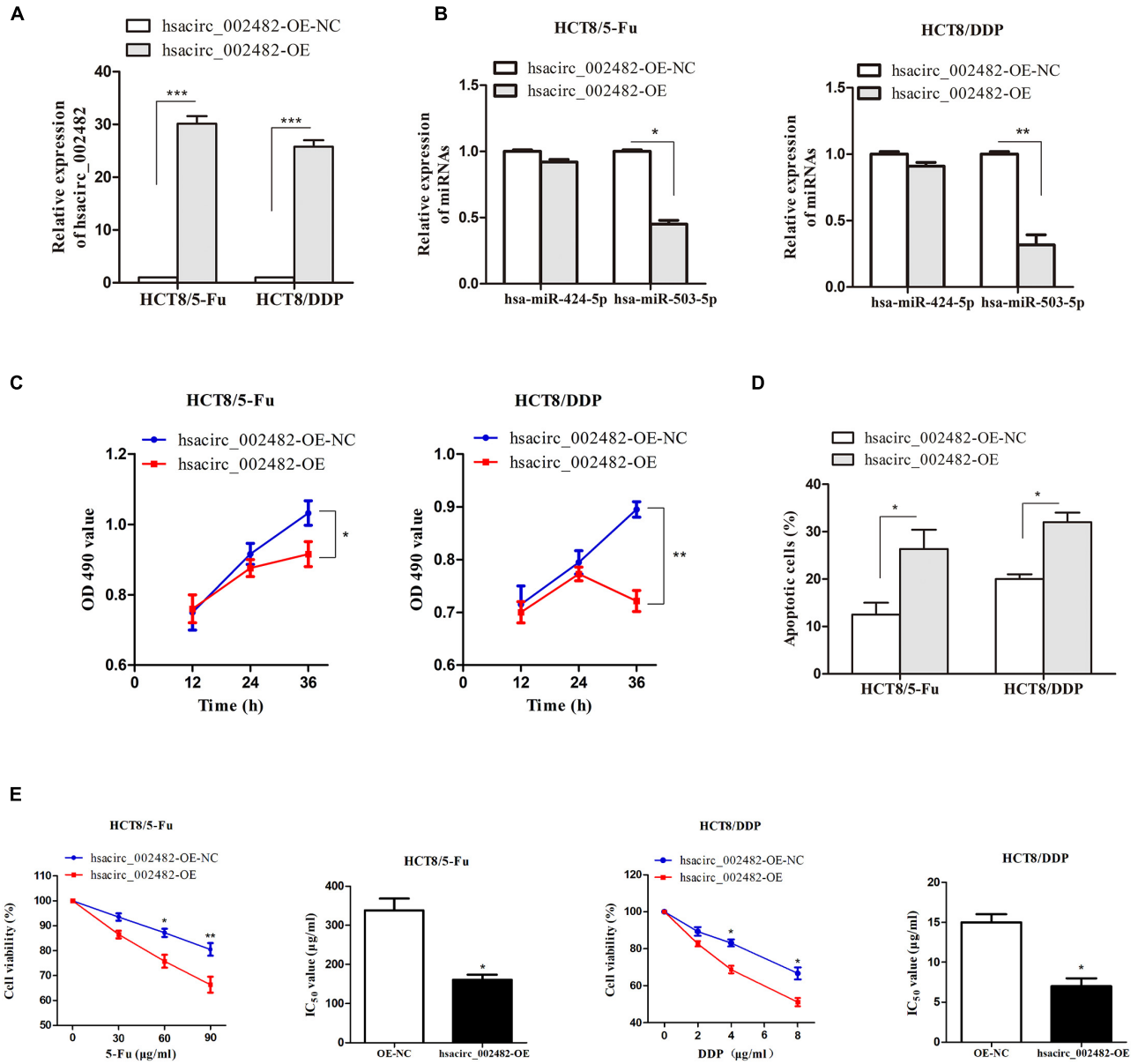

FIGURE 7 | Overexpression of hsacirc_002482 sensitizes CRC chemoresistant cells to 5-Fu and cisplatin. The expression of **(A)** hsacirc_002482 and **(B)** hsa-miR-424-5p and hsa-miR-503-5p in chemoresistant cells after hsacirc_002482 transfection. Overexpressing hsacirc_002482 **(C)** inhibited proliferation, **(D)** induced apoptosis, and **(E)** enhanced chemosensitivity of chemoresistant cells after treatment with chemotherapeutic drugs. $n = 3$, data are shown as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Gene Ontology analysis was performed to analyze the function of the host genes of dysregulated circRNAs; previous studies have reported that many factors were closely related to chemoresistance, such as mitochondria (Lee et al., 2021), nucleotide excision repair (Duan et al., 2020), DNA repair (Colomer et al., 2019), and cell apoptotic process (O'Connell et al., 2021), which were also confirmed in this study. In addition, KEGG pathway analysis was used to determine the main biochemical metabolic pathways and signaling pathways of DE-circRNAs. The DE-circRNAs in HCT8/5-Fu cells were mainly enriched in DNA repair pathways, including base excision repair, nucleotide excision repair, and mismatch repair. Accumulating evidence demonstrated that DNA repairs were significantly associated with chemoresistance of cancer. Sakthivel and Hariharan (2017) showed that the activation of various oncogenes, cancer stem cells, transcription factors, signaling pathways, and low oxygen environment in cancer cells could effectively repair DNA damage, resulting in repaired cancer cells getting more resistant to chemotherapy. Meng et al. (2015) also reported that abnormal activation of the Hedgehog signaling

pathway in tumors can cause chemoresistance through the DNA repair process. In addition, several studies have shown that the Hippo signaling pathway participated in chemoresistance of various cancers, such as osteosarcoma (Wang et al., 2016), bladder cancer (Xia et al., 2018), ovarian cancer (Xu et al., 2018), CRC (Wang et al., 2018), and breast cancer (Khanal et al., 2019). In the study, KEGG pathway analysis in HCT8/DDP cells showed that DE-circRNAs may affect many pathways such as Hippo, transforming growth factor-β (TGF-β), and mitogen-activated protein kinase (MAPK) signaling pathway, among them Hippo signaling pathway was the most enriched pathways, which may be related to the process of cisplatin resistance in CRC.

The expression levels of the common DE-circRNAs were confirmed using qRT-PCR, which were well consistent with the RNA-sequencing data. Additionally, compared with parental cells, hsacirc_023607 (upregulation) and hsacirc_007420 (downregulation) were found to have the largest expression fold change in chemoresistant cell lines. It has been reported that circRNAs could serve as sponges of miRNAs to regulate its expression (Thomson and Dinger, 2016; Zhong et al., 2018; Lai et al., 2020). A recent study showed that circCRIM1 prevented its inhibitory effect on the target gene FOXQ1 through competitive binding with miR-422a, promoted the metastasis of nasopharyngeal carcinoma, and developed resistance to docetaxel chemotherapy (Hong et al., 2020). In addition, Zhan et al. confirmed that hsa_circRNA_103809 can regulate the resistance to cisplatin of non-small cell lung cancer through the miR-377-3p/GOT1 axis *in vivo* and *in vitro* (Zhan et al., 2020). In the study, 951 miRNAs were predicted as target genes of common DE-circRNAs by miRanda and circinteractome database; we first chose hsacirc_023607 with the highest upregulation to study its correlation with drug resistance. However, silencing hsacirc_023607 does not affect CRC chemoresistance, indicating not all common DE-circRNAs related to drug resistance; further research is needed. Moreover, we filtered 133 targeted miRNAs related to cancer 5-Fu or cisplatin-resistance from 951 miRNAs. hsacirc_002482 was decreased in two drug-resistant cells and identified as the hub gene in the circRNA–miRNA network; hence, we selected it for further research. We found 29 miRNAs related to cancer resistance to 5-Fu or cisplatin were targeted by hsacirc_002482, in which hsa-miR-424-5p and hsa-miR-503-5p have been reported to regulate chemosensitivity of cisplatin and 5-Fu and also related to CRC resistance (Yang et al., 2017; Liu et al., 2021). In addition, the expressions of hsa-miR-424-5p and hsa-miR-503-5p were detected by qRT-PCR, and both were highly expressed in chemoresistance cell lines. To further investigate the biological functions of hsacirc_002482 in CRC chemoresistance, gain-of-function assays were conducted. We first detected the expression level of hsa-miR-503-5p and hsa-miR-424-5p and found hsacirc_002482 overexpression reduced the expression of hsa-miR-503-5p instead of hsa-miR-424-5p. Additionally, we also found hsacirc_002482 upregulation not only inhibited cell proliferation and promoted cell apoptosis but also significantly enhanced chemosensitivity of HCT8/5-Fu and HCT8/DDP cells. Thus, we speculated that the hsacirc_002482/hsa-miR-503-5p axis may play important roles in CRC chemoresistance.

In general, the study screened circRNAs profiles in chemosensitive and resistant CRC cell lines by RNA-sequencing. The common DE-circRNAs in HCT8/5-Fu and HCT8/DDP cells were screened out, and their expressions were validated by qRT-PCR. hsacirc_023607 and hsacirc_007420 were identified as the highest upregulation and downregulation circRNAs, respectively. Targeted miRNAs of common DE-circRNAs were predicted by bioinformatics methods. However, functional studies showed hsacirc_023607 has no effect on CRC chemoresistance. Thus, the networks of miRNAs related to cancer resistance to 5-Fu or cisplatin were constructed, in which hsacirc_002482 was regarded as the hub gene. Moreover, hsacirc_002482 overexpression could increase the chemosensitivity of HCT8/5-Fu and HCT8/DDP cells, suggesting hsacirc_002482 may play important roles in the development of CRC chemoresistance. However, further in-depth functional and mechanistic studies of hsacirc_002482 are required, which we will continue to undertake in the future.

DATA AVAILABILITY STATEMENT

We have uploaded our data to the GEO database (GSE173606).

AUTHOR CONTRIBUTIONS

QWu, FY, QWa, XX, and CZ designed the experiments. QWu, FY, and XX analyzed the RNA-sequencing data. FY, CZ, and XH performed the experiments. XX, QH, and ZX performed statistical analysis. All authors wrote the manuscript and reviewed drafts, and agreed with its submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.696948/full#supplementary-material

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Regulation of Long Non-coding RNA KCNQ1OT1 Network in Colorectal Cancer Immunity

Junjie Liu[1], *Wei Lv*[1], *Shuling Li*[1] *and Jingwen Deng*[2]*

[1]Guangzhou Panyu Central Hospital, Guangzhou, China, [2]Guangdong Provincial Hospital of Chinese Medicine, Guangzhou, China

Over the past few decades, researchers have become aware of the importance of non-coding RNA, which makes up the vast majority of the transcriptome. Long non-coding RNAs (lncRNAs) in turn constitute the largest fraction of non-coding transcripts. Increasing evidence has been found for the crucial roles of lncRNAs in both tissue homeostasis and development, and for their functional contributions to and regulation of the development and progression of various human diseases such as cancers. However, so far, only few findings with regards to functional lncRNAs in cancers have been translated into clinical applications. Based on multiple factors such as binding affinity of miRNAs to their lncRNA sponges, we analyzed the competitive endogenous RNA (ceRNA) network for the colorectal cancer RNA-seq datasets from The Cancer Genome Atlas (TCGA). After performing the ceRNA network construction and survival analysis, the lncRNA KCNQ1OT1 was found to be significantly upregulated in colorectal cancer tissues and associated with the survival of patients. A KCNQ1OT1-related lncRNA-miRNA-mRNA ceRNA network was constructed. A gene set variation analysis (GSVA) indicated that the expression of the KCNQ1OT1 ceRNA network in colorectal cancer tissues and normal tissues were significantly different, not only in the TCGA-COAD dataset but also in three other GEO datasets used as validation. By predicting comprehensive immune cell subsets from gene expression data, in samples grouped by differential expression levels of the KCNQ1OT1 ceRNA network in a cohort of patients, we found that $CD4^+$, $CD8^+$, and cytotoxic T cells and 14 other immune cell subsets were at different levels in the high- and low-KCNQ1OT1 ceRNA network score groups. These results indicated that the KCNQ1OT1 ceRNA network could be involved in the regulation of the tumor microenvironment, which would provide the rationale to further exploit KCNQ1OT1 as a possible functional contributor to and therapeutic target for colorectal cancer.

Keywords: long non-coding RNA, KCNQ1OT1, ceRNA network, colorectal cancer, immunity

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Shaveta Kanoria,
Wadsworth Center, United States

Reviewed by:
Stephen J. Bush,
University of Oxford, United Kingdom
Yu Zhou,
Wuhan University, China

***Correspondence:**
Jingwen Deng
dengjingwen@gzucm.edu.cn

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant cancer and is the second-highest contributor to the worldwide incidence of cancer-related deaths (Miller et al., 2019). CRC develops sporadically from some inflammatory bowel diseases or hereditary cancer syndromes. The development of colorectal cancer is based on the adenoma-carcinoma sequence. So far, the molecular mechanism of the adenoma-carcinoma sequence has been only partly identified. CRC prognosis depends on factors

related to the patient and treatment. The expertise of the treatment team is one of the most important determinants of the outcome. Early detection of CRC cells and cancer precursor cells significantly reduce morbidity and improve patient prognosis (Sung et al., 2021).

The mortality of colorectal cancer can be effectively reduced by screening for the cancer. The most common screening procedures include flexible sigmoidoscopy, double-contrast barium enema, fecal occult blood tests, and colonoscopy (Bibbins-Domingo et al., 2016). There is no consensus regarding which screening method is the best, and it appears that no one test is better than the other. Risk, cost, and effectiveness are the main factors to be considered when discussing different options (Issa and Noureddine, 2017). Undoubtedly, a complete colonoscopy has the advantages of allowing the entire colon to be assessed, material for a biopsy to be collected, and a polypectomy to be carried out all within the same examination time; however, it also has disadvantages of higher costs as well as risks, discomfort and inconvenience for the patient being examined. Therefore, it is for all practical purposes necessary to develop effective biomarkers of CRC for applications in screening, diagnosis and prognosis.

Most of the RNA transcribed from the human genome does not encode for proteins. Some of these non-coding RNAs (ncRNAs) have been found to dysregulate the normal expression of genes, including tumor suppressor genes and oncogenes. Therefore, ncRNAs are considered to be new promising targets for studying tumorigenesis. ncRNAs include long non-coding RNAs (lncRNAs), microRNAs (miRNAs), circular RNAs, and small interfering RNAs (siRNAs). An miRNA is a highly conserved non-coding RNA approximately 21–24 nucleotides in length, and interacts with target mRNAs to regulate gene expression. Some miRNAs have been reported to be involved in the occurrence and development of cancer (Hayes et al., 2014), and miRNA-based therapeutics have in fact reached the stage of clinical development (Toden et al., 2021). In recent years, the importance of lncRNA has gradually become recognized. An lncRNA is essentially an ncRNA with more than 200 base pairs. So far, some lncRNAs have been shown to play a major regulatory role in genetic regulation. Recent studies have shown multiple roles for lncRNAs in tumorigenesis (de Oliveira et al., 2019). The competitive endogenous RNA (ceRNA) hypothesis is related to lncRNAs and miRNAs, proposed by Salmena and others (Salmena et al., 2011), has been described as the "Rosetta Stele", used to decode the RNA language in order to regulate RNA crosstalk and regulate biological functions. Many studies have shown that miRNA-mediated ceRNA regulation plays a crucial role in the occurrence and development of cancer (de Oliveira et al., 2019). Long non-coding RNA KCNQ1 opposite strand/antisense transcript one gene (KCNQ1OT1) were markedly upregulated in gastric cancer tissues and cells (Zhong et al., 2021). High expression of lncRNA KCNQ1OT1 was significantly related to poor survival in patients with CRC in a pan-cancer meta-analysis. The upregulation of KCNQ1OT1 in CRC tissues and cell lines was also confirm the important role of KCNQ1OT1 in CRC (Lin et al., 2021). In our current work, we investigated the KCNQ1OT1 by mining the CRC RNA-Seq dataset in The Cancer Genome Atlas (TCGA), tested its prognostic potential in a CRC cohort and constructed the KCNQ1OT1-related lncRNA-miRNA-mRNA ceRNA network. We aimed to provide the rationales to the further exploitation of KCNQ1OT1 as a possible functional contributor to and therapeutic target for CRC.

MATERIALS AND METHODS

Differentially Expressed Genes From TCGA RNA-Seq Data

RNA-seq data of a colorectal cancer cohort (TCGA-COAD) was downloaded with gdc-client (version 1.3.0) from the data portal of TCGA (the dbGaP accession: phs000178. v11. p8. Release date: December 18, 2019). The RNA sequencing read counts of sample were obtained from TCGA. All these samples were sequencing by Illumina Genome Analyzer IIX. According to the bioinformatics pipeline for mRNA analysis in TCGA (https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline/), quality assessment was performed with FASTQC (version 0.11.8), the alignment was performed using a two-pass method with STAR (version 2.4.2a). The reads were aligned to the GRCh38 reference genome and then were quantified with HTSeq (version 0.6.1). CPM normalization was performed to correct library size differences between samples. As an initial filter, we retained only genes with $\log_2$CPM >1 in more than half of the samples. To compare expression levels between samples, for these genes alone, we then re-normalised raw count data by TMM (implemented in edgeR version 3.32.1) and transformed these by voom in limma (version 3.46.0) (Robinson and Oshlack, 2010; McCarthy et al., 2012; Ritchie et al., 2015). After carrying out this normalization, mRNAs, microRNA and lncRNAs differentially expressed in tumor group and solid normal tissues were identified. Thresholds for differential expression were set each as the adjusted p value less than 0.01 and $|\log_2$fold change$|>1$.

Construction of a lncRNA-miRNA-mRNA ceRNA Network

All of the differentially expressed genes were considered as candidates in the lncRNA-miRNA-mRNA ceRNA network construction, which was based on multiple factors such as binding affinity of miRNAs to their lncRNA sponges, RNA secondary structures and RNA-binding proteins, and the abundance and subcellular localization of ceRNA components (Qi et al., 2015). To construct the ceRNA network, the R package GDCRNATools (version 1.10.1) was used (Li et al., 2018). with five databases on miRNA-mRNA interactions including STarMir (version 2.2) (Kanoria et al., 2016), StarBase (version 2.0) (Li et al., 2014), miRcode (version 11) (Jeggari et al., 2012), spongeScan (version 1.0) (Furió-Tarí et al., 2016), and mirTarBase (version 7.0) (Chou et al., 2018) incorporated for the interactions analysis. The criteria for identifying competing lncRNA-mRNA interactions are: a) the strength of positive

association between expression of lncRNA and its target mRNAs, b) the hypergeometric probability of shared miRNAs on the lncRNA-mRNA pair, c) the strength of regulation similarity of all shared miRNAs on the lncRNA-mRNA pair. Based on above criteria, *Pearson*'s correlation was used to measure the association between expression of lncRNA and mRNA. Hypergeometric distribution was measured by *Fisher*'s exact test. Regulation similarity was calculated based on the total number of the lncRNA-mRNA shared miRNAs, the *Pearson*'s correlation between the miRNA with lncRNA, as well as miRNA with mRNA (Li et al., 2018). After the construction, the ceRNA network was plotted using Cytoscape (version 3.7.0) software (Smoot et al., 2011).

Survival Analysis

We investigated the prognostic values of the main differentially expressed lncRNAs in the ceRNA network for CRC patients. Kaplan-Meier curves for survival analysis were depicted to present the survival-related lncRNAs. A log-rank test was performed to compare the survival distributions of samples grouped by lncRNA differential expression levels of the patient cohort.

Functional Annotation

To identify the biological function of the ceRNA network possibly contributing to tumor development, we performed a functional annotation analysis with multiple pathway databases, including Gene Ontology (GO) (Ashburner et al., 2000), Reactome (Haw et al., 2011), and Speed2 (Signalling Pathway Enrichment using Experimental Datasets 2) (Rydenfelt et al., 2020). A p value of less than 0.05 was considered to indicate statistically significant enrichment. The R package clusterProfiler V3.11 (Yu et al., 2012) was used for the GO and Reactome pathway enrichment analysis and visualization of significant modules. The R package SPEED2 was used for checking the upstream pathway activity from the genes in the ceRNA network, and a Bates test was used to calculate the test statistics for pathway enrichment.

Optimization of the ceRNA Network

There were more than 100 genes in the ceRNA network (**Figure 2**), so for practical applicability, we confirmed the target structural accessibility and selected the critical lncRNAs associated with the survival of CRC patients as the hub genes. Only the mRNAs both correlated with the hub gene and in the original ceRNA network were used to reconstruct the optimized network. Target structural accessibility for miRNA target recognition was calculated and visualized using STarMirDB and Sfold (version 2.2) (Kanoria et al., 2016; Rennie et al., 2019). The optimized network was visualized using Cytoscape.

Network Signature Analysis

Gene set variation analysis (GSVA) was used to determine the network expression level of each single sample, analogously to a competitive gene set test (Hänzelmann et al., 2013). By performing GSVA scoring, we were able to estimate the variation in the gene enrichments of the networks of the samples, and to do so independently for tumor and normal tissues. GSVA scores are designed to range from −1 to 1, with negative scores indicating relative decreases in network expression while positive scores indicated elevations. For practical applicability, only lncRNAs and mRNAs in networks were listed as the gene set signature for the GSVA assessment. The R package GSVA (v3.11) was used to calculate the GSVA score of networks over the samples in the CRC transcriptome dataset.

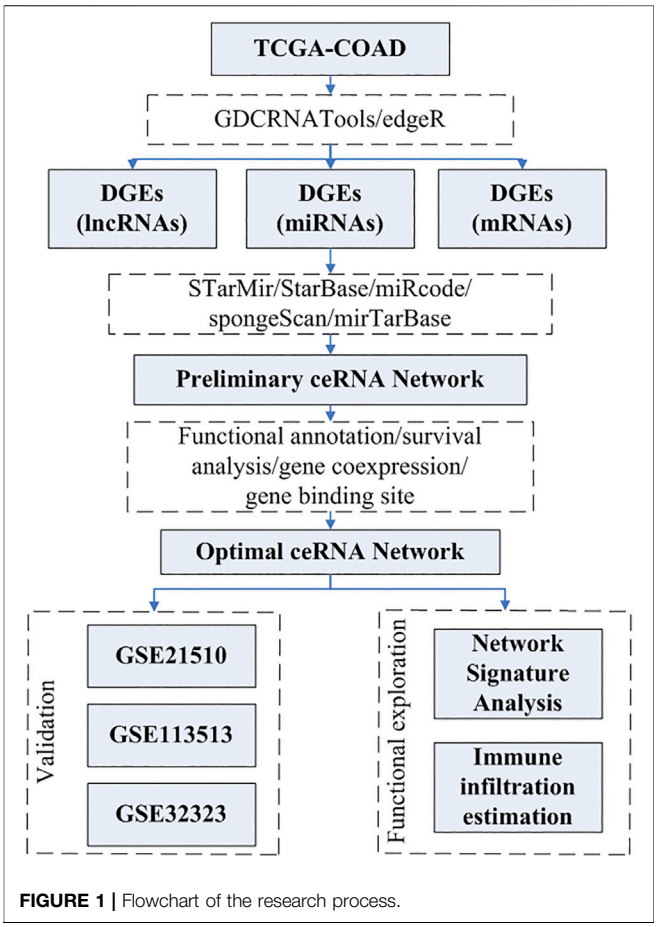

FIGURE 1 | Flowchart of the research process.

Estimation of Immune Cell Infiltration

Immune cells infiltrated in the tumor microenvironment play crucial roles in tumor invasion and metastasis. To estimate immune cell infiltration in samples with different network expression levels, the web-based tool ImmuCellAI was applied to calculate the abundance of 24 immune cell subsets *via* their gene expression profiles (Miao et al., 2020). The immune cell subsets estimated in this study included 18 T cells subsets: $CD4^+$T cells, $CD8^+$T cells, naïve $CD4^+$T cells, naïve $CD8^+$T cells, natural regulatory T (nTreg) cells, induced regulatory T (iTreg) cells, gamma delta T ($\gamma\delta$ T) cells, central memory T (Tcm) cells, effector memory T (Tem) cells, natural killer T (NKT) cells, T helper 1 (Th1) cells, T helper 2 (Th2) cells, and T helper 17 (Th17) cells, cytotoxic T (Tc) cells, exhausted T (Tex) cells, type 1 regulatory T (Tr1) cells, follicular T helper

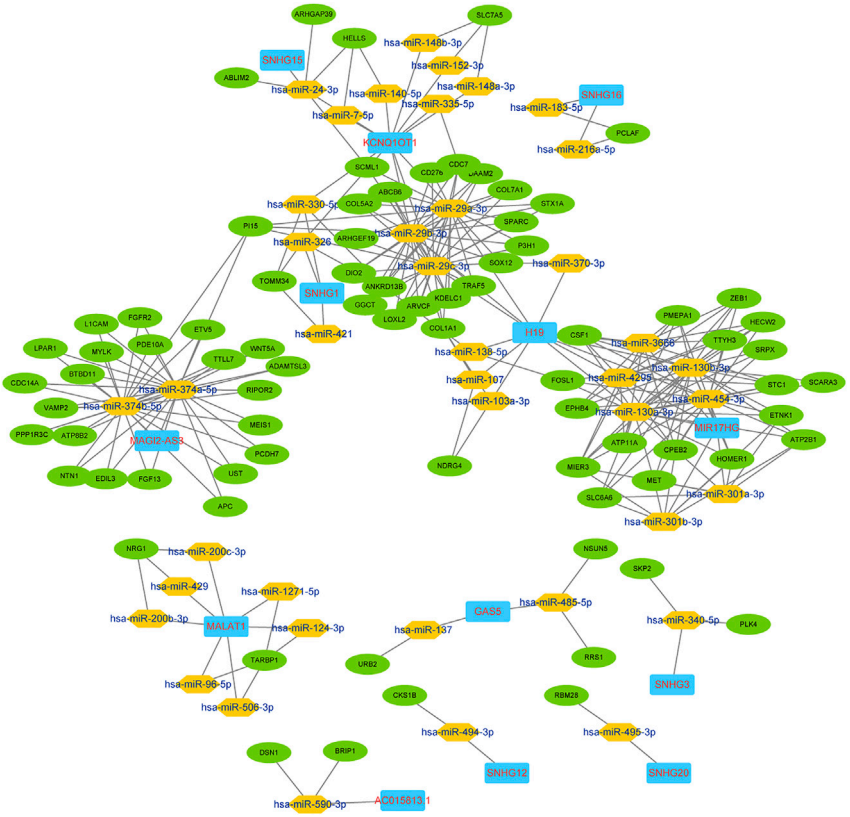

FIGURE 2 | lncRNA-miRNA-mRNA ceRNA preliminary network.

(Tfh) cells, mucosal-associated invariant T (MAIT) cells, and other six immune cell subests: B cells, natural killer (NK) cells, monocytes, macrophages, neutrophils and dendritic cells (DCs).

The pipeline of this study is depicted in the flowchart shown in **Figure 1**.

RESULTS

CRC Tumor Samples and Normal Samples Were Significantly Distinguished on the Basis of Differentially Expressed lncRNAs

We obtained the aligned read counts (GRCh38 (hg38) version) of tissue samples from 478 primary tumor, one metastatic tumor, one recurrent tumor and 41 normal solid tissue from TCGA. Samples from primary tumor, metastatic tumor and recurrent tumor were combined as tumor group. The age and sex of patients in normal group was matched to those in tumor group (**Supplementary Table 1**). There were total number of 60483 genes in this dataset. In prefiltering step, 14768 genes were kept for the downstream analyses. After TMM normalization and voom transformation, we explored the DGEs (differentially expressed genes) based on GLM (generalized linear model) likelihood ratio test, 2935 CRC tissue-specific mRNAs and 213 lncRNAs *via* the differential expression analysis of the TCGA-COAD dataset (**Supplementary Figures S1A,B**, **Supplementary Table S2**). The heatmaps of the differential expression of lncRNAs between CRC tissues and solid normal tissues are shown in **Supplementary Figure S1C**. The expression of 213 differentially expressed lncRNAs separated the tumor group and normal group clearly.

Construction of the lncRNA-miRNA-mRNA ceRNA Network

In the current work, certain lncRNAs and mRNAs were shown to co-express in the ceRNA networks. We built the ceRNA network based on the co-expression patterns of the lncRNAs, miRNAs and mRNAs. In total, 133 nodes and 288 edges constituted the ceRNA network. The preliminary network is shown in **Figure 2**.

Knowledge-Driven Pathway Analysis of ceRNA Network

According to the Reactome analysis, biological processes of the genes in the ceRNA network from tumor tissue were mainly related to MET pathways (**Figure 3A**). MET is a receptor tyrosine kinase (RTK), and like other related RTKs such as EGFR, MET can be activated by binding to its ligand, namely hepatocyte

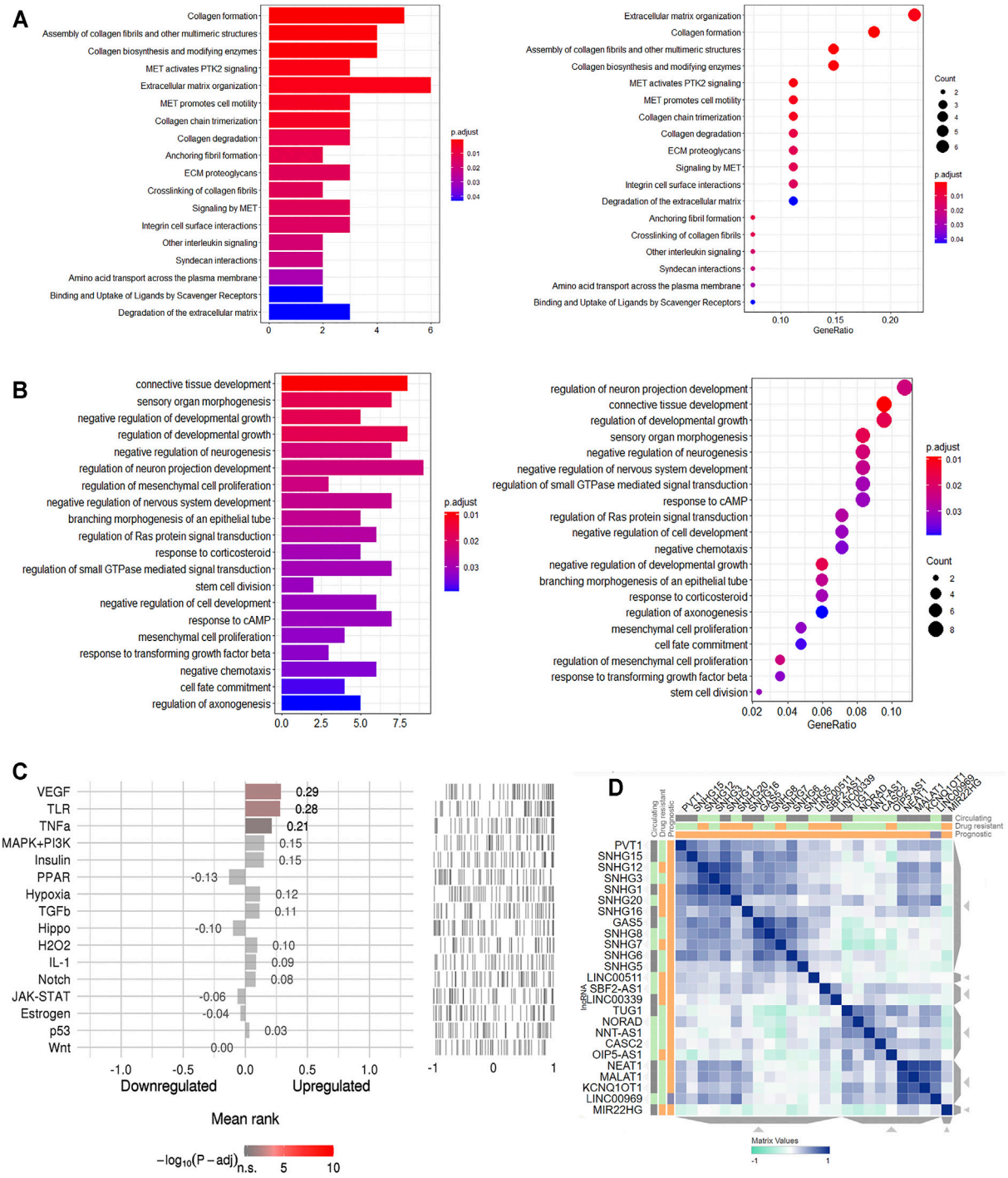

FIGURE 3 | Functional annotation for the genes in ceRNA network of CRC. **(A)**. Reactome pathway enrichment of the genes in ceRNA network of CRC. **(B)**. The top 20 enriched molecular in GO terms. **(C)**. Speed2 pathway activity ranking. **(D)**. CRC related lncRNAs similarity.

growth factor/scatter factor (HGF/SF), resulting in MET dimerization and *trans*-autophosphorylation.

A total of 106 GO terms were extracted using the GO analysis (**Supplementary Table S3**). Of the 106 GO terms, 97 were Biological Process terms, eight were Cellular Component terms and one was a Molecular Function term. The BP GO terms have all been confirmed to be related to the regulation of neurogenesis and mesenchymal cell proliferation (**Figure 3B**) with, for instance, GO:0050768 (negative regulation of neurogenesis), GO:0010975 (regulation of neuron projection development)

TABLE 1 | Survival analysis with the critical hub genes.

lncRNAs	HR	Lower 95	Upper 95	*p* value
MIER3	0.93122	0.62377	1.39021	0.726
MET	0.820336	0.549485	1.224696	0.331
ANKRD13B	1.255134	0.841125	1.872922	0.265
ATP11A	1.148337	0.769663	1.713318	0.498
TTYH3	1.224092	0.820421	1.826383	0.322
EPHB4	1.009199	0.676432	1.505668	0.964
FOSL1	1.160118	0.77757	1.730871	0.467
MIR17HG	1.021937	0.684606	1.525483	0.915
TARBP1	1.066937	0.714761	1.592637	0.751
COL1A1	1.112393	0.74518	1.660564	0.601
SCML1	0.95713	0.641016	1.429135	0.829
ATP2B1	0.920591	0.616832	1.373935	0.683
H19	1.108367	0.742886	1.653656	0.614
CPEB2	0.77813	0.520825	1.162552	0.217
PMEPA1	0.999453	0.669902	1.491122	0.998
KCNQ1OT1	1.493465	1.000447	2.229441	0.0497*
SOX12	1.331673	0.892524	1.986897	0.161
CDC7	1.131191	0.757923	1.688289	0.547
HECW2	1.059556	0.710069	1.581057	0.776
MALAT1	1.416188	0.948783	2.113852	0.0881
CSF1	1.324722	0.886839	1.978812	0.166
PI15	1.039925	0.696976	1.551622	0.847

and GO:0051961 (negative regulation of nervous system development) involving neurogenesis, and GO:0010464 (regulation of mesenchymal cell proliferation) and GO: 0051591 (mesenchymal cell proliferation) involving proliferation of human mesenchymal cells.

SPEED2 analyses allow one to infer upstream pathways. In the current work, the VEGF pathway was indicated from this analysis to be the signaling pathway that likely caused the genes in the ceRNA network to be deregulated (**Figure 3C**, **Supplementary Table S4**). We used another web-based tool LnCeVar-Cluster which provides cluster profiles between differential expressed lncRNAs and ceRNAs, especially the similarity profiles between differential expressed lncRNAs (Wang et al., 2020). The differential expressed lncRNAs clustering profile of TACG-COAD indicated that NEAT1, MALAT1, KCNQ1OT1 and LINC00969 had the similar expression pattern in CRC (**Figure 3D**). Functional annotations showed the ceRNA network genes to be closely related to the tumor pathogenesis in general.

KCNQ1OT1 Determined to Be the Critical Hub Gene for the Network Optimization

We chose the hub lncRNAs (degree >5) and their related miRNAs and mRNAs in the ceRNA network. We identified 22 lncRNAs involved in preliminary ceRNA networks (MIER3, MET, ANKRD13B, ATP11A, TTYH3, EPHB4, FOSL1, MIR17HG, TARBP1, COL1A1, SCML1, ATP2B1, H19, CPEB2, PMEPA1, KCNQ1OT1, SOX12, CDC7, HECW2, MALAT1, CSF1, PI15) (**Figure 2**). Then we analyzed the association of the hub lncRNAs with the clinically obtained survival data to identify the lncRNAs crucial to CRC prognosis. Only the lncRNA KCNQ1OT1 was significantly differentially expressed according to the log-rank test of survival analyses (**Table 1**). A Kaplan-Meier estimate showed poorer prognoses for patients with higher levels of KCNQ1OT1 (**Figure 4**).

Of the lncRNAs present in the preliminary ceRNA network, only one gene, namely KCNQ1OT1, was indicated to be associated with survival in the CRC cohort. Thus, a correlation analysis was performed based on this critical KCNQ1OT1 hub gene. Based on a hypergeometric test and correlation analysis (**Table 2**), the mRNAs and lncRNAs not significantly correlated with KCNQ1OT1 or without a direct link with KCNQ1OT1 were removed from the network. The KCNQ1OT1-miRNA-mRNA subnetwork consisted of one centroid lncRNA node, nine miRNA nodes and nine mRNA nodes. This KCNQ1OT1 ceRNA network was reconstructed and visualized using Cytoscape (**Figure 5**). The expression level of the lncRNA KCNQ1OT1 was positively correlated with the expression levels of the nine mRNAs (**Figure 6**). This result was consistent with the ceRNA theory that the lncRNA regulated other RNA transcripts by competing for shared microRNAs. We checked the expression profile of KCNQ1OT1 and other hub network genes in tumor group and normal group in TCGA data set (**Supplementary Table S2**, **Supplementary Figure S2**). An additional searching in The Genotype-Tissue Expression (GTEx) portal (dbGaP accession number phs000424. vN.pN) which is a comprehensive public dataset for tissue-specific gene expression and regulation in non-diseased cohort for verification of the expression of KCNQ1OT1 in colon was conducted (**Supplementary Figure S3**). The results showed that the gene expression of KCNQ1OT1 in both transverse colon and sigmoid colon was higher than in blood. And the gene expression of KCNQ1OT1 across all tissues showed that, although KCNQ1OT1 did not represent the highest expression in colon, its expression level was not low either, consistent with what we found in TCGA-COAD: the KCNQ1OT1 expression in normal tissue was low (CPM: median: 2.41, mean: 3.46). But its expression level in tumor tissue in TCGA-COAD was higher (CPM: median: 4.73, mean: 10.67). All this data showed that KCNQ1OT1 had enough expression to physiologically function as a miRNA sponge.

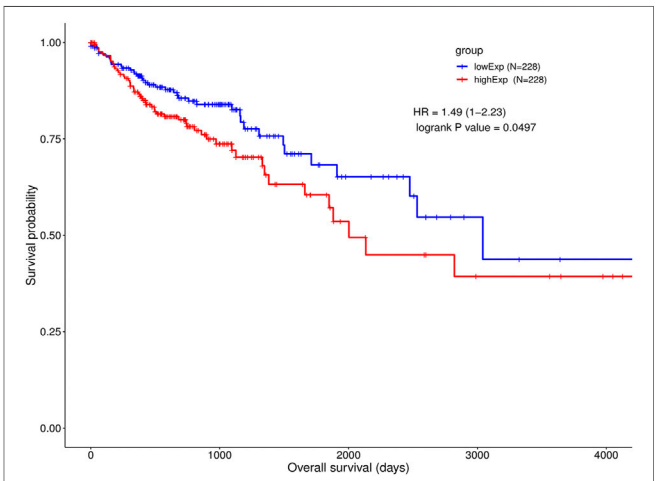

FIGURE 4 | Survival analysis of KCNQ1OT1 ceRNA network and gene co-expressions. Kaplan-Meier and ROC curves of CRC cohort in high and low KCNQ1OT1 expression level.

TABLE 2 | Hypergeometric test and Correlation analysis for mRNAs selection.

Genes	Fold enrichment	Hyper *P*	Shared miRNAs	Cor R	Cor *P*
ANKRD13B	3.763586957	0.00598274	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-326,hsa-miR-330–5p,hsa-miR-29a-3p	0.192288435	2.05E-05
COL7A1	6.02173913	0.008538913	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-29a-3p	0.204474578	6.30E-06
HELLS	9.032608696	0.001910986	hsa-miR-7-5p,hsa-miR-140–5p,hsa-miR-24–3p	0.231966962	3.36E-07
TRAF5	12.04347826	0.000505415	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-29a-3p	0.331376549	2.86E-13
SCML1	4.817391304	0.005491359	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-24–3p,hsa-miR-29a-3p	0.234030918	2.65E-07
ARHGEF19	12.04347826	0.000505415	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-29a-3p	0.197882747	1.20E-05
GGCT	12.04347826	0.000505415	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-29a-3p	0.14387579	0.001121
ABCB6	12.04347826	0.000505415	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-29a-3p	0.294248362	1.02E-10
CDC7	4.37944664	0.008147588	hsa-miR-29c-3p,hsa-miR-29b-3p,hsa-miR-335–5p,hsa-miR-29a-3p	0.113130038	0.008238

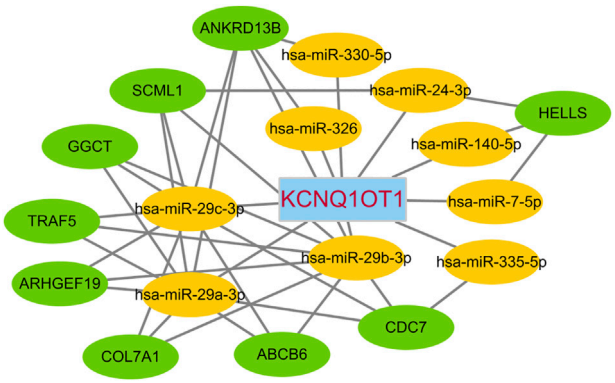

FIGURE 5 | KCNQ1OT1 ceRNA network.

Target structural accessibility for miRNA target recognition to lncRNA KCNQ1OT1 or mRNAs was calculated and visualized using Sfold and STarMirDB (**Supplementary Figures S4, S5**).

The KCNQ1OT1 ceRNA Network Signature Was Highly Expressed in CRC Transcriptomic Profiles

A GSVA assessment of network signatures in tumor and normal samples and using the TCGA-COAD dataset showed higher network signature GSVA scores for the tumor tissues than for the normal samples. This result was expected because we constructed this network from DEGs in the TCGA-COAD dataset. For validation, we applied the GSVA assessment of network signatures in three GEO CRC transcriptome datasets (GSE21510, GSE113513 and GSE32323). In these GEO datasets, KCNQ1OT1 ceRNA network signature GSVA scores were significantly higher for tumor samples than for normal samples, which indicated the disease specificity of this KCNQ1OT1 ceRNA network (**Figure 7**).

Different Immune Cell Infiltration Levels for High- and Low-GSVA-Score Tumor Samples

The immune cell infiltration levels for 24 immune cell subsets in tumor samples from the TCGA-COAD dataset are shown in **Figure 8**. Overall, the profiles of immune infiltration varied significantly between the tumor samples with high GSVA scores and those with low scores (**Figure 8A**). Also, lower $CD4^+/CD8^+$ T-cell ratios were found in the tumor samples with high network GSVA scores than in the tumor samples with low scores. In the high-score group, along with the increase in the levels of $CD8^+$ T cells in general was observed an increase in those of naïve $CD8^+$ T cells. Notably, in contrast, lower levels of cytotoxic T cells were found for the high-score group than for the low-score group. Also, along with the relatively low levels of $CD4^+$ T cells in the tumor samples with high scores, were relatively low levels of the T helper subsets Th2 and Th17. Other T cell subsets, namely Treg (nTreg and iTreg), Tex, Tem and MAIT cells were also downregulated. However, the B cells were upregulated. Regarding another lymphoid cell line, natural killer cell levels showed relatively low levels for the high-score group. Further, regarding the myeloid cell line, lower levels of monocytes, macrophages and neutrophils were also found for the high-score group.

Patient sex, tumor stage and age were also investigated. However, the network GSVA score was neither associated with sex ($p = 0.8$) nor with age ($p = 0.77$) nor with tumor stage ($p = 0.78$), as shown in **Supplementary Figures S6A–C**, respectively.

DISCUSSION

There has been increasing evidence for a link between dysregulation of lncRNAs and cancers (Gutschner and Diederichs, 2012). For instance, studies have shown the lncRNA MALAT1 to be associated with the development and metastasis of cancer cells (Gutschner et al., 2013; Tripathi et al., 2013). HOTAIR to be implicated in cancer metastasis regulation by targeting the chromatin repressor polycomb protein (Gupta et al., 2010), and linc00673 to activate WNT/β-catenin signaling and aggravate lung adenocarcinoma by binding between casein kinase 1ε (CK1ε) and DEAD box RNA helicase DDX3 (Guan et al., 2019).

Recent studies have also demonstrated important roles played by lncRNAs in the tumorigenesis of CRC. Colorectal cancer associated transcript 1 (CCAT1) was reported to be a specific biomarker for CRC (Xiang et al., 2014), and to be expressed at high levels not only in pre-malignant conditions but also throughout the various disease stages of CRC (Ozawa et al.,

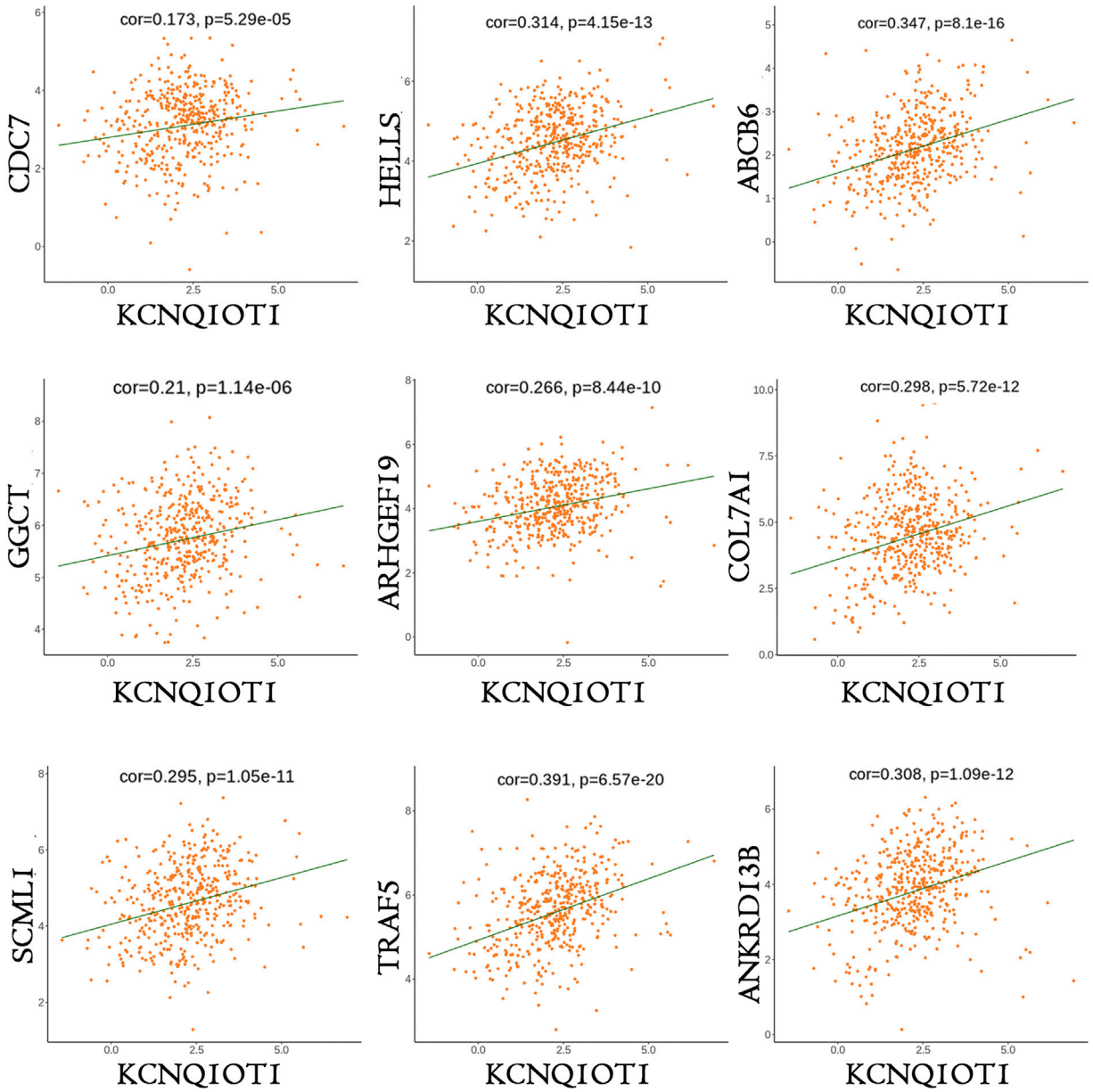

FIGURE 6 | Co-expressions of mRNAs and KCNQ1OT1 in KCNQ1OT1 ceRNA network.

2017). Further, CCAT2, a lncRNA derived from the human chromosome MYC-335 region, has been shown to enhance metastasis and invasion through the MYC pathway-regulating miRNAs miR-20a29 and miR-17–5p. These two CRC-specific lncRNAs were shown to be transcribed from the 8q24 region where previous studies have found common genetic variants related to the risk of CRC (Yang et al., 2019).

In the current work, we showed that the lncRNA KCNQ1OT1, an antisense lncRNA transcribed from the human chromosome 11p15.5 KCNQ1 locus, is related to the pathogenesis and progression of CRC. It has been shown in previous work to function as a *cis*-silencer of the imprinted KCNQ1 cluster and to be involved in the metastasis and proliferation of various tumors, such as hepatocellular carcinoma, cholangiocarcinoma, ovarian and breast cancer tumors (Feng et al., 2018; Luo and Jin, 2019).

In our research, there were 213 lncRNAs differentially expressed between tumor and normal tissue. Among these 213 lncRNAs, we identified 22 lncRNAs involved in ceRNA networks. We analyzed the survival association with these 22 lncRNAs. Only KCNQ1OT1 significantly associated with the clinically obtained survival data. We compared 228 patients expressing KCNQ1OT1 at high levels with 228 patients expressing it at low levels. According to our Kaplan-Meier survival analysis, the patients with overexpressed KCNQ1OT1 did not survive on

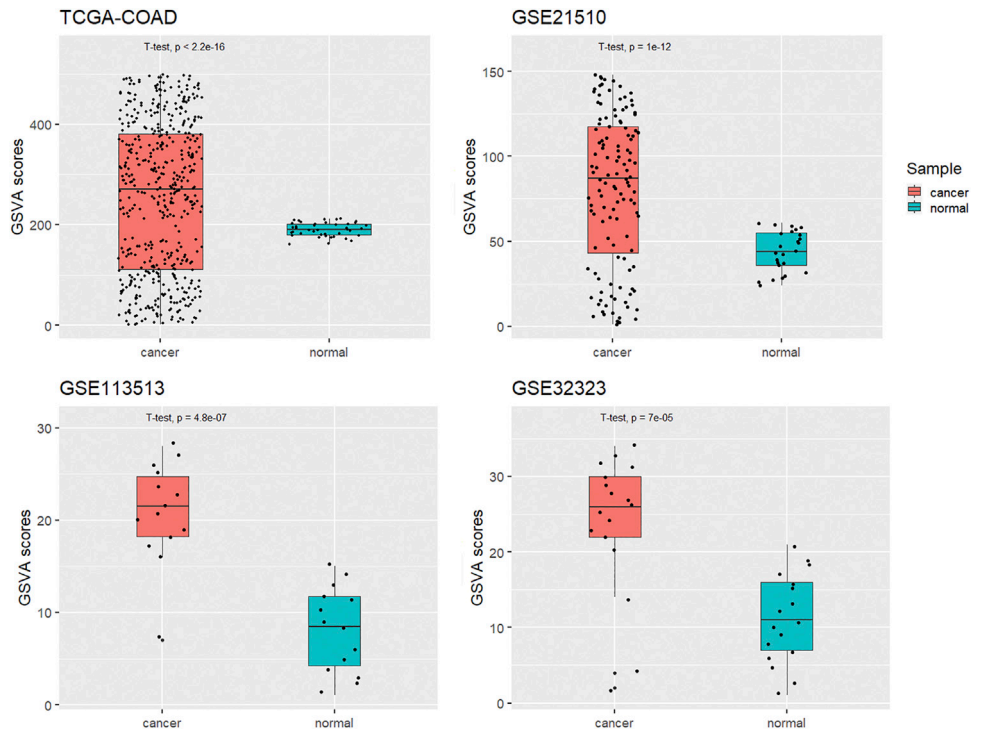

FIGURE 7 | GSVA scores of KCNQ1OT1 ceRNA network for cancer and normal samples in four different CRC datasets.

average for as long as did those displaying low KCNQ1OT1 expression. This result was consistent with the latest research (Lin et al., 2021). KCNQ1OT1 was reported to be upregulated in CRC tissue according to a study by Li and others (Li F. et al., 2019). KCNQ1OT1 knockdown in HCT116 and SW480 CRC cells downregulated the expression of Atg4B, which has been shown to cleave LC3 and promote the formation of autophagosomes (Hemelaar et al., 2003). The viability of these cells decreased after being treated with oxaliplatin, which implicated KCNQ1OT1 in inducing autophagy protection and chemo-resistance. Moreover, the relationship between the upregulation of KCNQ1OT1 and poor prognosis of CRC patients also suggested that higher KCNQ1OT1 levels in patients make them resistant to chemotherapy or other anti-cancer treatments (Li Y. et al., 2019). These results suggested that KCNQ1OT1 might become a promising therapeutic target for use in CRC patients.

The development of an understanding of RNA-RNA interactions is expected to provide deep insights into gene regulatory networks potentially implicated in various cancer diseases. Our optimized ceRNA network showed that the critical hub gene KCNQ1OT1 indirectly regulated 9 other mRNAs, namely those encoding ATP-binding cassette subfamily B member 6 (ABCB6), Rho guanine nucleotide exchange factor 19 (ARHGEF19), helicase lymphoid-specific (HELLS), gamma-glutamylcyclotransferase (GGCT), cell division cycle 7 (CDC7), sex comb on midleg-like 1 (SCML1), collagen type VII alpha 1 chain (COL7A1), TNF-receptor-associated factor 5 (TRAF5) and ankyrin repeat domain 13B (ANKRD13B) (**Figure 5**). According to our correlation analysis, these 9 mRNAs showed a co-expression relationship with KCNQ1OT1. A GeneCards (www.genecards.org) (Stelzer et al., 2016) analysis showed these gene products to be involved in some cancer-related pathways, for instance TRAF5 in the RANKL/RANK (receptor activator of NFKB (ligand)) signaling pathway, COL7A1 in ERK signaling, HELLS in the AMPK enzyme complex pathway and BRCA1 pathway, ARHGEF19 in RET signaling, and GGCT in a cancer metabolism pathway. These mRNAs and KCNQ1OT1 influence each other's level by competing for the same pool of microRNAs: miR-29c-3p, miR-29b-3p, miR-326, miR-330-5p, miR-29a-3p, miR-7-5p, miR-140-5p, miR-24-3p and miR-335-5p. Among these microRNAs, the binding sited of miR-7-5p, miR-29a-3p, miR-29c-3p, miR-140-5p, miR-326 and hsa-miR-335-5p with KCNQ1OT1 have been confirm by experimental studies (Hu et al., 2018; Sun et al., 2018; Cheng et al., 2020; Mu et al., 2020; Yao et al., 2020; Zhou et al., 2021).

As the GSVA method can be used to score a gene set signature and depends neither on the composition nor size of a dataset, we applied this method to measure the optimal network signature across different datasets. Samples with a high network score across the independent datasets were found, based on the results, to be particularly enriched in the CRC tumor group.

The identified subnetwork was reported previously to be significantly more reproducible between different disease cohorts then were individual marker genes (Chuang et al., 2007), and in our study, the results of all these datasets indicated dramatically higher network scores for the tumor

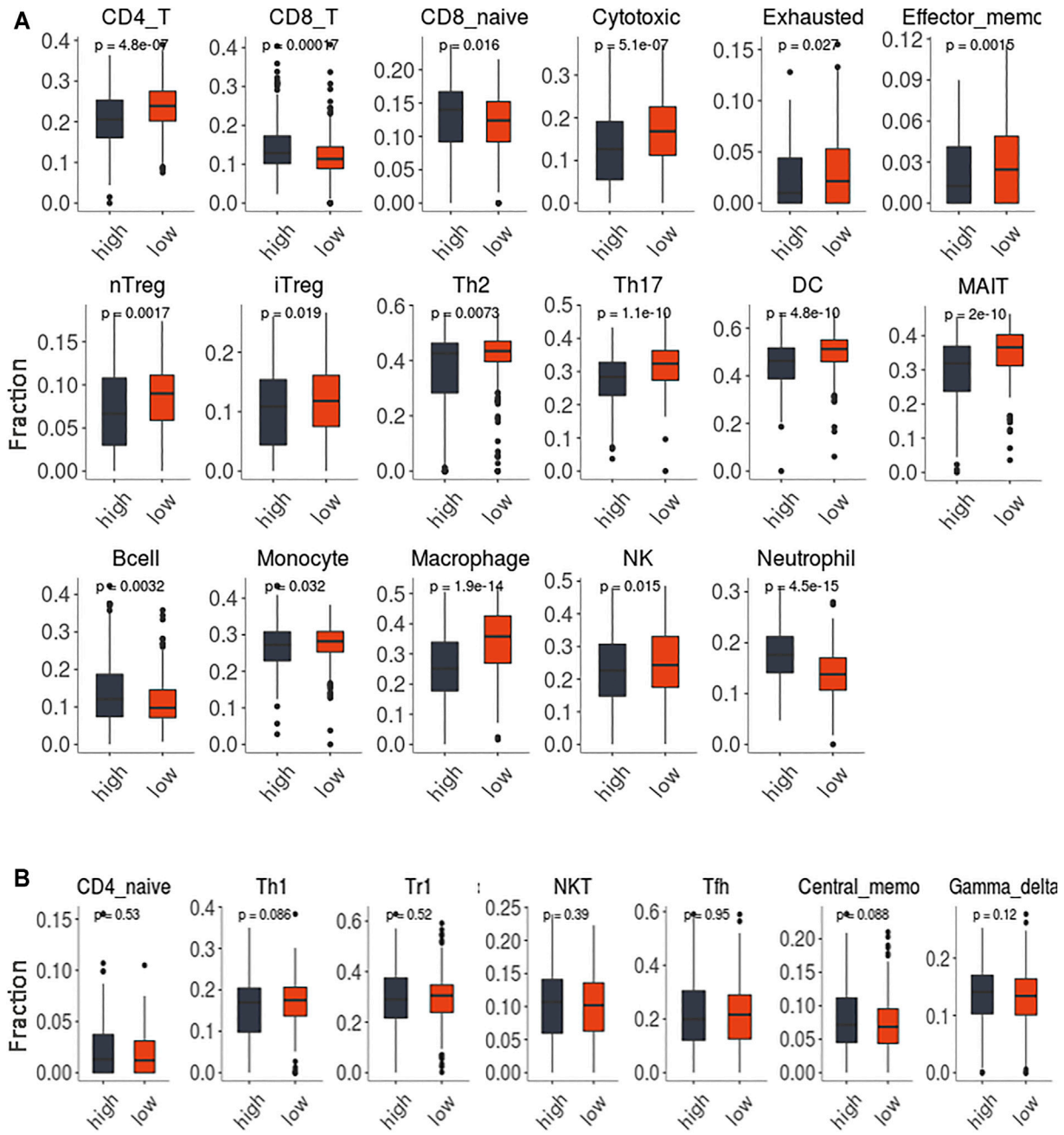

FIGURE 8 | The immune infiltration of 24 immune cells subsets in the tumor samples with high GSVA score and low scores. **(A)**. The comparisons with statistically significant difference. **(B)**. The comparisons without statistically significant difference.

samples than for the normal samples (**Figure 7**). These results suggested that the KCNQ1OT1 ceRNA network might play a role in the mechanism of CRC pathogenesis. This network signature could be a potentially way to distinguish CRC tissue form normal tissue.

To gain more knowledge about the function of the KCNQ1OT1 ceRNA network related to immune infiltration, we used ImmuCellAI to estimate the abundance of immune cells from individual samples. The estimation implied that the overall profiles of immune infiltration differed significantly between the tumor samples with high GSVA scores and those with low scores (**Figure 8A**, **Supplementary Table S7, S8**). Lower $CD4^+/CD8^+$ T-cell ratio s were indicated for the higher-score groups. Both the reduction of the $CD4^+$ T cell population and increase of the $CD8^+$ T cell population contributed to the lower ratio of $CD4^+/CD8^+$ T-cell. The $CD4^+/CD8^+$ T-cell ratio was considered as an immunostimulatory marker in the general population (Gojak et al., 2019). A low $CD4^+/CD8^+$

T-cell ratio has been shown to be an immune risk phenotype related to chronic inflammation, persistent immune dysfunction and immune senescence (Wikby et al., 2005). In some investigations, the $CD4^+/CD8^+$ T-cell ratio was described as a significant marker for prognostic prediction in HIV/AIDS patients (Castilho et al., 2019; Gojak et al., 2019; F; Li F. et al., 2019). Notably, in our study, the trend found for $CD8^+$ T cells was opposite that found for cytotoxic T cells (cytokine-produced $CD8^+$ T cells) in the network score comparisons. The generation of a functional cytotoxic T-cell response in general depends on the activation of Th1 cells. However, in the current work, the proportions of Th1 cells in the high- and low-GSVA-score groups showed no significant difference. Another possibility was that a recruitment of $CD8^+$ T cells in the tumor tissue accompanied the reduction of cytotoxic T cells, and that this recruitment reflected a suppression of the cytotoxic machinery of the infiltrates, suggesting that the dysfunctional status of the effector cells was due to the microenvironment in the samples with high network scores.

Beside the immune infiltration, the network score differences in sex, tumor stage and age were also investigated. Neither any sex bias nor age association with the network scoring was found according to the statistical analysis (**Supplementary Figure S6**).

Our findings investigated a network signature for the lncRNA KCNQ1OT1, which was shown in the current work to be a representative of the ceRNA network transcribed in CRC tissues. This ceRNA network could be a potential regulator in colorectal cancer immunity. These findings indicated an oncogenic role for KCNQ1OT1 and its downstream target mRNAs in the pathogenesis and progression of CRC.

CONCLUSION

The results of our study indicated that the KCNQ1OT1 ceRNA network could be involved in the regulation of the CRC tumor microenvironment, providing a rationale for the further exploitation of KCNQ1OT1 as a possible functional contributor to and therapeutic target for CRC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL wrote the manuscript. JD contributed the design of the study. WL and SL organized the references and databases. JD and JL explored the RNAseq data. JL and WL revised manuscript. All authors contributed to write and approve the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.684002/full#supplementary-material

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GLOSSARY

ABCB6 ATP binding cassette subfamily B member 6

ANKRD13B ankyrin repeat domain 13B

ARHGEF19 rho guanine nucleotide exchange factor 19

BP biological process

CC cellular component

CCAT1 colorectal cancer associated transcript 1

CDC7 cell division cycle 7

COL7A1 Collagen Type VII Alpha 1 Chain

ceRNA competitive endogenous RNA

CK1ε casein kinase 1ε

CRC colorectal cancer

DC dendritic cell

DEGs differentially expressed genes

γδ T gamma delta T cell

GGCT gamma-glutamylcyclotransferase

GO gene ontology

GSVA gene set variation analysis

GTEx The Genotype-Tissue Expression

HELLS helicase, lymphoid-specific

HGF hepatocyte growth factor

iTreg induced regulatory T cell

KM Kaplan-Meier

lncRNAs long non-coding RNAs

MAIT mucosal-associated invariant T cell

MF molecular function; miRNA, microRNA

ncRNAs non-coding RNAs

NK natural killer cell

NKT natural killer T cell

nTreg natural regulatory T cell

RANKL/RANK receptor activator of NFKB (ligand)

RTK receptor tyrosine kinase

SCML1 sex comb on midleg-like 1 (Drosophila)

SF scatter factor

siRNAs small interfering RNA

Speed2 Signalling Pathway Enrichment using Experimental Datasets 2

Tc cytotoxic T cell

TCGA The Cancer Genome Atlas

Tcm central memory T cell

Tex exhausted T cell

Tem effector memory T cell

Tfh follicular T helper cell

Th1 T helper 1 cell

Th2 T helper 2 cell

Th17 T helper 17 cell

TMM trimmed mean of M values

TRAF5 TNF receptor associated factor 5

Tr1 type 1 regulatory T cell.

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ORIGINAL RESEARCH
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Assessment of CircRNA Expression Profiles and Potential Functions in Brown Adipogenesis

Pengpeng Zhang[1], *Mingxuan Sheng*[1], *Chunyu Du*[1], *Zhe Chao*[2], *Haixia Xu*[1], *Xiaofang Cheng*[1], *Cencen Li*[1]* *and Yongjie Xu*[1,3]*

[1]*Department of Biotechnology, College of Life Sciences, Xinyang Normal University, Xinyang, China,* [2]*Institute of Animal Science and Veterinary Medicine, Hainan Academy of Agricultural Sciences, Haikou, China,* [3]*Institute for Conservation and Utilization of Agro-Bioresources in Dabie Mountains, Xinyang Normal University, Xinyang, China*

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Santosh Kumar,
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Nagarjuna Reddy Pamudurti,
Brandeis University, United States

***Correspondence:**
Cencen Li
licencen2009@126.com
Yongjie Xu
yongjx81@126.com

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Brown adipose tissue (BAT) is specialized for energy expenditure, thus a better understanding of the regulators influencing BAT development could provide novel strategies to defense obesity. Many protein-coding genes, miRNAs, and lncRNAs have been investigated in BAT development, however, the expression patterns and functions of circRNA in brown adipogenesis have not been reported yet. This study determined the circRNA expression profiles across brown adipogenesis (proliferation, early differentiated, and fully differentiated stages) by RNA-seq. We identified 3,869 circRNAs and 36.9% of them were novel. We found the biogenesis of circRNA was significantly related to linear mRNA transcription, meanwhile, almost 70% of circRNAs were generated by alternative back-splicing. Next, we examined the cell-specific and differentiation stage-specific expression of circRNAs. Compared to white adipocytes, nearly 30% of them were specifically expressed in brown adipocytes. Further, time-series expression analysis showed circRNAs were dynamically expressed, and 117 differential expression circRNAs (DECs) in brown adipogenesis were identified, with 77 upregulated and 40 downregulated. Experimental validation showed the identified circRNAs could be successfully amplified and the expression levels detected by RNA-seq were reliable. For the potential functions of the circRNAs, GO analysis suggested that the decreased circRNAs were enriched in cell proliferation terms, while the increased circRNAs were enriched in development and thermogenic terms. Bioinformatics predictions showed that DECs contained numerous binding sites of functional miRNAs. More interestingly, most of the circRNAs contained multiple binding sites for the same miRNA, indicating that they may facilitate functions by acting as microRNA sponges. Collectively, we characterized the circRNA expression profiles during brown adipogenesis and provide numerous novel circRNAs candidates for future brown adipogenesis regulating studies.

Keywords: circRNA, brown adipocyte, adipogenesis, obesity, high-throughput RNA sequencing

INTRODUCTION

In recent years, the number of obese people is increasing rapidly and becoming pandemic. As obesity is highly associated with metabolic syndromes, such as diabetes, cardiovascular diseases, and even cancer, it severely threatens public health (Blüher, 2019). Obesity develops when the energy intake is more than the energy expenditure. Human adipose tissue mainly includes white adipose tissue (WAT) and BAT. The WAT, which contains large lipid droplets, is the main place of energy storage, whereas BAT contains plenty of mitochondria and converts chemical energy into heat to maintain body temperature (Betz and Enerbäck, 2018). It had been considered that BAT only exists in infants, however recent reports detected functional BAT in adult humans (Leitner et al., 2017). The BAT activity positively correlates with human metabolic rate and reduces energy storage, thus strategies that enhance BAT development and increase BAT activity are considered as possible approaches to combat obesity (Bhatt et al., 2017).

Adipogenesis is the process through which preadipocytes differentiate into adipocytes. Previous studies have identified numerous protein regulators of brown adipogenesis, e.g. Pparγ, Prdm16, and Pgc1α (Shapira and Seale, 2019). In recent years, a lot of studies have been focusing on noncoding RNAs. It is known that many miRNAs (Gharanei et al., 2020) and lncRNA (Squillaro et al., 2020) regulate brown adipogenesis and thermogenesis. Recently, circRNA emerged as a new type of noncoding RNA. CircRNAs are covalently linked non-coding RNAs with neither 5′ caps nor 3' polyadenylated tails, thus it is more stable than linear RNAs (Li et al., 2018a). In 1979, circRNA was first observed in Hela cells by electron microscopy (Hsu and Coca-Prados, 1979). At that time, circRNA was considered as rare byproducts or spliced intermediates. However, with the routine use of high-throughput sequencing technology, circRNAs are expressed in various organisms (Memczak et al., 2015). CircRNAs are highly conserved between species and the expression patterns are tissue-specific and developmental stage-specific. Recent work suggests that circRNAs play vital roles in various biological activities, such as normal tissue development, pathological processes, and even disease progression. CircRNAs exert their function in several ways. First, circRNAs can bind miRNAs and inhibit their functions. Second, circRNAs can act as protein sponges to promote protein interaction or directly affect protein functions. Third, circRNAs can bind to RNA Pol II complex and act as cis-regulators of transcription. Fourth, a few circRNAs are reported to encode functional proteins (Zhang et al., 2019a).

Recent work indicated that circRNAs are involved in adipose tissue development and obesity. We previously reported that 3,771 circRNAs were expressed in white adipogenesis (Zhang et al., 2021a). Arcinas et al. detected thousands of circRNAs in mouse and human WAT. They identified circTshz2-1 and circArhgap5-2 were essential for adipogenesis (Arcinas et al., 2019). Liu et al. found circSAMD4A (hsa_circ_0004846) was significantly upregulated in obese people. CircSAMD4A affected pre-adipocytes differentiation by acting as the miR-138-5p sponge and regulated EZH2 expression (Liu et al., 2020). Wang et al. discovered that 9,311 circRNAs were expressed in duck pre-adipocytes and adipocytes. Circ-PLXNA1 could regulate adipogenesis by binding to miR-214 and affecting CTNNB1expression (Wang et al., 2020). Zhang et al. found circNrxn2 could promote WAT browning by binding to miR-103 and enhance FGF10 expression levels (Zhang et al., 2019b). Recently, circRNAs were reported to be differentially expressed during brown to white adipose tissue transformation in goats (Zhang et al., 2021b). However, the global expression patterns and functions of circRNAs in brown adipogenesis have not been reported.

BAT primary stromal vascular fraction (SVF) cells which contain a lot of primary BAT preadipocytes are widely used to study adipogenesis *in vitro* (Shan et al., 2016; Oguri et al., 2020). In the present study, we focus on determining the expression profiles and potential roles of circRNAs in brown adipogenesis. The primary SVF cells were isolated from mice interscapular BAT and circRNAs were detected during adipogenesis by RNA-seq. A great number of novel circRNAs were identified and their expression patterns were characterized. Then, we determined the tissue-specific and differentiation stage-specific circRNAs. We also demonstrated that the differential expression circRNAs (DECs) may potentially be involved in regulating brown adipogenesis by acting as miRNA sponges.

MATERIALS AND METHODS

BAT SVF Cells Culture

Primary BAT SVF cells were isolated from C57BL/6J background mice as described (Shan et al., 2016). Briefly, the interscapular brown fat was dissected from 8-week-old mice. Next, the brown fat was cut to fine pieces and digested with 1.5 mg/ml collagenase type I (catalog number SCR103, Sigma-Aldrich) at 37°C water bath for 50 min. The tissue was vortexed every 10 min. Then, the digestion was filtered through 100-μm and 70-μm cell strainers. The digestion was centrifuged at 400 g for 6 min to enrich SVF cells. The cells were seeded into a cell culture plate with growth medium (DMEM and 10% fetal bovine serum). To induce adipogenic differentiation, the growth medium was supplemented with 2.85 mM recombinant human insulin (catalog number I8830, Solarbio), 0.3 mM dexamethasone (catalog number D8040, Solarbio), and 0.63 mM 3-isobutyl-methylxanthine (catalog number I7018, Sigma-Aldrich). After 96 h, the medium was switched to growth medium, supplemented with 10 nM triiodothyronine (catalog number T6397, Sigma-Aldrich), and 200 nM insulin to induce mature adipocytes. For Oil red O staining, cells were fixed with 10% formaldehyde for 5 min and stained with staining solutions (catalog number G1262, Solarbio) according to the instructions.

RNA-Sequencing

Total RNA of the BAT adipocytes was collected using Trizol (catalog number 15596026, Thermo Fisher Scientific). The total RNA was treated with Epicentre Ribozero rRNA Removal kit (catalog number RZH1046, Epicentre) and RNase R (catalog number RNR07250, Epicentre) to remove ribosomal RNA and

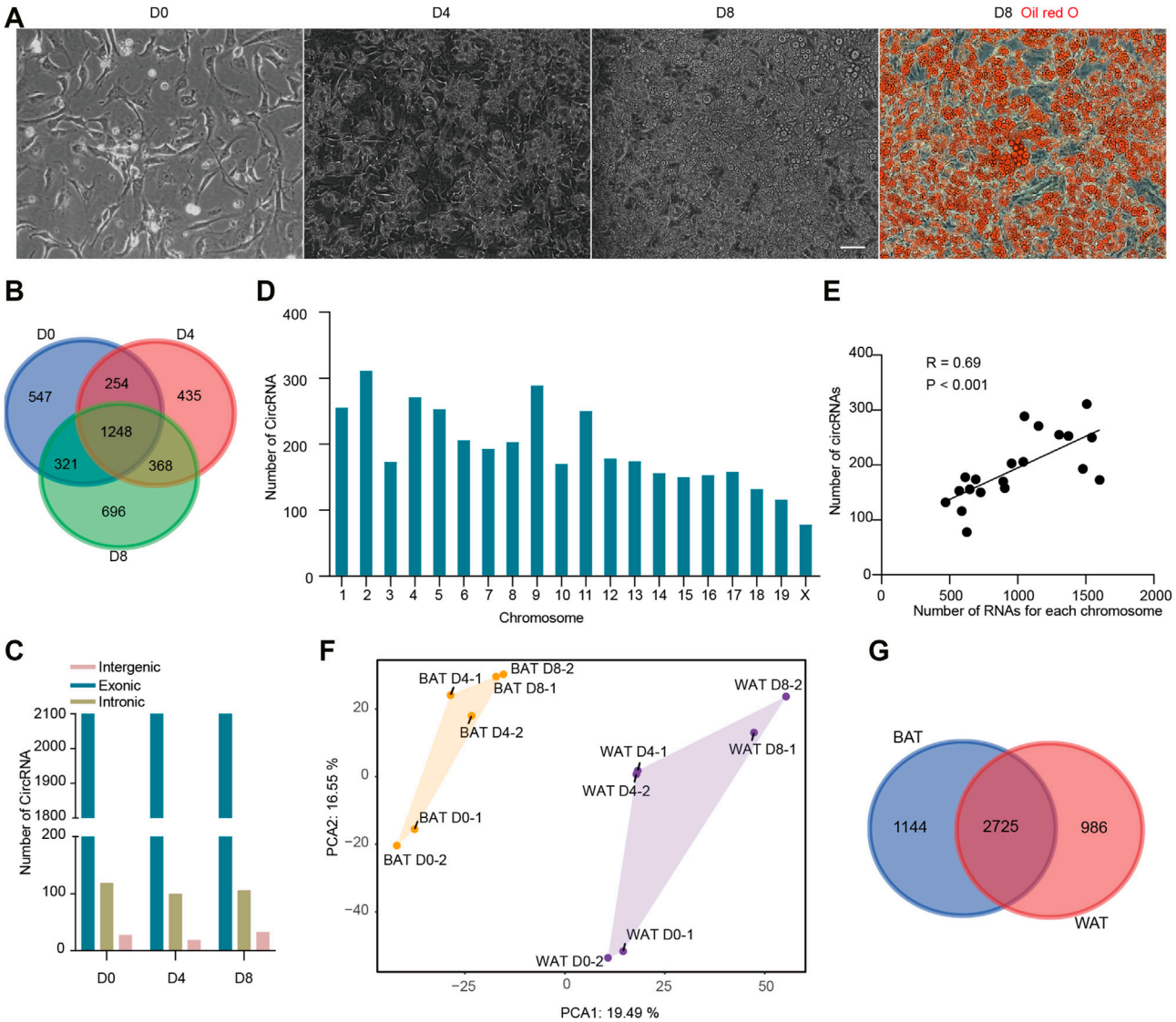

FIGURE 1 | Identification and characterization of circRNAs in brown adipogenesis. **(A)** Representative pictures of BAT pre-adipocytes differentiation. Scale bar = 20 μm. **(B)** The number of CircRNAs identified during BAT pre-adipocytes differentiation. **(C)** The number of circRNA based on back-splicing regions in each adipogenesis stage. **(D)** The number of circRNAs derived from each chromosome. **(E)** Correlation of circRNA and mRNA number from the same chromosome. (F) Principal Component Analysis (PCA) of circRNA expression during BAT and WAT pre-adipocytes differentiation. **(G)** Venn diagrams of circRNAs expressed in BAT and WAT adipogenesis.

linear RNA. Then, the sequencing libraries were prepared by NEBNext Ultra Directional RNA Library Prep Kit (catalog number E7760S, NEB, USA) following the manufacturer's instructions. At last, the libraries were sequenced on an illumine platform and 150 bp paired reads were generated.

Computational Analyses of CircRNA

CircRNAs were detected by CIRI2 as previously described (Zhang et al., 2018). Then the sequence data were mapped to mm9 with BWA-MEM algorithm (Li, 2013). CircRNA candidates mapped by at least two reads in both replicated were kept for subsequent study. Then, the DECs were identified with the likelihood ratio test from R package DESeq2 (version 1.10.1) (Hiraike et al., 2017). The DECs were clustered based on their expression patterns by the degPatterns function from the DEGreport package (Pantano, 2021).

RT-PCR

The cDNA was obtained by using random primers and a reverse transcription kit (catalog number RR037A, Takara). CircRNA sequences were amplified by divergent primers which were designed by Primer3 (https://primer3.ut.ee/) to cover the back-splicing sites. PCR products were extracted by agarose gel DNA extraction kit (catalog number 9762, Takara). Then the purified DNA was sent to perform Sanger sequencing. Then qPCR was performed to examine the expression levels of circRNAs on a

LightCycler 96 system (Roche, Germany) using TB Green Premix Ex II (catalog number RR820A, Takara) according to the instructions. The relative expression levels were normalized to 18S.

CircRNA Expression Patterns Analysis

The expression patterns of circRNAs were classified by the STEM program with No normalization/add 0 option (Ernst and Bar-Joseph, 2006). GO annotations were obtained from the STEM program with the default option.

CircRNA-miRNA Networks Construction

The circRNA sequences were obtained from package circPrimer (Zhong et al., 2018)and the potential miRNA binding sites were predicted by miRanda (version 3.3a) (John et al., 2004). To obtain more rigorous prediction results, the energy threshold was set to −7 kcal/mol and the score threshold was set to 150, while the other parameters were set to default values. Then, the circRNA-miRNA interaction networks were constructed by Cytoscape (version 3.8.2) (Shannon et al., 2003).

RESULTS

Identification of circRNAs in brown Adipogenesis

To detect circRNAs transcripts in brown adipogenesis, we cultured primary BAT SVF cells and induced them to differentiate. As shown in **Figure 1A**, the BAT SVF cells were in fibroblast-like spindle shape on day 0 (D0, proliferation stage), whereas the cells round-up on day 4 (D4, early differentiated stage) and were filled with large lipid droplets on day 8 (D8, fully differentiated stage) as indicated by Oil red O staining. Total RNA was collected on D0, D4, and D8 post differentiation. The circRNAs were enriched and then RNA-seq was performed. CIRI2, which is based on back splicing alignment, was used to detect circRNAs. Then the high confident circRNAs with at least 2 reads were detected in both 2 replicates were selected for subsequent analysis. In this way, a total of 3,869 distinct circRNA candidates were detected, indicating that a larger number of circRNAs were expressed in brown adipogenesis (**Supplementary Table S1**). Notably, up to 36.9% of the circRNAs were novel and not annotated in the circBase database (http://www.circbase.org/). We found that only 32.3% of circRNAs continue to be expressed, while 547, 435, and 696 circRNAs were specifically expressed in proliferation, early differentiated, and fully differentiated stages respectively (**Figure 1B**). We found that 48 circRNAs were not aligned to the annotated genome sequence, the other 3,821 circRNAs were derived from 2,046 gene locus. Based on our previous study, 20,968 genes were transcribed to mRNA during brown adipogenesis (with a minimum of 4 reads, GEO accession number GSE173710). Thus, as many as 9.61% of genes could produce circRNAs.

According to the back-splicing regions, circRNAs are classified into exonic, intronic, and intergenic circRNAs. We found the ratio of the three types of circRNAs were similar across brown adipogenesis, in which exonic circRNAs were identified as the major type which accounts for more than 93% of all circRNAs, then followed by intronic circRNAs and intergenic circRNAs (**Figure 1C**). Next, we examined the chromosome distribution of circRNAs. It is noticed that circRNAs were unevenly distributed. Chromosome 2 gave rise to 311 circRNAs which is the greatest, while chromosome X produced only 78 circRNAs (**Figure 1D**). We suspected that the number of circRNA was related to the length of the chromosome. Thus, we calculated the association between them, and a significant correlation was found ($R = 0.51$, $p < 0.05$). It is reported that both circRNAs and liner mRNAs are sliced from pre-RNAs, we further examined the association between circRNA and linear mRNA number from the same chromosome ($R = 0.69$, $p < 0.001$, **Figure1E**). These results suggested that the biogenesis of circRNA was related to linear mRNA transcription.

As mentioned earlier, BAT and WAT are functionally distinct adipose tissue. To compare the circRNA expression profiles, we collected the WAT SVF circRNA expression data from our previous study (GEO accession number GSE178502), in which the same mice were used as the current study and WAT SVF were isolated from inguinal WAT. Principal component analysis (PCA) analysis showed that samples derived from different tissues located far away, indicating BAT and WAT circRNA expression profiles were largely different (**Figure 1F**). Then we examined the tissue-specific circRNAs. As shown in (**Figure 1G**), 2,725 circRNAs were co-expressed in both adipose tissues, while 1,144 and 986 circRNAs were specifically expressed in BAT and WAT, respectively. Thus, almost 30% of the circRNAs were specific to BAT or WAT.

Alternative Back-Splicing of CircRNAs

In most cases, a host gene only produced one circRNA, however, these circRNAs only account for 31.8% of all the circRNAs expressed in BAT. The other circRNAs came from alternative back splicing (**Figure 2A**). Strikingly, some host genes even produced more than 10 circRNA isoforms, for example, Atrophin 2, which acts as transcriptional co-repressors and plays key roles during embryogenesis (Zoltewicz et al., 2004), produced up to 20 distinct circRNAs. The above results suggest that alternative back-splicing is an important source of circRNA diversity.

As reported, one gene can make several circRNAs by selectively using different splice donors or splice acceptors (Liang et al., 2017). For example, Nedd4 is an E3 ubiquitin ligase that is involved in adipogenesis by regulating Pparγ stability (Yao et al., 2019). Nedd4 could produce three distinct circRNA isoforms by using the same splice acceptor and different splice donors (**Figure 2B**). Another example is Glis3, which is a transcription factor and plays a key role in neonatal diabetes, type 1 and type 2 diabetes (Shan et al., 2016). Glis3 produced three circRNA isoforms (circ_0000942, circ_0007671, and circ_0007670) that also share the same acceptor. Another example is Nifa, which co-localizes with Pparγ and transcriptionally induces the brown fat gene expression during brown adipocyte differentiation (Hiraike et al., 2017). As shown in **Figure 2C**, Nifa produced four circRNA isoforms (circ_Nifa_1,

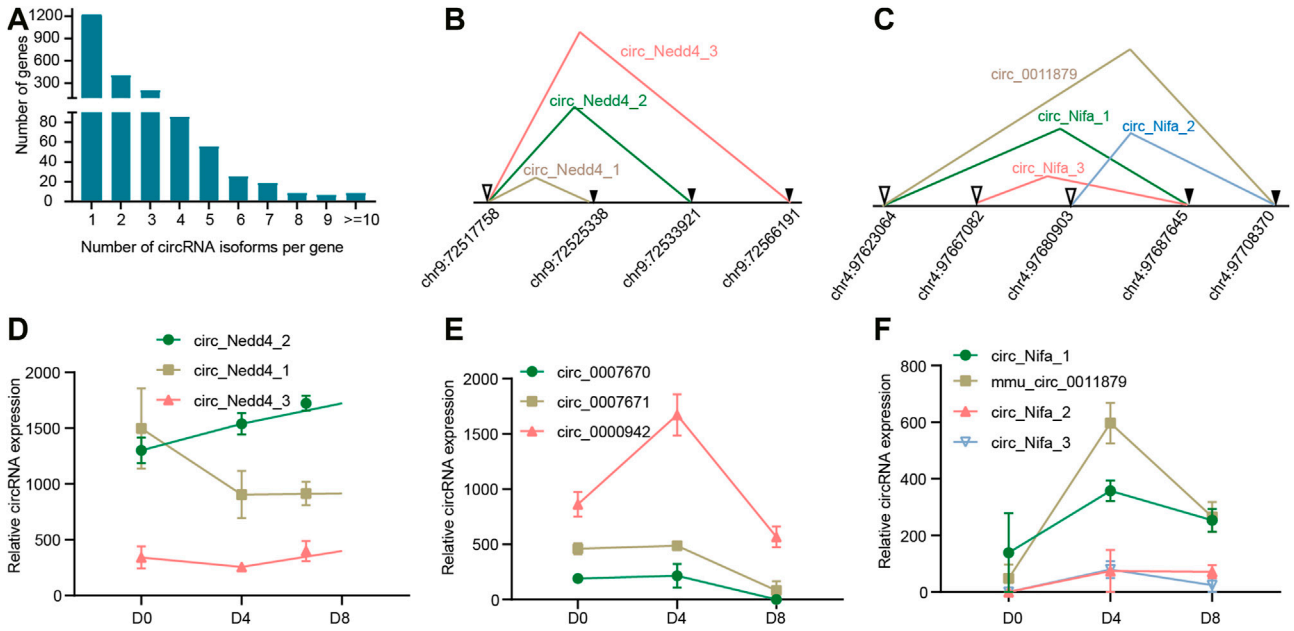

FIGURE 2 | Alternative back-splicing of circular RNAs. **(A)** Distribution of circRNAs variants among genes. **(B)** Generation model of circRNA variants derived from the same acceptor in Nedd4. Splicing acceptor is indicated with ▼, while splicing donor is indicated with▽. **(C)** Generation model of circRNA variants derived from same acceptor or same donor in Nifa. **(D–F)** Expression profile of circRNA isoforms for Nedd4, Glis3, and Nifa across brown adipogenesis.

circ_Nifa_2, circ_Nifa_3, and circ_0011879). The alternative splicing was very complex. circNifa1 and circNifa3 shared an acceptor, while circ_0011879 and circNifa2 shared another acceptor. Meanwhile, circ_0011879 and circNifa1 shared a donor.

Next, we explored the expression of these circRNA isoforms. For circRNAs derived from Nedd4, we found circNedd4_1 and circNedd4_2 were the dominant isoforms and the expression patterns of the three circRNA isoforms were completely different. CircNedd4_2 continually increased, while circNedd4_2 decreased on D4. CircNedd4_3 began to increase on D8 (**Figure 2D**). For the circRNAs derived from Glis3, circ_0000942 increased from D0 to D4, and then start to decrease. Circ_0007671 and circ_0007671 showed similar expression profiles, which kept unchanged until D4 and then decreased to the lowest expression level on D8 (**Figure 2E**). For the circRNAs derived from Nifa, circ_Nifa_1 and circ_0011879 were the dominant isoforms. Then expression patterns of them were similar, which showed an increase on D4 and then decreased (**Figure 2F**). In summary, alternative back-splicing expands the diversity of circRNAs, and expression among the back-splicing variants is different.

Expression Patterns of CircRNAs in BAT Adipogenesis

As mentioned earlier, BAT preadipocytes undergo a tremendous change of morphology during adipogenesis. To examine the overall circRNA expression levels, we did a boxplot and found that the levels of circRNAs during brown adipogenesis were comparable to each other (**Figure 3A**). To analyze the variation of circRNA expression profiles, we performed PCA. As shown in **Figure 3B**, the samples derived from the same group were located near each other, indicating the high repeatability of the results. As expected, the samples came from D4 and D8 located nearer than the D0, indicating the circRNA expression patterns were similar in differentiation stages, whereas they were largely different with the proliferation stage. Hierarchical clustering heatmap showed the correlation between different samples ranged from 0.88 to 0.98 (**Figure 3C**). Replicates in each stage were highly correlated, meanwhile, the D4 and D8 samples were clustered together, which was consistent with the PCA results.

To get an overview of circRNA expression patterns in brown adipogenesis, we carried out a time series analysis by STEM. 14 clusters were significantly enriched (with colored background, **Figure 3D**), which belong to 6 groups (with the same color). When we examined the GO enrichment results, we found green and yellow clusters were interesting, which correspond to the up and down regulated circRNAs. GO analysis for their parental gene showed the green cluster was associated with brown adipogenesis (**Supplementary Table S2**). For example, most genes in cluster 40 were associated with lipid metabolisms, such as sterol metabolic process, cholesterol metabolic process, and lipid catabolic process (**Figure 3E**). The genes in cluster 42 were enriched with GO terms of cold-induced thermogenesis, adaptive thermogenesis, and regulation of fat cell differentiation (**Figure 3F**). While in the yellow group (clusters 35 and 44), most genes were associated with cell mitosis. For instance, regulation of cell division, mitotic cell cycle process, and mitotic sister chromatid segregation (**Figures**

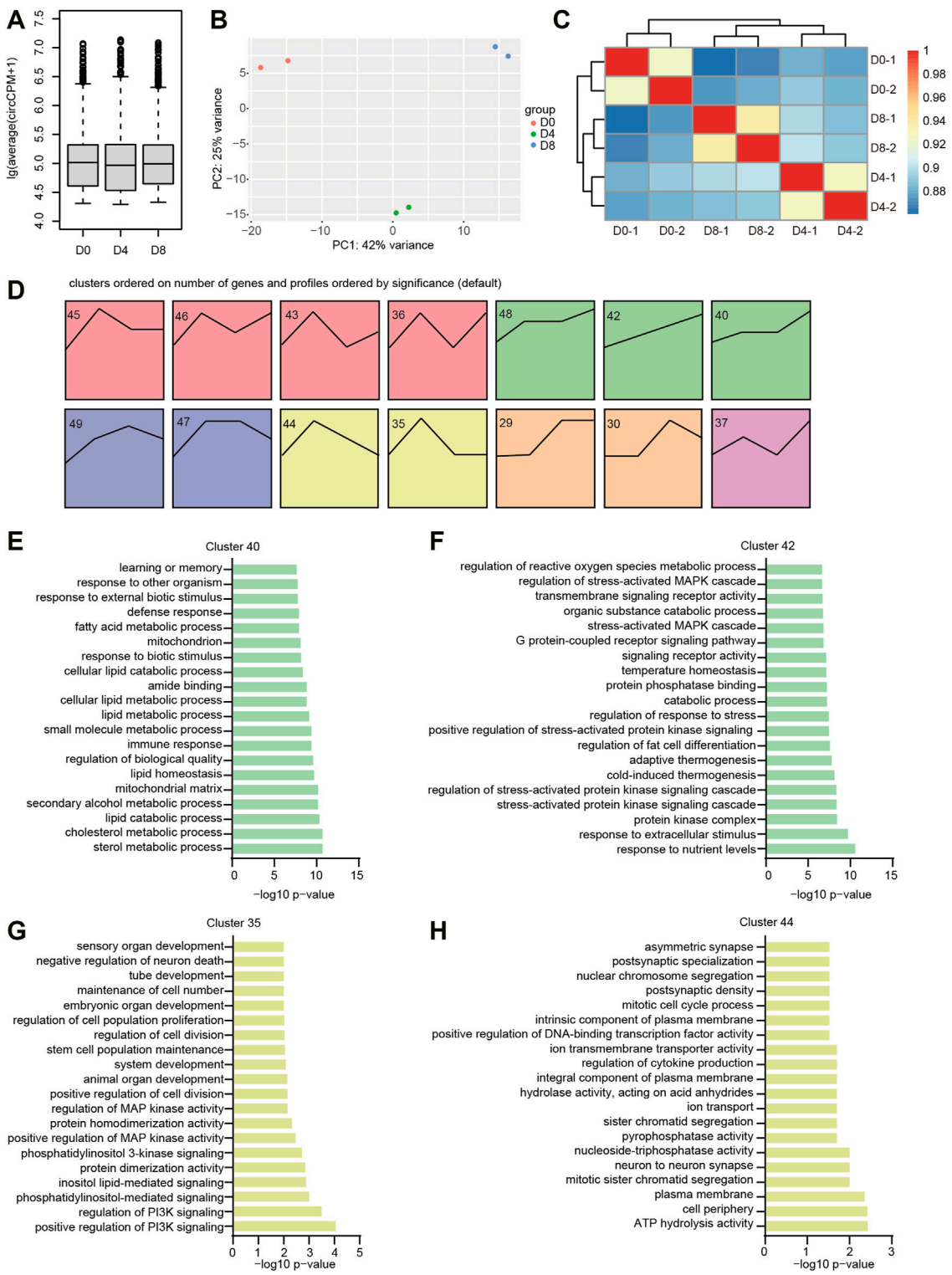

FIGURE 3 | The expression patterns of circRNA during brown adipogenesis. **(A)** Relative expression levels of circRNAs during brown adipogenesis. **(B)** PCA plot of circRNA expression during brown adipogenesis. **(C)** Correlation of circRNA expression calculated by DESeq2 rlog-normalized RNA-seq data. **(D)** Time series analysis of circRNA expression patterns. The number on the top left indicated the expression profile number. **(E–H)** GO terms enriched for the host genes of indicated circRNA clusters.

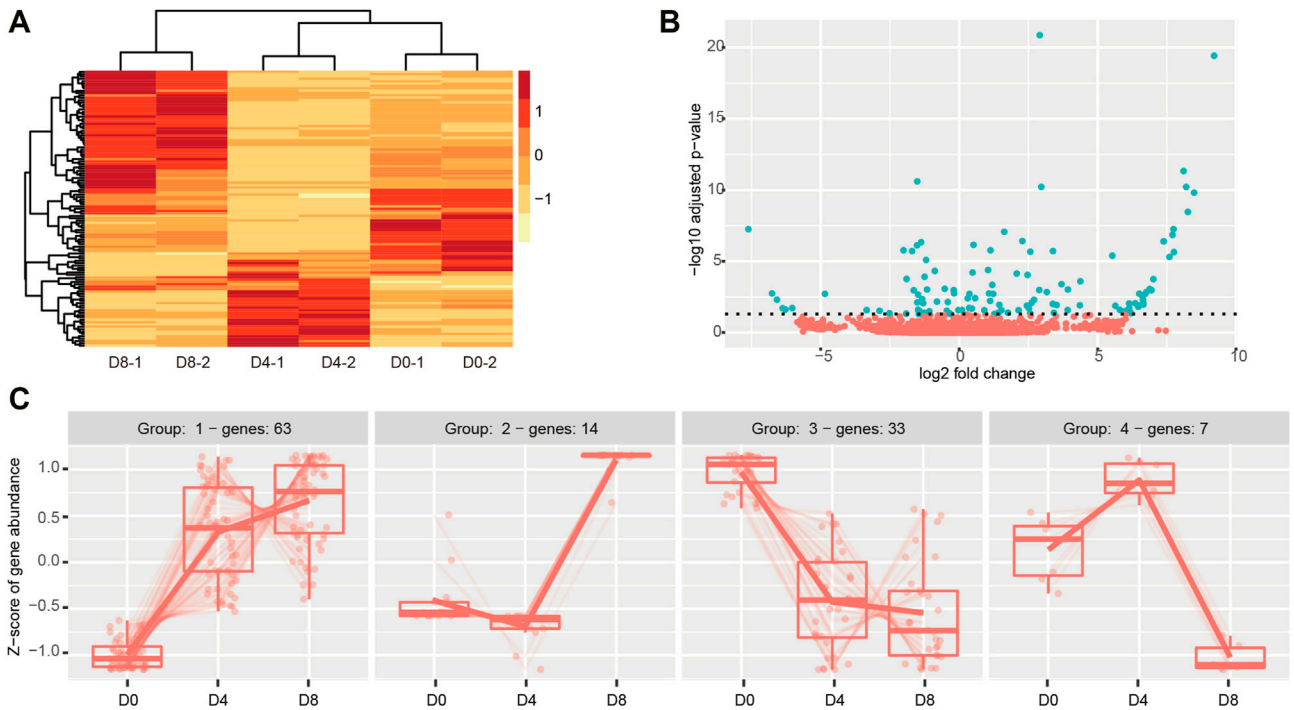

FIGURE 4 | Dynamic differentially expressed circRNAs in brown adipogenesis. **(A)** Heatmap of DECs across different stages of brown adipogenesis. **(B)** Volcano plot showing circRNA abundance across brown adipogenesis. The green color presents the significant changed DECs (padj <0.05). **(C)** Expression patterns of the DECs analyzed by degPatterns function from the DEGreport package (minc = 5).

3G,H). These results were consistent with the process of brown adipogenesis, in which pre-adipocytes exit the cell cycle, enter the differentiation process, and obtain the function of thermogenesis.

Differentially Expressed CircRNAs in brown Adipogenesis

To examine the DECs in the time-course of brown adipogenesis, we performed the Likelihood ratio test by DESeq2, and the cut-off was set as adjusted $p < 0.05$. As a result, 117 DECs were identified, with 77 upregulated and 40 downregulated (**Supplementary Table S3**). Notably, much more up-regulated circRNAs were found in the D8 group than in the D0 and D4 groups (**Figure 4A**). The volcano plot showed log 2 fold change ranged from −7.5 to 10 (**Figure 4B**). The top upregulated circRNA was circ_Acss3_1, which could not be detected on D0, then it increased to 479 circCPM on D4 and reached an extremely high level on D8 (2428 circCPM).

It has been suggested that circRNA may positively or negatively regulate host gene expression (Shao et al., 2021). To explore the relationship between DECs and their host genes, we collected mRNA expression profiles in brown adipogenesis from our previous study (GEO accession number GSE173710). Then we calculated the correlation of them by Pearson correlation test. We found 40 circRNA-mRNA pairs were significantly correlated ($p < 0.05$, **Supplementary Table S4**). Interestingly, all of them showed positive correlations, which are consistent with our previous study in white adipogenesis (Zhang et al., 2021a). These results suggested that these DECs may potentially regulate their host genes expression in brown adipogenesis.

It is reported that some of the circRNAs showed highly evolutionary conservation between humans and mice (Jeck et al., 2013). To evaluate the conserved circRNAs, we obtained the human circRNA sequences from circBase (Glažar et al., 2014). Then, alignments were conducted using the NCBI-BLAST-2.11.0+ (https://www.ncbi.nlm.nih.gov/books/NBK131777/) to identify the regions of the mouse circRNAs that corresponded to the human circRNAs. Among the 117 mouse DECs, 85 of them aligned to human circRNAs (E value $<10^{-5}$, **Supplementary Table S5**). In addition, it should be noted that the number of the conserved circRNAs may be underestimated, as circRNAs are tissue-specific and their expression profile in human BAT is not reported yet. These results indicated that a high ratio of the DECs is conserved between mice and humans.

According to the previous study, the circSAMD4A (derived from host gene SAMD4A, also known as SAMD4) regulated white pre-adipocytes differentiation (Liu et al., 2020). In the current study, we also found the circRNA was differentially expressed in brown adipogenesis, suggesting that it may also be involved in brown adipogenesis. Another reported circRNA derived from Arhgap5 (Arcinas et al., 2019) was also detected in our study, but it was not significantly differentially expressed in brown adipogenesis.

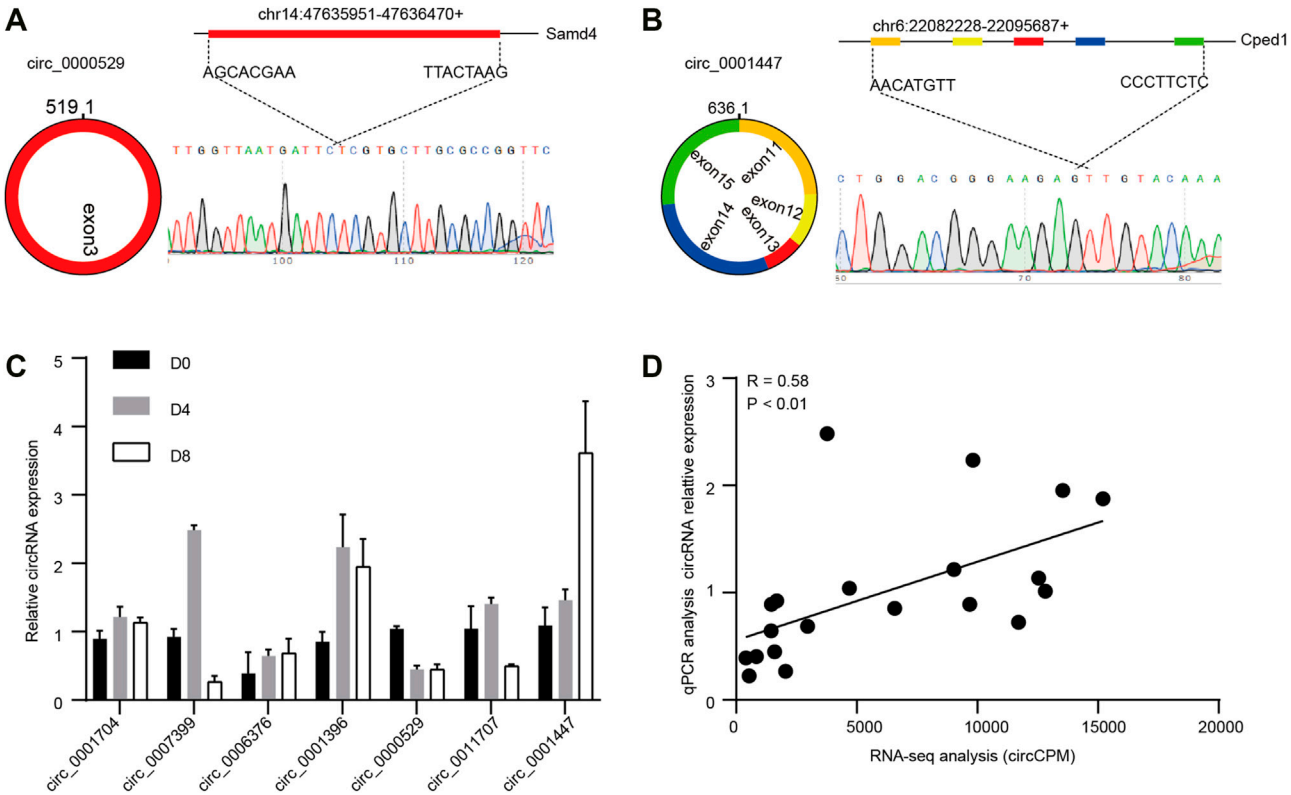

FIGURE 5 | Verification of circRNAs. Representative circRNA generation models and Sanger sequencing results of circRNAs **(A)** circ_0000529, **(B)** circ_0001447. **(C)** Relative circRNA expression levels were detected by qPCR. (D) Correlation analysis of RNA-seq results and qPCR results (n = 3).

Then, we classified the genes that exhibited similar change across adipogenesis by using DEGreport which uses a hierarchical clustering approach based on pair-wise correlations. The DECs were classified into four groups (**Figure 4C**; **Supplementary Table S3**). The genes included in each group ranged from 7 to 63. The largest one was group1, which contained 63 circRNAs with a continuously increasing trend during adipogenesis. Then, it was group 3, which contained 33 circRNAs and kept on decreasing in adipogenesis. Group 2 contained 14 circRNAs which showed a sharp increase after D4. Group 4 contained only 7 circRNAs which were found to increase on D4 and then declined on D8. Although GO analysis showed no terms were significantly enriched, we identified many DECs parental genes which are essential in adipogenesis and brown adipocyte bioenergetics, such as Pparγ, Zbtb16, Sik2, Snrk, Nfia, and Tead1.

Validation of circRNAs

To determine the authenticity of the RNA-seq results, seven of the DECs were selected randomly to detect the back-splicing sites by Sanger sequencing. The circRNA sequence and genome locus were obtained by circPrimer (version 2.0). Then, we performed RT-PCR using divergent primers (version 4.1.0, **Supplementary Table S6**) and all the circRNAs were successfully amplified. We collected the corresponding DNA products and performed Sanger sequencing, as shown in **Figures 5A,B**, the expected back-splicing sites were detected. Then, the relative circRNAs expression levels were detected by qPCR. Compared to RNA-seq results, the expression trend of most circRNAs was similar. (**Figure 5C**). Further, we found the qPCR and RNA-seq results were significantly correlated (R = 0.580, $p < 0.01$, **Figure 5D**).

Prediction of microRNA-circRNA Interaction

Recently, it has been reported that circRNAs can act as miRNA sponges and repress miRNA functions (Hansen et al., 2013). As miRNAs control a large set of biological processes in adipogenesis, circRNAs may be involved in the process through miRNAs (Zhong et al., 2019). To validate the hypothesis, we chose the top 10 abundant DECs to predict the potential circRNA-miRNA interactions with miRanda. We found all of them were predicted to contain miRNA binding sites, except circ_0001511. To reduce false positives, we only kept the miRNAs that could be detected in brown adipogenesis (GEO accession number GSE45499) (Chen et al., 2013). In the end, we predicted 49 miRNAs may interact with the DECs. Cytoscape was used to construct the miRNA-circRNA interaction networks (**Figure 6**; **Supplementary Table S7**). Notably, each circRNA contained several different miRNA binding sites and a total of 97 miRNA binding sites were identified. More interestingly, most

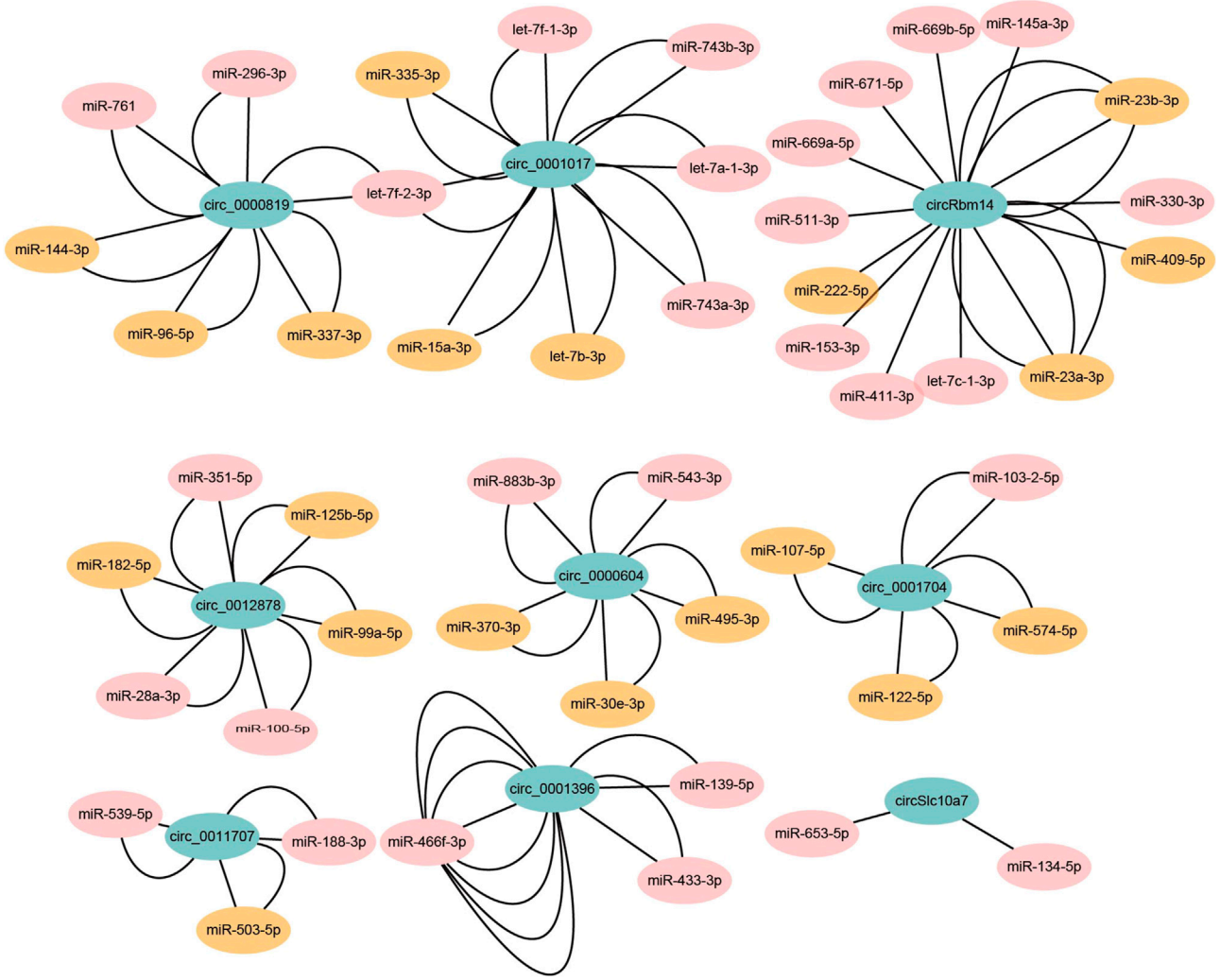

FIGURE 6 | The predicted circRNA-miRNA interaction networks. The potential miRNA binding sites of circRNAs were predicted by miRanda. The edge number represents the number of miRNA binding sites in the indicated circRNA sequences. The miRNAs with brown color indicate that these miRNAs are associated with adipogenesis or metabolic diseases according to HMDD (version 3.2) or previous study.

of the circRNAs contained multiple binding sites for the same miRNA, for example, circ_001396 was predicted to contain as many as 8 binding sites for mir-466f-3p, which greatly increased the possibility of their interaction.

The Human microRNA Disease Database (HMDD) collects experiment-supported miRNAs associated with disease (Huang et al., 2019). We further explored the potential biological functions of the identified miRNAs. According to HMDD (version 3.2) and published data, 20 of the 49 miRNAs were revealed to be associated with adipogenesis or metabolic diseases, including obesity, mitochondrial metabolism disease, type 2 diabetes, and non-alcoholic fatty liver disease (**Figure 6**; **Supplementary Table S8**). For example, let-7, miR-337, miR-503, and miR-182 were identified in the networks. They were reported to play regulatory roles in adipogenesis or thermogenesis. In addition, miR-15a and miR-30e may be involved in mitochondrial metabolism disease and obesity, respectively. In summary, the DECs may potentially interact with these miRNAs to exert their regulatory functions.

DISCUSSION

Previous studies had shown that a lot of circRNAs were expressed in adipose tissue. During Yak adipocytes differentiation, 7,203 circRNA were detected (Zhang et al., 2020). In human WAT, 6,925 circRNAs were detected, while in mouse WAT 2,380 circRNAs were detected (Arcinas et al., 2019). We previously showed 3,771 circRNA were detected during mouse white adipogenesis. However, the circRNA expression in BAT has not been reported. In the present work, the expression profiles of circRNAs were investigated during brown adipogenesis. As

many as 3,671circRNAs were identified and 36.9% of them were not annotated yet. This is the first study that reports the expression of circRNAs expression profiles in brown adipogenesis. Compare to our previous work studying circRNA in white adipogenesis, we found 1,144 of the circRNAs were specifically expressed in BAT. Further, we showed many circRNA were differentialy expressed during brown adipogenesis. These results are consistent with the idea that circRNAs are tissue-specific and developmental stage specific.

The functions of many circRNAs remain unclear at present. However, accumulated reports suggest they can regulate parental gene expression through various mechanisms (Shao et al., 2021). As both circRNAs and linear RNAs are generated from pre-RNA, they compete with each other for splicing, thus decrease the expression levels of parental genes (Ashwal-Fluss et al., 2014). On the other hand, circRNAs can increase parental gene expression. For example, circEIF3J and circPAIP2 bind to RNA Pol II and U1 snRNA, then activate parental genes transcription. Circ-Sirt1 can compete with miR-132/212 to bind Sirt1, leading to the enhancement of Sirt1 expression (Kong et al., 2019). CircRNA sisR-4 promoted parental gene expression by activating enhancer (Tay and Pek, 2017). Based on the idea that circRNAs may regulate parental genes expression, we predicted their potential functions. According to the annotation of the parental genes, downregulated circRNAs were enriched in GO terms related to cell proliferation and cell cycle, whereas the up-regulated circRNAs were enriched in cell differentiation and thermogenesis. These results were consistent with the activity of brown adipogenesis. At the early stage of brown adipogenesis, preadipocytes undergo a post confluent mitosis and exit the cell cycle. Then the pre-adipocytes are committed to adipocytes. A batch of adipose-related genes began to accumulate (Ntambi and Young-Cheul, 2000). In addition, we found many parental genes of the DECs participated in adipogenesis modulation. For example, Pparγ is indispensable for adipogenesis (Brun et al., 1996), which produced five circRNA isoforms and one of them showed a continuously increasing expression. Circ_0001335 derived from Nsd2 which inhibits H3K27me3 and increases expression of C/EBPα and Pparγ, thus promoting adipogenesis (Brun et al., 1996). Fkbp5 and Fndc3b are essential for adipogenesis (Muraoka et al., 2009; Li et al., 2018b), both can also produce circRNAs. Meanwhile, some of the DECs parental genes are involved in regulating brown adipocyte bioenergetics. Parental gene Zbtb16 is a transcription factor that is induced upon cold exposure. It can increase the expression of BAT marker genes and β-oxidation genes (Plaisier et al., 2012). Another circRNA parental gene was Sik2. Sik2/TORC2 signaling cascade regulates PGC-1α and UCP-1 gene expression in BAT (Muraoka et al., 2009). In addition, several other circRNA parental genes, such as Snrk (Li et al., 2018b), Nfia (Hiraike et al., 2017), and Tead1 (Tharp et al., 2018) are also involved in BAT-specific genes expression and bioenergetics. In summary, we find many parental genes are key adipogenesis regulators.

It is reported that circRNA can post-transcriptional modulate gene expression by binding miRNA. Several circRNAs have been reported to affect adipogenesis. In obese individuals, CircSAMD4A expression increased. It could bind with miR-138-5p and induce adipogenesis (Yang et al., 2011; Liu et al., 2020). In bone marrow mesenchymal stem cells, CDR1as increases adipogenesis by binding with miR-7-5p (Chen et al., 2020). In the current study, we identified that 9 of the top 10 abundant DCEs contained miRNA binding sites. We found circ_0001017 was predicted to contain several Let-7 binding sites. Let-7 is one of the well-studied miRNAs which inhibits pre-adipocytes clonal expansion and terminal differentiation via targeting HMGA2 (Sun et al., 2009). MiR-337-3p can inhibit TWIST1 and promote transcription of genes participating in brown fat metabolism (Vonhögen et al., 2020). In circ_0000819, two miR-337-3p binding sites were identified. In addition, we found that circ_0001017 may interact with miR-503 which was reported to regulate brown adipogenesis by inhibiting BMPR1a (Man et al., 2020). Thus, these DECs may be involved in brown adipogenesis through post-transcriptional regulation by binding with miRNAs.

CONCLUSION

Collectively, we characterized circRNA expression patterns during brown adipogenesis. We identified a large number of novel circRNAs and found alternative back splicing is an important source of circRNA diversity. We found many of the circRNAs are brown adipogenesis and differentiation stage specific. We also predicted that adipose circRNAs may regulate adipogenesis by acting as microRNA sponges. These novel circRNAs may potentially serve as new regulators of BAT development.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Xinyang Normal University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

PZ designed the experiments and wrote the manuscript, PZ, MS, CD, and XC performed the experiments, PZ, ZC, YX, CL, and HX analyzed the data. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Role of Non-Coding RNAs in Post-Transcriptional Regulation of Lung Diseases

*Dharmendra Kumar Soni and Roopa Biswas**

Department of Anatomy, Physiology and Genetics, School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, United States

Non-coding RNAs (ncRNAs), notably microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), have recently gained increasing consideration because of their versatile role as key regulators of gene expression. They adopt diverse mechanisms to regulate transcription and translation, and thereby, the function of the protein, which is associated with several major biological processes. For example, proliferation, differentiation, apoptosis, and metabolic pathways demand fine-tuning for the precise development of a specific tissue or organ. The deregulation of ncRNA expression is concomitant with multiple diseases, including lung diseases. This review highlights recent advances in the post-transcriptional regulation of miRNAs and lncRNAs in lung diseases such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, and idiopathic pulmonary fibrosis. Further, we also discuss the emerging role of ncRNAs as biomarkers as well as therapeutic targets for lung diseases. However, more investigations are required to explore miRNAs and lncRNAs interaction, and their function in the regulation of mRNA expression. Understanding these mechanisms might lead to early diagnosis and the development of novel therapeutics for lung diseases.

Keywords: microRNA, long noncoding RNA, asthma, chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis

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****Correspondence:***
Roopa Biswas
roopa.biswas@usuhs.edu

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INTRODUCTION

Non-coding RNAs (ncRNAs) are non-protein-coding RNA transcripts and were initially believed as "non-functional parts" and/or "junk RNAs" and/or "dark matter" of the human genome. But, the discoveries of the transcribed regions and protein-coding genes, i.e., approximately 85 and 2%, respectively, reveal that only a small portion of the human transcriptome encode for protein and the majority are non-protein-coding (Lander et al., 2001; Djebali et al., 2012; Hangauer et al., 2013; Jensen et al., 2013). This assessment has subverted the aforementioned conception and highlights the significance of ncRNAs, which leads to a paradigm shift and scientific revolution in RNA biology and regulation. Today, there is enormous evidence proving the function of ncRNAs as versatile key regulators of epigenetics, transcription, post-transcription, and translation (Cech and Steitz, 2014; Peschansky and Wahlestedt, 2014; Zhang et al., 2019). The pivotal role of ncRNAs in the regulation of nearly all biological activities, from tissue repair to organ development and immunity, is well-

Abbreviations: CF, Cystic Fibrosis; COPD, Chronic obstructive pulmonary disease; IPF, Idiopathic pulmonary fibrosis; LncRNA, Long non-coding RNA; miRNA, miR, MicroRNA.

established. Consequently, deregulation in ncRNA networks has been associated with a broad spectrum of pathological conditions and human diseases including lung diseases (Taft et al., 2010; Esteller, 2011; Beermann et al., 2016; Groot et al., 2018; Bao et al., 2019; Bhatti et al., 2021).

Lung diseases are a leading public health concern and cause substantial morbidity and mortality, globally (Schluger and Koppaka, 2014; Glass and Rosenthal, 2018). Undoubtedly, this necessitates in-depth knowledge of the lung disease etiology and pathophysiology, with a focus on inventing more efficacious therapeutic approaches. In recent decades, several reports have established the association of ncRNAs in various lung diseases and their pivotal functions in lung development and homeostasis (Lu et al., 2018; Pattarayan et al., 2018; Wang et al., 2019), expediting a new paradigm for lung disease diagnosis, control, and treatment. Here, we provide a comprehensive overview of the post-transcriptional regulation of ncRNAs, with special emphasis on microRNAs (miRNAs) and long ncRNAs (lncRNAs), in lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and idiopathic pulmonary fibrosis (IPF). Alterations of miRNA and lncRNA expression level in the disease state compared to the normal state could be exploited to identify biomarkers and targets for drug development. Understanding how post-transcriptional mechanisms regulate lung diseases will lead to the development of candidate therapeutic targets for the early diagnosis and treatment of lung diseases.

MicroRNAs and Long ncRNAs

The ncRNA repertoire encompasses myriads of RNA species and according to their regulatory roles are broadly classified into two categories, housekeeping and regulatory ncRNAs. The regulatory ncRNAs further consist of diverse groups of ncRNAs with the two-utmost noteworthy, microRNAs (miRNAs, transcripts between 19 and 25 nucleotides) and long ncRNAs (lncRNAs, transcripts >200 nucleotides). MiRNAs generally negatively regulate gene expression in a sequence-specific way at the post-transcriptional stage either through the target messenger RNA (mRNA) cleavage and degradation, and/or by inhibition of translation. On the other hand, lncRNAs are divided into different types and regulate either negatively or positively each stage of gene expression *via* the interactions with DNA, RNA, or protein and through various mechanisms (Fatica and Bozzoni, 2014; Chew et al., 2018). The biogenesis, characteristics, types, and mechanism of action of miRNAs and lncRNAs have been described in multiple articles (Denli et al., 2004; Han et al., 2004; Du and Zamore, 2005; Kim, 2005; Bartel, 2009; Kugel and Goodrich, 2012; Fatica and Bozzoni, 2014; Quinn and Chang, 2016; Kopp and Mendell, 2018; Zhang et al., 2019; Statello et al., 2021). Collectively, both of these ncRNA species have an imperative role in development and homeostasis as well as in diseases.

Non-Coding RNAs in Lung Disease

Emerging evidence suggests that in the respiratory system, ncRNAs are accountable for normal lung development and maintenance of lung homeostasis. Thus, deregulation of miRNAs and lncRNAs causes pathophysiological alteration of the respiratory system leading to the initiation, progression, and development of various types of lung diseases. In the following sections, we have described the emerging roles and mechanistic functions of miRNA and lncRNA in various lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and idiopathic pulmonary fibrosis (IPF).

Asthma

Asthma is a multifaceted heterogeneous disease, primarily characterized by chronic inflammation, hyperresponsiveness, and transient airflow obstruction of the airways. The global increase in the incidence of asthma has been reported in all age groups, and approximately 300 million people are affected by asthma (Cevhertas et al., 2020; Stern et al., 2020). Therefore, the management of asthma as well as developing novel therapies is vital.

Several ncRNAs regulate airway inflammation and are associated with the pathophysiology of asthma. For example, upregulation of miR-221 and miR-485-5p are reported in the blood sample of asthmatic children (Liu et al., 2012). In a murine model of asthma, miR-221 and miR-485-5p regulate interleukin-5 (IL-5) by targeting sprouty-related protein with an EVH1 domain-2 (*Spred-2*), which negatively regulates the Ras/ERK pathway involved in a variety of cellular processes, including airway inflammation and hypersensitivity (Liu et al., 2012). Elevated level of miR-1248 has been reported in the serum of asthmatic patients and it induces increased expression of IL-5 and upregulation of Th2 cytokine through the direct interaction with *IL-5* (Panganiban et al., 2012). The imbalance of Th1/Th2 cytokines has been found as a predominant factor associated with asthma, where increased expression of Th2 cytokines, mainly IL-4, IL-5, IL-9, and IL-13 promote the serum immunoglobulin E (IgE) and eosinophilia that stimulate a variety of cellular processes, including mucus hypersecretion, airway inflammation, and hypersensitivity (Ngoc et al., 2005; Zhu et al., 2016). On the other hand, Th1 cytokines, such as IFN-γ and IL-12, play an antagonist role in IgE synthesis as well as other Th2 responses. Thus, restoration of Th1/Th2 balance *via* inhibition of Th2 cytokines and activation of Th1 cytokines is one of the critical aspects in the treatment of asthma. One of the most studied miRNAs in asthma is miR-21. Several studies with asthmatic mice model and in asthmatic children indicate the upregulation of miR-21 negatively regulates IL-12p35, signal transducer and activator of transcription 4 (STAT4), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and histone deacetylase 2 (HDAC2), and positively regulates phosphoinositide 3-kinase (PI3K), which may promote increased expression of Th2 cytokines and inhibit Th1 cytokines expression (Lu et al., 2009; Wu S.-Q. et al., 2014; Wu XB. et al., 2014; Liu et al., 2015; Perry et al., 2015; Sawant et al., 2015; Elbehidy et al., 2016; Kim et al., 2017; Hammad Mahmoud Hammad et al., 2018). Thereby, the role of miR-21 in controlling the Th1/Th2 ratio, airway hypersensitivity, and cell proliferation and migration is established in different asthmatic models. In the bronchial epithelium of neutrophilic asthmatic, miR-629-3p is

upregulated and induces neutrophil chemoattractant IL-8, which suggests its role in the airway neutrophilia and disease pathogenesis through the regulation of proinflammatory and wound-repair pathways (Maes et al., 2016). In lung tissues from allergic asthma patients and ovalbumin (OVA)-induced mice, upregulation of miR-943-3p and downregulation of its target secreted frizzled-related protein 4 (*SFRP4*) enhances airway inflammation progression and remodeling *via* the activation of Wingless/Integrase I (Wnt) signaling pathway (Shen et al., 2019). The importance of WNT signaling has been shown in the development of the organism, context-dependent transcription of targets genes, maintaining equilibrium among proliferation and differentiation of airway smooth muscle (ASM) progenitor cells, and asthma pathogenesis (Sharma et al., 2010; Choy et al., 2011; Wang et al., 2013; Carraro et al., 2014; Barreto-Luis et al., 2017).

The decreased expression of miR-181b-5p is reported in plasma and epithelium of eosinophilic asthmatic and it has been demonstrated that miR-181b-5p negatively regulates proinflammatory cytokines, IL-1β and C-C motif chemokine ligand (CCL)-11 (eotaxin-1) expression by binding to its target secreted phosphoprotein-1 (SPP-1), which is associated with the recruitment of eosinophils into airways (Huo et al., 2016). In the lungs of asthmatic mice, miR-20b promotes the elevation of CCL-2 concentration and accumulation of myeloid-derived suppressor cells, which suppresses the Th2 response and airway inflammation in a transforming growth factor-beta (TGF-β)-dependent manner (Ma et al., 2017a; Ma et al., 2017b). MiR-221-3p is downregulated in the airway epithelium and sputum of asthmatics and its downregulation suppresses inflammatory cytokine, chemokine CCL-24 (eotaxin-2) and CCL-26 (eotaxin-3) expression, which are involved in the migration of eosinophils into the airways, by inducing the expression of its target chemokine C-X-C motif ligand (CXCL)-17, an anti-inflammatory chemokine (Zhang K. et al., 2018). This suggests the protective role of miR-221-3p against airway eosinophilic inflammation. The reduced expression of miR-485 is observed in the mouse model of chronic asthma. Consistently, overexpression of miR-485 leads to reduced proliferation of airway smooth muscle cells (ASMCs) and induces apoptosis by targeting Smad ubiquitin regulatory factor 2 (*Smurf2*) (Wang et al., 2018). Smurf2 modulates the TGF-β/decapentaplegic homolog (Smads) signaling pathway, which is shown to be associated with the remodeling of the airway in asthma (Qu et al., 2012). The association of miR-142-3p with WNT signaling and maintaining equilibrium among proliferation and migration of ASMCs has been observed in bronchial biopsies of asthmatics (Bartel et al., 2018). In bronchial epithelial cells from asthmatic patients, decreased level of miR-744 induces cell proliferation by targeting *TGF-ß1* and regulating the Smad3 pathway (Huang et al., 2019). The downregulation of miR-30a and upregulation of its target autophagy-related 5 (*ATG5*) is reported in lung tissues from asthmatic children and in mice treated with OVA, and promotes fibrogenesis, autophagic flux, and airway remodeling (Li et al., 2020).

In addition to miRNAs, lncRNAs are also associated with the regulation of airway inflammation and asthma. For example, in the rat model of asthma, upregulation of brain cytoplasmic RNA 1 (BCYRN1) lncRNA targets canonical transient receptor potential 1 (*TRPC1*), which is implicated in the pro-proliferative and pro-migratory role of BCYRN1 and induces proliferation and migration of ASMCs (Zhang XY. et al., 2016). TRPC1 has been reported as an important molecular counterpart of Ca2+ channels in ASMCs and as a critical component contraction and proliferation of vascular smooth muscle cells (Ong et al., 2003; Dietrich et al., 2006). A similar study, using rat model asthma, demonstrated that miR-150 downregulates BCYRN1 and reduces proliferation and migration of ASMCs (Zhang X.-y. et al., 2017). PVT1 lncRNA is upregulated in severe asthmatic patients who are insensitive to corticosteroids, and induces IL-6 expression and proliferation of ASMCs (Austin et al., 2017). The upregulation of TCF7 lncRNA and TIMMDC1 and the role of TCF7 in the regulation of TIMMDC1 expression and proliferation and migration of ASMCs have been established in asthmatics (Fan et al., 2019). Similarly, TUG1 and MALAT1 lncRNAs induces proliferation and migration of ASMCs *via* targeting miR-590-5p (TUG1/miR-590-5p/FGF1 axis) and miR-150 (miR-150-eIF4E/Akt signaling), respectively (Lin J. et al., 2019; Lin L. et al., 2019). The upregulation of the lncRNA antisense non-coding RNA in the INK4 locus (ANRIL)/miR-125a axis is found especially in the plasma of bronchial asthmatics at exacerbation (BA-E) compared to bronchial asthmatics at remission (BA-R) and control groups (Ye et al., 2020). Furthermore, there is a positive correlation between this regulatory axis and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-17) in bronchial asthmatics. A brief summary of ncRNAs, miRNAs and lncRNAs, associated with asthma are shown in **Table 1**. From these studies, it is clear that through the various mechanisms including post-transcriptional regulation miRNAs, lncRNAs and associated molecules play a pivotal role in the genesis and development of asthma. Further analyses of the function, and mechanism of action of ncRNAs will lead to therapeutic targets for asthma.

Chronic Obstructive Pulmonary Disease (COPD)

COPD is a heterogenous persistent lung disease, caused by progressive and irreversible airflow obstruction. COPD has a high rate of morbidity and mortality, accounting for 3.2 million deaths globally, and is considered the third leading cause of death (WHO, 2021). Among the environmental factors, the recurrent exposure of noxious particles and gas irritants such as cigarette smoke to the lungs are among the main causes of the development of COPD. However, genetic and epigenetic factors also play an important role in the pathogenesis of COPD, as this disease is reported in only 20% of smokers.

The involvement of ncRNAs in the pathogenesis and development of COPD is established by several studies. For example, upregulation of miR-15b and downregulation of its

TABLE 1 | List of ncRNAs and their targets and functions in asthma.

ncRNA	Source	Expression	Target/regulator	Function	Reference
miR-21	doxycycline-induced lung-specific IL-13 transgenic mice	up	IL-12p35	modulates IL-12 and Th cell polarization	Lu et al. (2009)
miR-1248	serum from asthmatics	up	IL-5	positive regulator to increase IL-5	Panganiban et al. (2012)
miR-21 miR-126	bronchial epidermal cells from asthmatic treated with or without inhaled corticosteroids (ICS)	up	IL-13	positively correlates with IL-13	Wu et al. (2014b)
miR-21	lung tissue from allergic asthmatic mice model	up	IL-12, STAT4	develops allergic asthma	Wu et al. (2014a)
miR-21	HASM cells	up	PTEN	triggers cell proliferation and migration	Liu et al. (2015)
miR-155	HASM cells from asthmatic	up	COX-2	positively correlates with COX-2	Comer et al. (2015)
miR-21	serum from asthmatic children without ICS, steroid sensitive (SS) asthma children and steroid resistant (SR) asthma children	up	IL-12p35	negatively correlates with serum IL-12p35 and FEV1, while positively correlates with both sputum and blood eosinophils	Elbehidy et al. (2016)
miR-146a	plasma from asthmatic children	up	EGFR	inhibits proliferation and promotes apoptosis of BSMCs	Zhang et al. (2016b)
miR-181b-5p	epithelial cells and plasma from asthmatic	down	SPP1	regulates IL-13-induced IL-1β and CCL11	Huo et al. (2016)
miR-21	lung cells from severe, steroid-insensitive allergic asthmatic mice model	up	phosphatase and tensin homolog	Ant-21 treatment reduces PI3K activity and restores HDAC2 as well as suppresses airway hyperresponsiveness and restores steroid sensitivity	Kim et al. (2017)
miR-155	lung tissue from allergic asthmatic mice model	up	IL-33	regulates ILC2s and IL-33	Johansson et al. (2017)
miR-371 miR-138 miR-544 miR-145 miR-214	$CD4^+$ T cells from asthmatic	up	Runx3	regulates Runx3 in a combinatorial manner and modulates Th1/Th2 balance	Qiu et al. (2017)
miR-98	peripheral B cells from allergic asthmatic	up	TSP1, IL-13	suppresses TSP1	Chen et al. (2017)
miR-21 miR-146a	plasma from asthmatic children with ICS	up	IL-13	miR-21 positively correlates with IL-13 and eosinophil percentage, while miR-146a only correlates to eosinophil percentage	Hammad Mahmoud Hammad et al. (2018)
miR-221-3p	bronchial brushings, induced sputum, and plasma from steroid-naive asthmatic	down	CXCL17	regulates CCL24, CCL26, and airway eosinophilic inflammation	Zhang et al. (2018b)
miR-485	ASMCs from mouse model of chronic asthmatic	down	Smurf2	regulates cell proliferation and apoptosis	Wang et al. (2018)
miR-192	plasma and $CD4^+$ T cells from acute asthmatic children	down	CXCR5	blocks T follicular helper cells activation pathway	Zhang et al. (2018a)
miR-943-3p	lung tissues from allergic asthmatics and OVA-induced mice	up	SFRP4	enhances airway inflammation progression and remodeling	Shen et al. (2019)
miR-744	bronchial epithelial cells from asthmatic	down	TGF-ß1	induces cell proliferation through mediating Smad3 pathway	Huang et al. (2019)
miR-30a	lung tissues from asthmatic children and OVA-induced mice	down	ATG5	induces fibrogenesis, autophagic flux and airway remodeling	Li et al. (2020)
lncR-BCYRN1	ASMCs from rat asthmatic model	up	TRPC1	induces cell proliferation and migration	Zhang et al. (2016a)
lncR-BCYRN1	ASMCs from rat asthmatic model	up	miR-150	regulates cell proliferation and migration	Zhang et al. (2017c)
lncR-PVT1	ASMCs from severe asthmatics	up	IL-6	regulates IL-6 and cell proliferation	Austin et al. (2017)
lncR-TCF7	ASMCs from asthmatics	up	TIMMDC1	regulates cell growth and migration	Fan et al. (2019)
lncR-MEG3	peripheral blood CD4 + T cells from asthmatics	up	miR-17	regulates RORγt and affects Treg/Th17 balance	Qiu et al. (2019)
lncR-TUG1	ASMCs from rat asthmatic model	up	miR-590-5p	regulates cell proliferation and migration	Lin et al. (2019a)
lncR-MALAT1	ASMCs from asthmatics	up	miR-150	derepresses eIF4E, activates Akt signaling, and regulates cell proliferation and migration	Lin et al. (2019b)
lncR-ANRIL	plasma from bronchial asthmatics	up	miR-125a	positive correlations with pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-17)	Ye et al. (2020)

target *SMAD7*, which is an inhibitory SMAD in TGF-β signaling, is reported in lung tissues of COPD patients compared with smokers without obstruction, and thereby regulates TGF-β signaling pathway and pathogenesis of COPD (Ezzie et al., 2012). TGF-β is a profibrogenic cytokine and the impairment in TGF-β signaling in COPD patients induces fibrotic airway remodeling that could promote a decline in lung function (Morty et al., 2009). MiR-135b is upregulated in lung tissues of mice exposed to cigarette smoke, and regulates the IL-1 pathway by targeting IL-1R1 (Halappanavar et al., 2013). Several studies have

shown the involvement of IL-1 signaling in chronic inflammation, remodeling of airways, and pathogenesis of COPD (Osei et al., 2020). The upregulation of miR-223 in lung tissues of COPD patients and in mice exposed to cigarette smoke is inversely correlated to the expression of its target *HDAC2* and leads to the upregulation of CX3CL1 (Leuenberger et al., 2016). Declined HDAC activity permits the acetylated chromatin to be unbound to histones and this step allows chromatin access for transcription factors and transcription of various inflammatory cytokines and chemokines (Barnes et al., 2005). Elevated expression of miR-195 is observed in lung tissues of COPD patients and mice exposed to cigarette smoke, which causes downregulation of its target PH domain and leucine-rich repeat protein phosphatase 2 (*PHLPP2*) and increases Akt phosphorylation, leading to increased expression of IL-6 and TNF-α (Gu et al., 2018). Earlier studies suggest the role of PHLPP2 in direct dephosphorylation and inactivation of Akt, which has multifunctional activities and is a potential regulator of various cellular processes involved in the pathogenesis of COPD (Bozinovski et al., 2006; Gao et al., 2009; Nowak et al., 2015). The upregulation of miR-664a-3p and downregulation of its target four and a half LIM domains 1 (*FHL1*), which acts as a transcription factor and implicated in various cellular mechanisms, in lung tissue and peripheral blood mononuclear cells (PBMCs) of COPD patients positively correlated with forced expiratory volume in one second (FEV1)/forced vital capacity (FVC)% and has a role in cigarette smoke-induced COPD (Zhong et al., 2019). A recent report demonstrates that the upregulation of miR-130 in BEAS-2B cells treated with cigarette smoke extract (CSE) and in mice exposed to CSE, negatively regulates Wnt/β-catenin signaling by targeting Wnt1 and modulating β-Catenin, and lymphoid enhancer-binding factor (LEF) (Wu et al., 2020). Earlier, the role of β-Catenin is shown in cell proliferation and injury repair (Zemans et al., 2011; Tanjore et al., 2013). Further, it has been demonstrated that activation of Wnt/β-catenin signaling may potentially attenuate COPD pathogenesis (Kneidinger et al., 2011; Uhl et al., 2015).

Reduced expression of miR-34c in lung tissues of COPD patients modulates the expression of SERPINE1, which is a protease and fibrinolysis inhibitor (Savarimuthu Francis et al., 2014). The authors suggest that SERPINE1 has other functions apart from antiproteases in the lung that may play important role in emphysema progression. Nuclear factor-kappaB (NF-κB) is a crucial transcription factor and persistent stimulation of the NF-κB signaling pathway provokes the exaggerated synthesis of pro-inflammatory mediators such as IL-8 and TNF-α, which leads to airway impairment in COPD patients (Edwards et al., 2009). The downregulation of miR-149-3p in the blood of smokers with COPD activates TLR-4/NF-κB signaling and upregulates IL-1β and TNF-α by targeting *TLR-4* (Shen et al., 2017). Moreover, miR-145-5p expression is reduced in lung tissues of smokers without or with COPD and regulates p53-mediated apoptotic signaling, NF-κB signaling, TNF-α, IL-6, and IL-8 by targeting kruppel like factor 5 (*KLF5*) (Dang et al., 2019). Consistently, overexpression of miR-145-5p attenuates CSE-stimulated apoptosis and inflammation in human bronchial epithelial cells (HBECs) (Dang et al., 2019). The role of p53-mediated signaling pathways has been shown in CSE-induced cell apoptosis (Lee and Choi, 2018). KLF5 belongs to a family of zinc-finger (ZF) containing transcription factors and is implicated in the regulation of a wide range of cellular processes such as cell proliferation, apoptosis, inflammation, migration, and differentiation (Dong and Chen, 2009). The downregulation of miR-29b is found in lung tissues and plasma from COPD patients, which regulates CSE-induced IL-8 expression by targeting bromodomain protein 4 (*BRD4*) (Tang et al., 2019). The role of BRD4 has been shown in direct or indirect regulation of gene transcription (Devaiah et al., 2016a; Devaiah et al., 2016b). Further, studies also demonstrated that inhibition of BRD4 significantly decreases the level of proinflammatory cytokines, which suggests its important role in the inflammatory process (Nicodeme et al., 2010; Tian et al., 2017). Thus, signify the vital function of the miR-29b-BRD4 axis in airway inflammation and pathogenesis of COPD.

In addition to miRNAs, the association of lncRNA is also shown in the pathogenesis and development of COPD. For example, in the lung tissues of COPD patients, TUG1 is upregulated and its silencing reduces α-SMA and fibronectin expression and stimulates the proliferation of TGF-β induced-BEAS-2B and HFL1 cells (Tang W. et al., 2016). The upregulation of lncRNA-ENST00000502883.1 is found in B cells and $CD4^+$ T cells from COPD patients and it is shown that it affects PBMC recruitment *via* regulation of CXCL16 (Qu et al., 2018). CXCL16 functions as a chemoattractant for Th1 cells and it is considered as a systemic inflammatory marker for COPD (Shashkin et al., 2003; Donnelly and Barnes, 2006; Eagan et al., 2010). The nuclear enriched abundant transcript 1 (NEAT1) is upregulated in plasma from COPD patients and negatively correlates with miR-193a and positively correlates with GOLD stage and the expressions of TNF-α, IL-1β, IL-6, and IL-17 (Ming et al., 2019). NEAT1-induced inflammatory cascades and oxidative stress lead to severe lung injury, which establishes NEAT1 is positively correlated with COPD severity and inflammation and its potential in the prediction of disease susceptibility and acute exacerbation risk. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is upregulated in lung tissues of COPD patients (Hu et al., 2020). In the same study, *in vitro* experiments with TGF-β-treated human lung fibroblasts showed that MALAT1 downregulation stimulates cellular viability and inhibits mesenchymal protein expression by regulating the mTOR pathway, which is involved in lung cell senescence in COPD. The downregulation of HOXA cluster antisense RNA 2 (HOXA-AS2) is found in lung tissues from COPD patients and further studies in CSE-treated human pulmonary microvascular endothelial cells (HPMECs) demonstrated that the downregulation of HOXA-AS2 suppresses cell proliferation *via* Notch1 signaling (Zhou et al., 2020). This implies that upregulation Notch1, which is implicated in various cellular processes such as cell proliferation, differentiation, and apoptosis, stimulates HOXA-AS2-dependent cell proliferation and mitigates the cell viability injury. The lung cancer-associated transcript 1 (LUCAT1) is elevated in the serum of COPD patients (Zhao et al., 2021).

TABLE 2 | List of ncRNAs and their targets and functions in COPD.

ncRNA	Source	Expression	Target/ regulator	Function	Reference
miR-15b	lung tissues from smokers with and without COPD	up	SMAD7	regulates TGF-β signaling	Ezzie et al. (2012)
miR-199a-5p	lung tissues from COPD patients	up	HIF-1α	regulates HIF-1α	Mizuno et al. (2012)
miR-135b	lungs tissues from mice exposed to cigarette smoke	up	IL-1R1	regulates IL-1 pathway	Halappanavar et al. (2013)
miR-34c	lung tissues from COPD patients	down	SERPINE1	regulates TGF- β signaling	Savarimuthu Francis et al. (2014)
miR-223	lung tissues from COPD patients and mice exposed to cigarette smoke	up	HDAC2	upregulates CX3CL1	Leuenberger et al. (2016)
miR-218	serum from smokers without or with COPD	down	TNFR1	upregulates MUC5AC, IL-6, IL-8, TNFR1, and p-p65	Xu et al. (2017)
miR-181c	lung tissues from COPD patients and mice exposed to cigarette smoke	down	CCN1	increases inflammatory response, neutrophil infiltration, ROS generation, and inflammatory cytokines induced by CS	Du et al. (2017)
miR-149-3p	blood from smokers without or with COPD	down	TLR-4	activates TLR-4/NF-κB signaling and upregulates IL-1β and TNF-α	Shen et al. (2017)
miR-195	lung tissues from COPD patients and mice exposed to cigarette smoke	up	PHLPP2	increases Akt phosphorylation, IL-6 and TNF-α in BEAS-2B cells	Gu et al. (2018)
miR-3202	blood from smokers without or with COPD	down	FAIM2	upregulates INF-γ, TNF-α and FAIM2 and downregulates Fas and FasL in T lymphocytes	Shen et al. (2018)
miR-664a-3p	lung tissue and PBMCs from COPD patients	up	FHL1	positively correlates with FEV1/FVC%	Zhong et al. (2019)
miR-145-5p	lung tissues from smokers without or with COPD	down	KLF5	conferred protection against CSE-induced airway epithelial cell apoptosis and inflammation	Dang et al. (2019)
miR-29b	lung tissues and plasma from COPD patients	down	BRD4	regulates CSE-induced IL-8	Tang et al. (2019)
miR-130	cigarette smoke extract (CSE)-treated BEAS-2B cells and CS-exposed mice	up	WNT1	negatively regulates Wnt/β-catenin signaling by modulating Wnt1, β-Catenin, and LEF1	Wu et al. (2020)
lncR-TUG1	lung tissues from COPD patients	up	α-SMA and fibronectins	Knockdown of lncRNA TUG1 promotes BEAS-2B and HFL1 cell proliferation after TGF-β treatment	Tang et al. (2016b)
SAL-RNA1 SAL-RNA2	lung tissues from COPD patients	down up	SIRT1/FoxO3a, p53, p21	regulate AECII senescence	Gu et al. (2017)
lncR-ENST00000502883.1	B cells and $CD4^+$ T cells from COPD patients	up	CXCL16	effects PBMC recruitment	Qu et al. (2018)
lncR-NEAT1	plasma from COPD patients	up	miR-193a	positively correlates with GOLD stage and TNF-α, IL-1β, IL-6 and IL-17	Ming et al. (2019)
lncR-ENST00000447867 NR-026690	$CD4^+$ T cells from acute exacerbations of COPD patients	up	RAPGEF3	affect RAPGEF3 as miRNA sponges	Qi et al. (2019)
lncR-ANRIL	Plasma from acute exacerbations of COPD patients	down	TNF-α, IL-1β, IL-17A, LTB-4	associates with lower acute exacerbation risk, decreased inflammatory cytokines, and mild GOLD stage	Ge et al. (2019)
lncR-MALAT1	lung tissues from COPD patients	up	mTORC1	downregulation of MALAT1 induces cellular viability following TGF-β stimulation in HFL1 cells	Hu et al. (2020)
lncR-HOXA-AS2	lung tissues from COPD patients	down	Notch1	regulating HPMECs proliferation	Zhou et al. (2020)
lncR-LUCAT1	serums from COPD patients	up	miR-181a-5p	LUCAT1 silencing alleviates CSE's effects on 16HBE cell proliferation and apoptosis	Zhao et al. (2021)

Further studies in CSE-treated 16HBE cells show that LUCAT1 downregulates its target, miR-181a-5p, upregulates inflammatory cytokines (IL-1β, IL-6, and TNF-α), and regulates cell proliferation and apoptosis *via* the Wnt/β-catenin pathway (Zhao et al., 2021). The role of activated Wnt/β-catenin pathway in induction of inflammatory cytokines (IL-1β, IL-6, and TNF-α) and cell proliferation and apoptosis are well characterized (Masckauchan et al., 2005; Aumiller et al., 2013; Jang et al., 2017). This suggests LUCAT1 plays an important role in the regulation of inflammatory cytokines and the Wnt/β-catenin pathway, thus have a crucial function in the pathogenesis of COPD. **Table 2** summarizes the list of ncRNAs, miRNAs and lncRNAs with their targets and functions in COPD. Collectively, these studies suggest that miRNAs, lncRNAs, and their interaction and regulation have a significant role in the pathogenesis and development of COPD.

TABLE 3 | List of ncRNAs that directly or indirectly target and regulate CFTR in cystic fibrosis.

ncRNA	Source	Target/ regulator	Function	Reference
miR-101, miR-145, miR-384, miR-494, miR-600	A549, Beas-2B, bronchial brushing, Caco-2, Calu-3, CFBE41o-, differentiated primary cell cultures, 16HBE14o-, HBEpiC, HEK293, PANC-1	CFTR	directly target and regulate CFTR	Gillen et al. (2011); Megiorni et al. (2011); Hassan et al. (2012); Oglesby et al. (2013); Ramachandran et al. (2013); Viart et al. (2015); Fabbri et al. (2017); Lutful Kabir et al. (2018); Dutta et al. (2019); Finotti et al. (2019); Sultan et al. (2020); Fabbri et al. (2021)
miRNA-138	differentiated primary cell cultures, Calu-3, HEK293, HeLa	SIN3A	regulates CFTR	Ramachandran et al. (2012)
miR-9	CFBE41o, 16HBE14o-	ANO1/ TMEM16A	modulates mucus hydration and chloride efflux activity	Sonneville et al. (2017)
lncR-BGas	CFPAC, 1HAEo-, 16HBE14o-, CFBE41o-	CFTR	directly targets and regulates CFTR	Saayman et al. (2016)

Understanding these mechanisms will lead to novel therapeutic interventions and approaches for better management of COPD.

Cystic Fibrosis (CF)

CF is the most common genetic autosomal recessive lethal disease. It is caused by loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene leading to aberrant translation, protein mis-folding, and/or trafficking (Riordan et al., 1989; Cutting, 2015). Impairment of CFTR, a crucial chloride ion channel, results in ionic disequilibria and concurrently, airway dehydration and mucus accumulation. This further leads to chronic airway infections and inflammation and eventually, fatal deterioration in lung function.

Growing evidence supports the role of miRNAs in the direct or indirect regulation of CFTR and/or CFTR-related genes/proteins (**Table 3**). For example, several miRNAs including miR-101, miR-145, miR-384, miR-494, miR-600 are directly concomitant with CFTR dysregulation in airway epithelial cells like A549, Beas-2B, bronchial brushings, Caco-2, Calu-3, CFBE41o-, differentiated primary cell cultures, 16HBE14o-, HBEpiC, HEK293, PANC-1 (Gillen et al., 2011; Megiorni et al., 2011; Hassan et al., 2012; Oglesby et al., 2013; Ramachandran et al., 2013; Viart et al., 2015; Fabbri et al., 2017; Lutful Kabir et al., 2018). These studies suggest that the regulation of CFTR expression by miRNAs in different cell types is diverse, tissue-specific, and time-dependent. Antisense targeting of miR-145-5p through peptide nucleic acid (PNA) upregulates CFTR expression (Fabbri et al., 2017; Finotti et al., 2019). Consistently, suppression of miR-145 has been shown to restore F508del CFTR expression (Lutful Kabir et al., 2018; Dutta et al., 2019). Further, a recent study shows PNA masking of the miR-145-5p binding site of CFTR mRNA upregulates CFTR at both mRNA and protein levels (Sultan et al., 2020). Similar PNA targeting of miR-101-3p also upregulates CFTR (Fabbri et al., 2021). The indirect association is also determined between miRNA and CFTR. For example, miRNA-138 interacts with its target switch-independent 3 homolog A (*SIN3A*), a transcriptional regulatory protein, and downregulates CFTR (Ramachandran et al., 2012). Further, the same study showed that controlling miR-138/SIN3A expression restores F508del-CFTR expression. The upregulation of miR-9 in CF cells downregulates its target anoctamin 1 (*ANO1*) alias calcium-activated chloride channel (transmembrane protein 16A, TMEM16A) and preventing the inhibition of ANO1 *in vitro* and *in vivo* CF models *via* the miR-9 target site blocker (TSB) elevates chloride efflux, mucociliary clearance, and migration rate of cells (Benedetto et al., 2017; Sonneville et al., 2017).

MiRNAs that regulate CF lung disease *via* the regulation of inflammation, airway obstruction, or infection are listed in **Table 4**. For example, the elevated expression of miR-155 in the CF lung epithelium leads to downregulation of SH-2 containing inositol 5′ polyphosphatase 1 (SHIP1), an inositol 5-phosphatase, and thereby induces IL-8 expression *via* regulation of phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) signaling (Bhattacharyya et al., 2011). Further, the RNA-binding protein tristetraprolin (TTP), a zinc finger protein also known as ZFP36, suppresses miR-155 expression in CF lung epithelial cells *via* upregulation of miR-1, while KH-type splicing regulatory protein (KSRP), the KH domain-containing splicing factor, upregulates miR-155 *via* promoting enhanced biogenesis (Bhattacharyya et al., 2013). Moreover, miR-155 targets the regulatory associated protein of mTOR complex 1 (*RPTOR*) and activates TGF-β signaling, and upregulates connective tissue growth factor (CTGF) in CF lung epithelial cells, thereby promoting fibrosis. (Tsuchiya et al., 2016). RPTOR is implicated in the modulation of the mammalian target of rapamycin complex 1 (mTORC1) activity that controls cell growth and survival whereas CTGF is a fibrotic factor that stimulates amplified fibrogenesis and airway remodeling. Furthermore, miR-16 rescues the F508del-CFTR trafficking defects probably through downregulation of heat shock protein 90 (HSP90) (Kumar et al., 2015).

In endobronchial brushings from CF patients, reduced expression of miR-126 upregulates the Target of Myb1 (TOM1) and regulates NF-κB-mediated IL-8 secretion (Oglesby et al., 2010). TOM1 belongs to a family of proteins containing an N-terminal VHS (Vps27p/Hrs/STAM) domain, and it has been demonstrated that TOM1 negatively regulates IL-1β- and TNF-α–induced signaling pathways while its upregulation leads to suppression of NF-κB (Yamakami and Yokosawa, 2004). Moreover, TOM1 through the interaction with Toll-interacting protein (Tollip) regulates intracellular

TABLE 4 | List of ncRNAs and their targets (other than CFTR) and functions in cystic fibrosis.

ncRNA	Source	Expression	Target/ regulator	Function	Reference
miR-126	Bronchial brushing, 16HBE14o-, CFBE41o-, HEK293	down	TOM1	regulates NF-κB regulated IL-8 secretion	Oglesby et al. (2010)
miR-155	IB3-1, IB3-1/S9	up	SHIP1	upregulates IL-8 *via* regulation of PI3K/Akt signaling	Bhattacharyya et al. (2011)
miR-146a	16HBE14o- cells	down	MUC5AC	negative feedback role in the control of MUC5AC production	Zhong et al. (2011)
miR-145	Nasal epithelium cells, HEK293	up	SMAD3	downregulates SMAD3	Megiorni et al. (2013)
miR-155	IB3-1, IB3-1/S9	up	TTP, KSRP	TTP and KSRP regulate miR-155 biogenesis	Bhattacharyya et al. (2013)
miR-31	Differentiated primary cell cultures	down	IRF1	regulates a deteriorator of antimicrobial proteins, cathepsin S	Weldon et al. (2014)
miR-93	IB3-1, CuFi-1, NuLi-1	down	IL-8	regulates IL-8 *via* direct interaction	Fabbri et al. (2014)
miR-17	Bronchial brushing, 16HBE14o-, CFBE41o-, HEK293	down	IL-8	regulates IL-8	Oglesby et al. (2015)
miR-16	IB3-1, IB3-1/S9, CFPAC-1	basal comparable levels	HSP90	regulates F508del-CFTR trafficking defects	Kumar et al. (2015)
miR-199a-5p	Human and murine macrophages from lungs	up	CAV1	AKT/miR-199a-5p/CAV1 pathway as a regulator of innate immunity	Zhang et al. (2015)
miR-155	IB-3, IB3-1/S9	up	RPTOR	upregulates CTGF and regulates CF lungs fibrosis	Tsuchiya et al. (2016)
miR-1343	A549, 16HBE14o-, Caco-2	down	TGF-β	increases levels of activated TGF-β, pSMAD2 and pSMAD3	Stolzenburg et al. (2016)
miR-145	Primary cells from CF and non-CF patients	up	TGF-β	mediates TGF-β inhibition of CFTR synthesis and function	Lutful Kabir et al. (2018)
miR-199a-3p	CFBE41o-	down	IKKβ	increases IKKβ, NF-κB activity, and IL-8	Bardin et al. (2018)

trafficking (Yamakami et al., 2003; Katoh et al., 2004). These findings suggest miR-126, which directly targets TOM1, represents a crucial role in the regulation of innate immune responses and endosomal trafficking of ubiquitinated proteins in the CF lung. miR-146a negatively regulates Mucin 5AC (MUC5AC) expression, which is one of the foremost constituents of airway mucus, probably through the c-Jun N-terminal kinase (JNK) and NF-κB signaling in the neutrophil elastase (NE)-induced 16HBE14o-cells (Zhong et al., 2011). These results indicate the manipulation in miR-146a expression could regulate the excessive synthesis of mucus and thereby, CF pathogenesis. A recent study demonstrated that the inhibition of miR-146a induces increased expression of IL-6 in lipopolysaccharide (LPS)-stimulated CF macrophages (Luly et al., 2019). This study indicates that miR-146a dysregulation leads to dysfunctional CF macrophages, which results in impaired host defense and overproduction of inflammatory responses, and contributes to the progression and severity of CF. Several miRNAs including miR-509-3p, miR-494, and miR-126 regulate NF-kB, which in turn regulate CFTR expression and function (McKiernan et al., 2013; Ramachandran et al., 2013). Mir-31 downregulation increases cathepsin S, an inhibitor of antimicrobial proteins, through targeting the transcription factor *IRF-1* in CF pulmonary epithelial cells (Weldon et al., 2014). This results in the excessive accumulation of cathepsin S, which leads to the protease burden of the CF lung, and thereby the miR-31/IRF-1/CTSS pathway contributes to pulmonary inflammation in the CF airways (Weldon et al., 2014). The exogenous overexpression of miR-17 and miR-1343 downregulates IL-8 and TGF-β, respectively, in CF airway epithelial cells (Oglesby et al., 2015; Stolzenburg et al., 2016). MiR-199a-3p negatively regulates the NF-kB signaling pathway and IL-8 *via* its target inhibitor of nuclear factor kappa-B kinase subunit beta (*IKKβ*), which is implicated in the NF-κB pathway, and downregulation of miR-199a-3p contributes to pulmonary inflammation in the CF airways (Bardin et al., 2018).

Besides miRNAs, lncRNAs also regulate CF lung disease. For example, a number of lncRNAs including TLR8-AS1, HOTAIR, XIST, and MALAT are differentially expressed in bronchial brushings of CF patients (McKiernan et al., 2014). *Pseudomonas aeruginosa* infected CF bronchial epithelial cells exhibit dysregulation of several lncRNAs including MEG9 (maternally expressed 9) and BLACAT1 (bladder cancer-associated transcript 1) (Balloy et al., 2017). However, more investigations are required to understand the role and molecular mechanism of these lncRNAs in CF. Further, a recent study has illustrated the differential expression of lncRNAs in CF lung airway and parenchyma tissues, that affect multiple signaling pathways and cell membrane functions (Kumar et al., 2019). Interestingly, the suppression of lncRNA BGas rescues CFTR expression and function through the interaction with HMGB DNA-distorting proteins, members of the high mobility group (HMG) superfamily that lead to modifications of local chromatin and DNA structure of intron 11 of the CFTR gene (Saayman et al., 2016). Collectively, these observations underscore the promising associations of certain ncRNAs, both miRNAs and lncRNAs, in the direct or indirect regulation of CFTR expression and function, as well other aspects

of CF disease phenotypes such as inflammation, airway obstruction, or infection as summarized in **Tables 3**, **4**. The comprehensive knowledge of their roles and mechanisms in the pathogenesis and regulation of CF disease may represent a novel therapeutic approach for cystic fibrosis.

Idiopathic Pulmonary Fibrosis (IPF)

IPF is a lethal progressive fibrotic disease of the lung interstitium, and is mainly characterized by persistent epithelial injury, scar tissue accumulation, increased fibroblast proliferation, amplified production of extracellular matrix (ECM), and excessive inflammation (Martinez et al., 2017; Mora et al., 2017). However, the exact etiology and pathogenesis of this disease is still not very well-defined.

In recent decades, the association between the pathogenesis of IPF and ncRNAs is recognized by an increasing number of studies. For example, miR-199a-5p is upregulated in lungs and lung myofibroblasts from IPF patients and bleomycin (BLM)-induced mouse models and activates lung fibroblast and fibrosis through targeting caveolin-1 (*CAV-1*), a major mediator of pulmonary fibrosis, and modulation of TGF-β signaling, which is involved in activation of fibroblasts proliferation and induction of EMT in alveolar epithelial cells (Lino Cardenas et al., 2013). MiR-21 is upregulated in peripheral blood from IPF patients, and inhibition of miR-21 in rat models upregulates its target a disintegrin-like and metalloproteinase with thrombospondin type 1 motif (*ADAMTS-1*), which downregulates pulmonary collagen type 1 (Col1) and collagen type 3 (Col3) contents and reduces the progression of IPF (Liu et al., 2016). The increased expression of miR-142-5p and reduced expression of miR-130a-3p are observed in macrophages from IPF patients and BLM-induced mouse models (Su et al., 2015). Thus, inhibition of miR-142-5p and overexpression of miR-130a-3p suppress lung fibrosis through stimulation of the STAT6 pathway by targeting peroxisome proliferator-activated receptor γ (*PPARγ*, a STAT6 coordinator) and suppressor of cytokine signaling 1 (*SOCS1*, a STAT6 inhibitor), respectively, which facilitates macrophage activation and contribute to extensive tissue fibrosis.

MiR-26a is downregulated in A549 cells and BLM-induced mouse models, and its overexpression diminishes epithelial-mesenchymal transition (EMT) through targeting high mobility group AT-hook 2 (*HMGA2*), a main positive regulatory factor in EMT (Liang et al., 2014a). The downregulation of miR-326 is reported in the lungs of IPF patients and BLM-induced mouse models (Das et al., 2014). Consistently, the overexpression of miR-326 suppresses TGF-β1 expression and diminishes the fibrotic response by downregulation of profibrotic genes (Ets1, Smad3, and matrix metalloproteinase 9 (MM9) and upregulation of antifibrotic genes (Smad7) (Das et al., 2014). The downregulation of miR-486-5p is found in lung tissues of IPF patients, and its overexpression in mouse models reduces lung fibrosis through targeting *SMAD2*, a crucial mediator of pulmonary fibrosis and implicated in TGF-β1 signaling (Ji et al., 2015). The downregulation of miR-323a-3p is found in lung epithelium from IPF patients, and its overexpression in IPF mouse models reduces fibroproliferation *via* directly targeting its targets *TGFA* and *SMAD2* and modulation of various profibrotic signaling such as TGF-α, TGF-β, and apoptosis (Ge et al., 2016). In addition, miR-323a-3p downregulates CASP3, which prevents programmed cell death. MiR-221 is downregulated in tissues from human IPF, and in adenocarcinoma A549 and human bronchial epithelium (HBE) cell lines (Wang et al., 2016). Consistently, overexpression of miR-221 in these cell lines suppresses HMGA2 as well as phosphorylated-Smad3, which modulate TGF-β1 signaling, and leads to attenuation of EMT and lung fibrosis. The reduced expression of miR-29c is observed in alveolar epithelial cells from IPF patients, and overexpression of miR-29c in mice model reduces apoptosis, increases epithelial renewal, and thereby reduces lung fibrosis through targeting forkhead box O3a (*Foxo3a*), which is a transcription factor and play a crucial role in the induction of apoptosis (Xie et al., 2017). MiR-30a is downregulated in IPF patients, and further *in vitro* analyses indicate that overexpression of miR-30a directly targets ten-eleven translocation 1 (*TET1*) that modulates dynamin-related protein1 (Drp-1) promoter hydroxymethylation and thereby, show antifibrotic effect and defensive role against pulmonary damage (Zhang S. et al., 2017). The downregulation of miR-18a-5p is reported in pleural mesothelial cells (PMCs) and BLM-induced mouse models, and overexpression of miR-18a-5p downregulates its target *TGF-βRII* and reduces EMT of PMCs and sub-pleural pulmonary fibrosis (Zhang Q. et al., 2017). MiR-155 is downregulated in lung fibroblasts from IPF patients, and lung macrophages and fibroblasts from BLM-induced mouse models (Kurowska-Stolarska et al., 2017). In the same study, it is shown that overexpression of miR-155 decreases the exacerbated fibrotic response through downregulating its target liver X receptor α (*LXRα*), an oxysterol-activated transcription factor, and thereby, decreased production of collagen and TGF-β (Kurowska-Stolarska et al., 2017). The downregulation of miR-30a-5p is shown in exosomes from bronchoalveolar lavage fluid (BALF) of IPF elderly patients and A549 cells, its overexpression downregulates α-smooth muscle actin, and fibronectin expression by targeting TGF-β activated kinase 1/MAP3K7 binding protein 3 (*TAB3*), which is implicated in various cellular processes such as immune and inflammatory responses, altered fibrosis, and tissue repair and remodeling involved in IPF pathogenesis (Liu et al., 2018a). MiR-708-3p is downregulated in plasma and tissues from IPF patients, and overexpression of miR-708-3p attenuates lung fibrogenesis through directly modulating its target a disintegrin and metalloproteinase 17 (*ADAM17*), which regulates immune responses, fibrosis, and tissue regeneration, and by GATA/STAT3 signal pathway that is implicated in fibroblast-myofibroblast differentiation (Liu et al., 2018b). A recent report shows that miR-186 is downregulated in lung tissues of IPF patients, and delivery of miR-186 by human bone marrow mesenchymal stem cell-derived extracellular vesicles (BMSC-EVs) reduces fibroblast activation by downregulating its target SRY-related HMG box transcription factor 4 (*SOX4*) and thereby Dickkopf-1 (DKK1) (Zhou et al., 2021). SOX4 acts as a transcription factor and is associated with lung development and cell survival, whereas DKK1 is an inhibitor of the Wnt

TABLE 5 | List of ncRNAs and their targets and functions in IPF.

ncRNA	Source	Expression	Target/regulator	Function	Reference
miR-199a-5p	lungs and lung myofibroblasts from IPF patients, BLM-induced mouse models	up	CAV1	mediates TGF-β induced lung fibroblast activation	Lino Cardenas et al. (2013)
miR-26a	A549 cells, BLM-induced mouse models	down	HMGA2	induces EMT	Liang et al. (2014a)
miR-26a	lungs from IPF patients, BLM-induced mouse models, MRC-5 cells	down	Smad4	reveals positive feedback loop between miR-26a and p-Smad3	Liang et al. (2014b)
miR-92a	lung fibroblasts from IPF patients, BLM-induced mouse models	down	WISP1	increases WISP1	Berschneider et al. (2014)
miR-326	lungs from IPF patients, BLM-induced mouse models, multiple human cell lines	down	TGF-β1	regulates TGF-β1 expression and other profibrotic genes (Ets1, Smad3, Smad7, and MM9)	Das et al. (2014)
miR-9-5p	lungs from IPF patients, BLM-induced mouse models	up	TGFBR2, NOX4	suppresses pro-fibrogenic transformation of fibroblasts and prevents organ fibrosis	Fierro-Fernandez et al. (2015)
miR-29c	lung tissue from IPF patients	down	type I collagen	dysregulates PP2A/HDAC4 axis and increases type I collagen expression	Khalil et al. (2015)
miR-130a-3p	macrophages from IPF patients, BLM-induced mouse models	down	PPARγ	regulates macrophage profibrogenic gene expression	Su et al. (2015)
miR-142-5p	macrophages from IPF patients, BLM-induced mouse models	up	SOCS1	regulates macrophage profibrogenic gene expression	Su et al. (2015)
miR-486-5p	lung tissues from IPF patients, silica-induced mouse models, BLM-induced mouse models	down	SMAD2	promotes lung fibrosis	Ji et al. (2015)
miR-21	Peripheral blood from IPF patients, BLM-induced rat models	up	ADAMTS-1	increases of pulmonary Col1 and Col3 contents and promotes progression of pulmonary fibrosis	Liu et al. (2016)
miR-26a	A549 and MLE-12 cells	down	Lin28B	induces EMT by inhibition of let-7d	Liang et al. (2016)
miR-27a-3p	lung fibroblasts from IPF patients	down	α-smooth muscle actin, Smad2, Smad4	functions *via* a negative-feedback mechanism in inhibiting lung fibrosis	Cui et al. (2016)
miR-29a	clinical specimens from IPF, MRC-5 cells	down	LOXL2, SERPINH1	causes overexpression of LOXL2 and SERPINH1	Kamikawaji et al. (2016)
miR-29c	lung fibroblasts, IPF lungs	down	Fas	causes resistance to Fas-mediated apoptosis	Matsushima and Ishiyama (2016)
miR-34a,b,c	type II AECs from IPF patients	up	E2F1, c-Myc, cyclin E2	regulates cellular senescence	Disayabutr et al. (2016)
miR-130b-3p	lungs from IPF patients	down	IGF-1	contributes to fibroblasts activation and dysregulated epithelial-mesenchymal crosstalk	Li et al. (2016)
miR-185, miR-186	lung from IPF patients, A549 and HCC827 cells	down	COL5A1	induces EMT and collagen V overexpression	Lei et al. (2016)
miR-221	tissues from human IPF, A549, HBE	down	HMGA2	induces EMT and pulmonary fibrosis	Wang et al. (2016)
miR-323a-3p	lung epithelium from IPF patients, BLM-induced mouse models	down	TGF-α, TGF-β, caspase-3	releases inhibition of various profibrotic pathways to promote fibroproliferation	Ge et al. (2016)
miR-338-5p	BLM-induced mouse models	down	SMO	induces EMT and contributes to fibrotic phenotype	Zhuang et al. (2016b)
miR-338-5p	BLM-induced mouse models	down	LPA1	promotes pulmonary fibrosis	Zhuang et al. (2016a)
miR-18a-5p	PMCs, BLM-induced mouse models	down	TGF-βRII	induces EMT of PMCs and sub-pleural pulmonary fibrosis	Zhang et al. (2017a)
miR-27b	lung fibroblasts from IPF patients, BLM-induced mouse models	down	TGF-βRI, SMAD2	stimulates fibroblast activation	Zeng et al. (2017)
miR-29c	AEC from IPF patients, BLM-induced mouse models	down	Foxo3a	increases apoptosis and reduces epithelial renewal	Xie et al. (2017)
miR-30a	IPF patients, MRC-5 cells	down	TET1	increases the TET1 and reduces Drp-1 promoter hydroxymethylation	Zhang et al. (2017b)
miR-34a	AEC from IPF patients, BLM-induced mouse models	up	p53	promotes lung epithelial injury and pulmonary fibrosis	Shetty et al. (2017)
miR-34a	lungs and lung myofibroblasts from IPF patients, BLM-induced mouse models	up	β-galactosidase, senescence markers	induces a senescent phenotype in lung fibroblasts	Cui et al. (2017)
miR-155	lung fibroblasts from IPF patients, lung macrophages and fibroblasts from BLM-induced mouse models	down	LXRα	increases exacerbated lung fibrosis, collagen deposition, TGF-β production	Kurowska-Stolarska et al. (2017)

(Continued on following page)

TABLE 5 | (*Continued*) List of ncRNAs and their targets and functions in IPF.

ncRNA	Source	Expression	Target/regulator	Function	Reference
miR-30a-5p	exosomes from BALF of IPF patients, A549 cells	down	TAB3	increases TAB3, α-smooth muscle actin and fibronectin expression	Liu et al. (2018a)
miR-708-3p	plasma and tissues from IPF patients	down	ADAM17	promotes fibrogenesis	Liu et al. (2018b)
miR-186	lung tissues from IPF patients	down	SOX4	miR-186 delivered by BMSC-EVs could suppress fibroblast activation	Zhou et al. (2021)
lncR-H19	BLM-induced mouse models	up	miR-29b	upregulates COL1A1 and Acta2 and promotes fibrogenesis	Tang et al. (2016a)
lncR-TERRA	blood from IPF patients, BLM-induced mouse models A549 and MLE-12 cells	up	genes/components associated with telomeres and mitochondria	regulates telomeric and mitochondrial functions	Gao et al. (2017)
lncR-PCF	lungs from IPF patients, BLM-induced rat models, RLE-6TN cells	up	miR-344a-5p	promotes pulmonary fibrogenesis	Liu et al. (2017)
lncR-PFRL	lungs and lung fibroblasts from BLM-induced mouse	up	miR-26a	contributes to progression of lung fibrosis by modulating the reciprocal repression between miR-26a and Smad2	Jiang et al. (2018)
lncR-H19	BLM-induced mouse models	up	miR-196a	promotes fibrogenesis	Lu et al. (2018)
lncR-PFAR	lungs and lung fibroblasts from BLM-induced mouse	up	miR-15a	modulates of YAP1-Twist expression	(Zhao et al., 2018; Sun et al., 2019)
lncR-H19	tissues from IPF patients, BLM-induced mouse models, HBE and A549 cells	up	miR-140	promotes pulmonary fibrosis *via* regulatory network of lncRNA H19-miR-140-TGF-β/Smad3 signaling	Wang et al. (2019)
lncR-ZEB1-AS1	lungs from BLM-induced rat models, RLE-6TN cells	up	miR-141-3p	promotes EMT progress and fibrogenesis	Qian et al. (2019)

signaling pathway that have a significant role in lung development and differentiation and IPF pathogenesis and progression (Menezes et al., 2012; Guan and Zhou, 2017; Pan et al., 2017).

Several studies have also demonstrated the involvement of lncRNAs in the pathogenesis of IPF. For example, elevated levels of the lncRNA H19 in BLM-induced mouse models upregulates COL1A1 and Acta2, prominent factors linked with IPF pathogenesis, through direct targeting of miR-29b, and consequently knockdown of H19 attenuates fibrogenesis (Tang Y. et al., 2016). The telomeric repeat-containing RNA (TERRA) is upregulated in the blood of IPF patients, and further *in vivo* and *in vitro* studies demonstrate its role in fibrogenesis by the regulation of telomeric and mitochondrial functions (Gao et al., 2017). Dysfunctional telomerase activity and mitochondria under oxidative stress elicit apoptosis of epithelial cells and other processes linked with IPF progression. The increased expression of lncRNA PCF in the lungs of IPF patients induces pulmonary fibrosis by directly targeting miR-344a-5p and regulating map3k11 that elicit the proliferation of activated epithelial cells (Liu et al., 2017). Pulmonary fibrosis-regulatory lncRNA (PFRL) regulates the reciprocal repression of miR-26a and Smad2, which elicits the proliferation of activated epithelial cells, and contributes to the collagen deposition and progression of lung fibrosis (Jiang et al., 2018). In lungs and lung fibroblasts from mice, lncRNA pulmonary fibrosis-associated RNA (PFAR) functions as a competitive endogenous RNA (ceRNA) for miR-15a and in the regulation of yes-associated protein 1 (YAP1)-Twist expression, which is an important transcriptional effector in the Hippo pathway and implicated in the organ fibrosis process (Zhao et al., 2018; Sun et al., 2019). Upregulated lncRNA H19 in tissues from IPF patients downregulates miR-140 and modulates TGF-β/Smad3 signaling, and further *in vivo* and *in vitro* experiments show that the knockdown of H19 diminishes pulmonary fibrosis (Wang et al., 2019). The elevated expression of Zinc-finger E-box binding homeobox 1 antisense RNA 1 (ZEB1-AS1) and its positive correlation with the expression of ZEB1, which is a master regulator of EMT, is found in BLM-induced rats and TGF-β1-induced RLE-6TN cells (Qian et al., 2019). Subsequent experiments demonstrated that silencing of lncRNA ZEB1-AS1upregulates its target miR-141-3p and suppresses progression of EMT and fibrogenesis. Therefore, cumulatively these data clearly depict the regulatory functions, particularly the post-transcriptional regulation of ncRNA, miRNAs and lncRNAs, in onset, progression, and development of IPF. **Table 5** summarizes the list of ncRNAs, their targets, and functions associated with IPF. Further, additional in-depth studies will lead to therapies for early diagnosis, control, and treatment of IPF.

DISCUSSION

Here, we summarize the emerging roles, post-transcriptional regulations, and mechanistic functions of ncRNAs, with emphasis on miRNAs and lncRNAs, in lung diseases that are a major public health concern. According to a recent report, only in the year 2017, lung diseases globally affected nearly 545 million people and caused 3.9 million deaths with an increase of 39.8 and 18.0%, respectively, since 1990 (Collaborators, 2020). Thus, lung

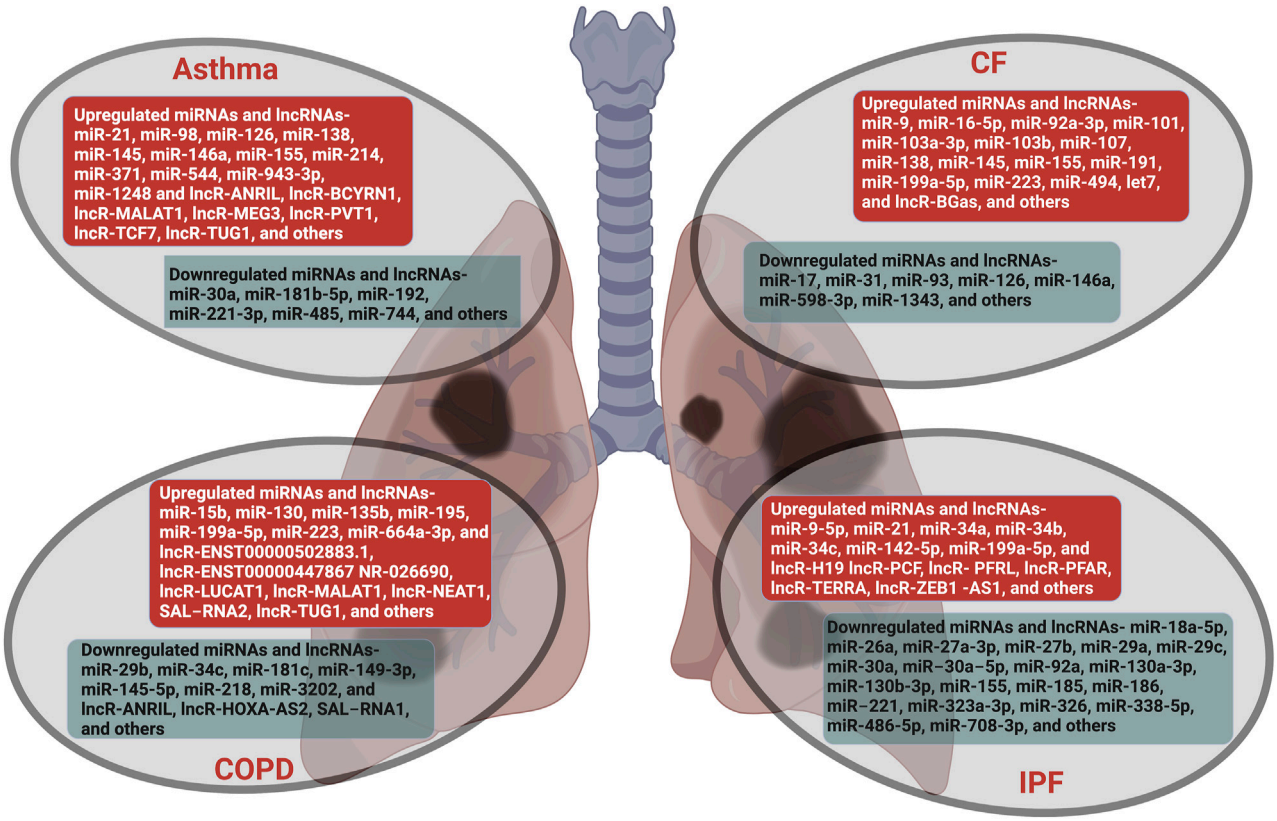

FIGURE 1 | Lung diseases regulated by non-coding RNAs. The miRNAs and lncRNAs that are aberrantly expressed in lung diseases such as asthma, COPD, CF and IPF are listed. The ncRNAs that are upregulated are in red boxes and those that are downregulated are in green boxes.

diseases are a predominant cause of substantial morbidity and mortality worldwide and demand an exhaustive understanding of etiology and pathophysiology. Recent studies have established the association and regulatory function of ncRNAs in lung development and maintenance of lung homeostasis. The deregulation of ncRNAs causes pathophysiological alteration and contributes to the onset, progression, and development of various types of lung diseases such as asthma, COPD, CF, and IPF (**Figure 1**)

Alterations of miRNA and lncRNA expression level in the disease state compared to the normal state expedite a new paradigm for the diagnosis and appraisal of drug action. As presented in this review, disease-specific dysregulated miRNAs/lncRNAs are identified in various types of lung cells and tissues, which together with the higher stability of miRNAs mark them as clinical diagnostic biomarkers. However, a major challenge is the invasive procedures used for obtaining lung biopsies. Recent reports indicate detection of miRNAs/lncRNAs in body fluids such as blood, serum, plasma, BAL fluid, saliva, sputum, and urine, which have tremendous potential for relatively non-invasive diagnosis and prognosis of lung disease as well as appraisal of drug action. However, the utility of these biospecimen as a clinical diagnostic biomarker is yet to be examined and established with a larger patient cohort in various lung diseases.

Notably, ncRNA-based therapeutics have great potential in the treatment of lung diseases. Collectively, the studies summarized here show that enormous efforts have been made to deliver mimic or antisense oligonucleotide (ASO, including inhibitor, miRNA sponge, and target site blocker (TSB)) to overexpress or suppress specific genes that are downregulated or upregulated, respectively, in the diseased state and contribute to the pathogenesis and pathophysiology of lung diseases. However, in order to translate this treatment strategy from lab to clinical settings, some challenges including cell/tissue-specific delivery, stability and binding affinity, and off-target effects need to be addressed. Recent progress in generating modified derivatives of nucleic acid as potential drugs include numerous chemical strategies, such as the addition of 2′-O-methyl (2-O′-Me) or phosphorothioate-like groups, locked nucleic acids (LNA), miRNA sponges, nanoparticles, morpholinos, or peptide nucleic acids (PNA) as well as strategies for efficient delivery, such as viral vectors, polymers-, peptides-, and lipid-based delivery systems. Despite these efforts, there is still a need for more extensive studies to evaluate the effect of chemical modifications in *in vivo* systems and develop more consistent cell/tissue-specific delivery strategies.

Our current knowledge suggests that the identification of disease-specific miRNAs/lncRNAs and comprehensive knowledge of post-transcriptional regulation mechanisms will

help understand their role and mode of functioning in the pathogenesis of lung diseases. Concurrently, the development of safe and cell/tissue-specific delivery systems will help to translate ncRNAs-based therapeutics from lab to clinical settings. Hence, we are optimistic that the continued elucidation of the function of ncRNAs encompasses the great potential to uncover diagnostic and prognostic tools and candidate therapeutic targets for lung diseases in the near future.

AUTHOR CONTRIBUTIONS

DS and RB prepared the manuscript.

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miR-224-5p and miR-545-5p Levels Relate to Exacerbations and Lung Function in a Pilot Study of X-Linked MicroRNA Expression in Cystic Fibrosis Monocytes

Paul J. McKiernan[1†], *Kevin P. Molloy*[1†], *Arlene M. A. Glasgow*[2]*, *Noel G. McElvaney*[1] *and Catherine M. Greene*[2]

[1]Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin, Ireland, [2]Department of Clinical Microbiology, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin, Ireland

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***Correspondence:**
Arlene M. A. Glasgow
arleneglasgow@rcsi.ie

†These authors have contributed equally to this work and share first authorship

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Altered microRNA expression patterns in bronchial brushings from people with versus without cystic fibrosis (CF) relate to functional changes and disease pathophysiology. The expression of microRNAs encoded on the X chromosome is also altered in peripheral blood monocytes of p. Phe508del homozygous versus non-CF individuals. Here we investigate whether levels of the top seven X-linked microRNAs (miR-224-5p, miR-452-5p, miR-450b-5p, miR-542-3p, miR-450a-5p, miR-424-5p, and miR-545-5p) that are significantly increased over 1.5 fold in CF versus non-CF monocytes correlate with lung function. $CD14^+$ monocytes were isolated from males and females with (n = 12) and without cystic fibrosis (n = 12) and examined for the expression of X-linked microRNAs by qRT-PCR array. MicroRNA target mRNA levels were quantified using qRT-PCR. Clinical correlations with lung function data were analysed in the CF cohort. Increasing levels of miR-545-5p correlated moderately with FEV1% predicted (r = -0.4553, p > 0.05) and strongly with exacerbation rate (r = 0.5858, p = 0.0483). miR-224-5p levels were significantly higher in the severe (FEV1 <40%) versus mild (FEV1 ≥80%, p = 0.0377) or moderate (FEV1 40–79%, p = 0.0350) groups. MiR-224-5p expression inversely correlated with lung function (FEV1%: r = -0.5944, p = 0.0457) and positively correlated with exacerbation rates (r = 0.6139, p = 0.0370). These data show that peripheral blood monocyte miR-545-5p and miR-224-5p levels correlate with exacerbation rate, whilst miR-224-5p levels also correlate with lung function in cystic fibrosis.

Keywords: x-linked miRNAs, cystic fibrosis, x chromosome, miR-224-5p, miR-545-5p, Smad4, biomarkers, monocytes

INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel. The most prevalent *CFTR* mutation is p. Phe508del, which results in protein misfolding, retention in the endoplasmic reticulum, and consequently reduced functional CFTR at the cell surface. In addition to the primary *CFTR* defect there are other important factors that can contribute to CF lung disease pathology including, for example, intrapulmonary proteases, mucus hypersecretion and microRNA (miRNA) expression in bronchial epithelium (Cantin et al., 1989; Voynow et al., 1998; Voynow et al., 1999; Oglesby et al., 2010), amongst others. MiRNAs are non-coding regulatory RNAs that control protein expression levels. Functionally altered miRNA expression profiles are evident in people with CF and *in vitro* CF cell models (De Santi et al., 2020) and have been reviewed elsewhere (Glasgow et al., 2018).

There is a clinical need to find better strategies for monitoring early lung disease in people with CF in order to identify those at risk for more progressive lung disease and thereby allow earlier intervention (Laguna et al., 2018). The processes of bronchoscopy and bronchoalveolar lavage fluid sampling that are commonly used to determine lung inflammation and damage are highly invasive. Identifying less invasive biomarkers of lung function decline and/or the intrapulmonary inflammatory or infective milieu are highly sought after for CF and other chronic inflammatory lung diseases (Vencken et al., 2015). As fine-tuners of many molecular processes, miRNAs are known to be altered in many disease states, both in cellular expression levels and extracellularly in body fluids such as plasma or sputum (Cui et al., 2019). Given that they can be quantified with high accuracy via qPCR, and offer the prospects of less invasive sampling, miRNAs are attractive candidate biomarkers.

It is commonly recognized that females with CF have worse outcomes than men, for example lower lung function, earlier colonization with respiratory pathogens such as *Pseudomonas aeruginosa*, and greater frequency of exacerbations (Demko et al., 1995; Konstan et al., 2007; Levy et al., 2008). Sex hormones, in particular estrogen, have been implicated in the gender bias of CF lung disease (Chotirmall et al., 2010; Chotirmall et al., 2012; Holtrop et al., 2021). However, there is a lack of studies on the contribution of other major determinants of sex differences, e.g., the sex chromosomes, to CF pathology. The X chromosome is relatively rich in miRNAs, encoding approximately 10% of the total microRNAome. Previously we reported that X chromosome-encoded miRNAs are functionally increased in CF monocytes (McKiernan et al., 2018). Therein miR-224-5p was the most highly increased X–linked miRNA in CF versus non-CF monocytes; its validated target, *SMAD4,* was reciprocally decreased in the same samples. Here we further examined the expression pattern of the mostly highly differentially expressed X-linked miRNAs in monocytes from males and females with and without CF to determine whether they correlate with lung function and exacerbation rates in people with CF.

RESULTS

Study Group Demographics

Following informed consent in line with a protocol approved by Beaumont Hospital Ethics Committee (13/108), twenty-four individuals were recruited into this study; 12 were p. Phe508del homozygous individuals (**Table 1**) confirmed by genotyping, and 12 were non-CF controls, with no underlying lung disease with a mean age of 23.5 ± 5.1 years and 27.3 ± 3.6 years, respectively. Samples were obtained from CF patients at the time of their routine outpatient clinic review (i.e., samples were not from exacerbating inpatients). An exacerbation was defined as worsening of the patients respiratory symptoms requiring treatment with antibiotics. **Table 1** shows the CF patient characteristics including infection/colonisation status for pathogens associated with CF lung disease pathophysiology. There were no statistically significant differences between the CF male and CF female cohort in colonisation status for any individual pathogen (**Supplementary Figure S1**), or total number of pathogens (**Supplementary Figure S2**). **Supplementary Figure S3** shows that forced expiratory volume in 1 s percent predicted (FEV1% predicted) and exacerbation rate (defined as the number of exacerbations in the current year) were not different between the CF males and CF females.

X-Linked miRNA Profiles

Microarray profiling of 86 miRNAs located on the X chromosome was carried out using miScript PCR Array on peripheral blood $CD14^+$ monocytes from the CF and non-CF study populations as originally described (McKiernan et al., 2018). Differences in miRNA expression were observed between individuals and when compared by gender and CF pathology, CF females were most distantly related to CF males with respect to the expression of the X-linked miRNAs measured (**Figure 1**).

Expression of Some miRNAs Located on the X Chromosome Is Altered in Cystic Fibrosis Versus Non-CF Monocytes

qRT-PCR array data were analysed to determine whether a relationship existed between CF lung disease pathology and the expression of miRNA on the X chromosome in monocytes. Seven X chromosome miRNAs were increased ≥1.5 fold in CF versus non-CF controls. **Supplementary Table S1** shows details of the fold-change increase and p value for these differentially expressed (DE) miRNAs as first reported (McKiernan et al., 2018). The full microarray profiling data of X-miRNAs in CF versus non-CF monocytes is shown in **Supplementary Table S2**. No miRNAs were significantly decreased in the CF versus non-CF samples. No miRNAs were differentially expressed ≥1.5 fold between CF males and CF females (other than miR-452-5p which was 1.64-fold higher in CF males), or all males versus all females (data not shown).

TABLE 1 | Demographics and colonisation status of the CF cohort.

CF ID	Gender	Age (Years)	*PA*[a]	Mucoid *PA*	*SA*[b]	*Asp*[c]	*Can*[d]	Others
CFm_01	M	28	+	+	+	-	+	—
CFm_02	M	22	+	-	+	+	+	—
CFm_03	M	27	+	+	+	-	+	MRSA[e]
CFm_04	M	18	-	-	+	-	+	—
CFm_05	M	18	+	-	+	-	+	—
CFm_06	M	24	+	-	+	-	+	Bcc[f]
CFf_01	F	20	+	+	+	-	-	—
CFf_02	F	18	-	-	+	-	+	MRSA, SM[g]
CFf_03	F	21	+	+	+	-	+	—
CFf_04	F	23	+	-	-	+	-	—
CFf_05	F	29	+	+	-	+	+	—
CFf_06	F	34	+	+	-	+	+	—

[a]Pseudomonas aeruginosa.
[b]Staphylococcus aureus.
[c]Aspergillus species.
[d]Candida species.
[e]*Ilmethicillin-resistant* Staphylococcus aureus.
[f]Burkholderia cepacia *complex*.
[g]Stenotrophomonas maltophilia.

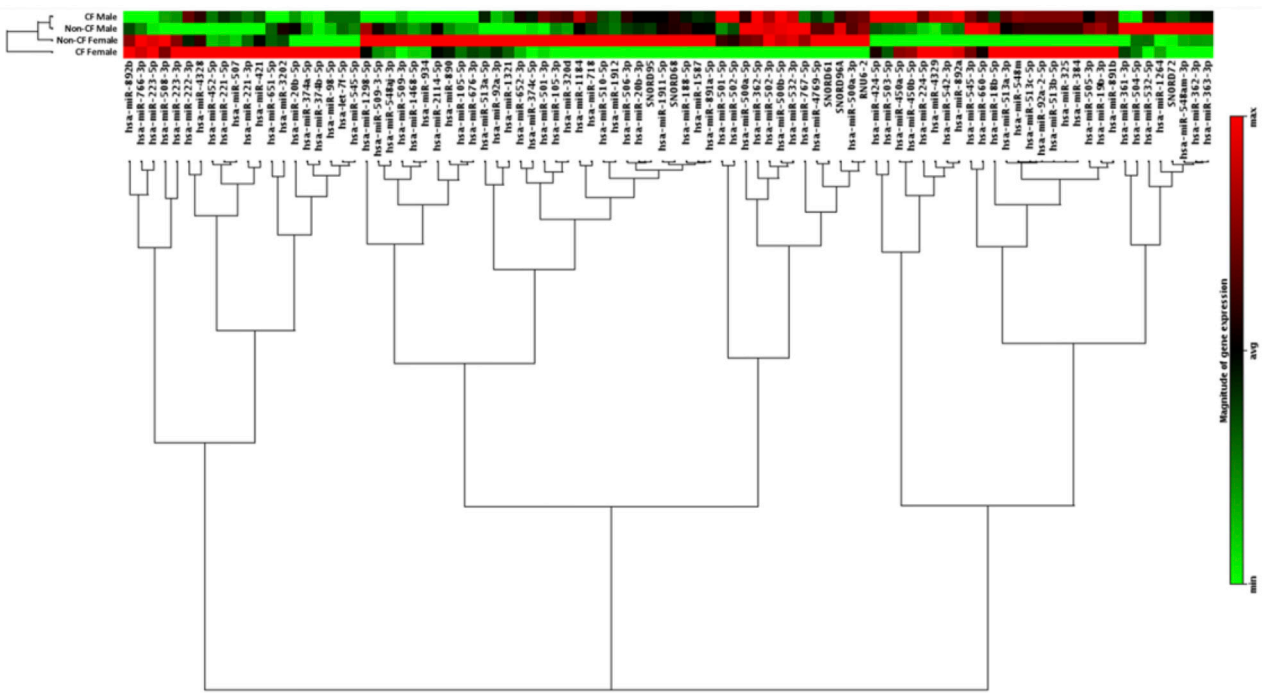

FIGURE 1 | Expression of X-chromosome located miRNA in CD14$^+$ monocytes from people with and without CF, annotated by gender and CF pathology. Hierarchical clustering dendrogram showing expression patterns of X-chromosome located microRNAs in males and females with and without CF (n = 6 each). CD14$^+$ monocytes were obtained from the peripheral blood of each individual. RNA was isolated from each sample and the expression of X-chromosome located miRNAs was examined by qRT-PCR array. Colour density is proportional to magnitude of expression.

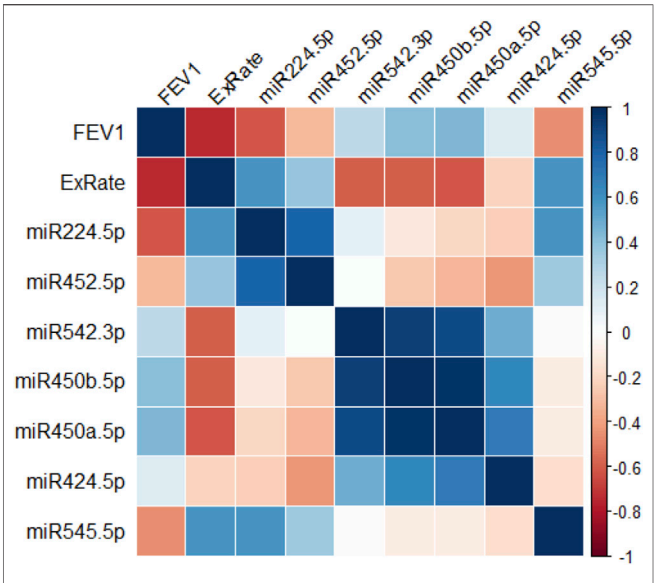

FIGURE 2 | X-linked miRNA expression, lung function and exacerbations. Correlogram of the relationships between levels of top 7 altered X-linked miRNA in peripheral blood monocytes and lung function parameters in people with CF (n = 12). r values are depicted as a color scale ranging from red (−1) to blue (+1).

miR-224-5p and miR-545-5p Correlate With Lung Function in People With Cystic Fibrosis

The relationship between the DE X-linked miRNA in CF monocytes and lung function data was examined. In this study, due to the small number of samples, we focused on miRNAs with the greatest fold-change difference rather than the lowest p-value. **Figure 2** is a correlation matrix of lung function (FEV1% predicted, exacerbation rate) and each of these miRNAs. MiR-224-5p shows the strongest inverse correlation with FEV1% predicted, and strongest positive correlation with exacerbation rate. After miR-224-5p, miR-545-5p demonstrates the next strongest, similar pattern.

In order to examine this further, the expression of miR-545-5p or miR-224-5p and its validated target *SMAD4* in monocytes from people with CF and markers of lung function were examined by Spearman's rank correlation analysis. To assess any relationship between the miR-224-5p validated target, *SMAD4*, in monocytes from people with CF and lung function, *SMAD4* expression was examined with respect to FEV1% predicted. *SMAD4* expression only weakly positively correlated with FEV1% predicted (r = 0.31) and the effect was not significant. There was no correlation between *SMAD4* and exacerbation rate (data not shown). Increasing levels of miR-545-5p were associated with decreasing lung function and increased exacerbation rate (**Figures 3A,B**). Although its expression was only moderately negatively correlated with FEV1% predicted (r = -0.4553, $p > 0.05$), the positive correlation between miR-545-5p levels and exacerbation rate was strong (r = 0.5858) and statistically significant (p = 0.0483).

All individuals with CF were clustered into three groups depending on lung function as measured by FEV1% predicted (**Figure 3C**). These were mild (FEV1 ≥80%), moderate (FEV1 40–79%) and severe (FEV1<40%). There was no significant difference in miR-224-5p expression between the "mild" and "moderate" groups. However, the expression of miR-224-5p was significantly higher in the severe versus mild (adjusted p value: 0.0377) and moderate (adjusted p value: 0.0350) groups (**Figure 3C**). Finally, a significant strong negative correlation (r = -0.5944, p = 0.0457) was observed between FEV1% predicted and miR-224-5p expression, indicating that low FEV1% predicted values are associated with high miR-224-5p expression (**Figure 3D**). Conversely, a significant strong positive correlation (r = 0.6139, p = 0.0370) between exacerbation rate and miR-224-5p expression was observed (**Figure 3E**) indicating that increasing numbers of exacerbations are associated with high miR-224-5p expression. Multiple linear regression was also performed to examine the relationship between clinical parameters and the combination of miR-224-5p and miR-545-5p levels, however no statistical significance was found (**Supplementary Figure S4**).

DISCUSSION

It is known that altered miRNA expression in CF bronchial brushings relates to functional changes that can contribute to CF lung disease pathophysiology. Prior to the present study, it was unknown whether expression levels of miRNA encoded on the X chromosome are associated with CF lung disease. Here we demonstrate that in people with CF, miR-224-5p expression in peripheral blood $CD14^+$ monocytes inversely correlates with lung function and positively correlates with exacerbation rate. MiR-545-5p levels are also related to exacerbation rate, albeit less robustly. Collectively the data highlight miR-224-5p as a potential biomarker for CF lung decline.

We previously reported that there is clear difference in the expression of X-linked miRNAs in CF versus non-CF monocytes (McKiernan et al., 2018). Here, two of the top seven miRNAs with the highest expression in the CF group, miR-224-5p and miR-545-5p, were examined further. Expression of both X-linked miRNAs was significantly correlated with exacerbation rate in CF; for miR-224-5p there was also a clear correlation with FEV1% predicted. High levels of miR-224-5p were associated with poor lung function; specifically, low FEV1% predicted values and high numbers of exacerbations per year. In this study an exacerbation was defined as worsening of the patient's respiratory symptoms requiring treatment with antibiotics. These results raise the possibility that this miRNA may be a biomarker for lung function decline in CF or other chronic inflammatory lung diseases. Despite the well-documented sexual dimorphisms in CF pathology, no statistically significant sex differences were observed in lung parameters in this small patient cohort, potentially due to the heterogeneous nature of CF lung disease. No individual miRNAs were differentially expressed between CF males and females, which may indicate that the increased expression of X-linked miR-224-5p and miR-545-5p in CF monocytes is a result of the same mechanisms in both sexes,

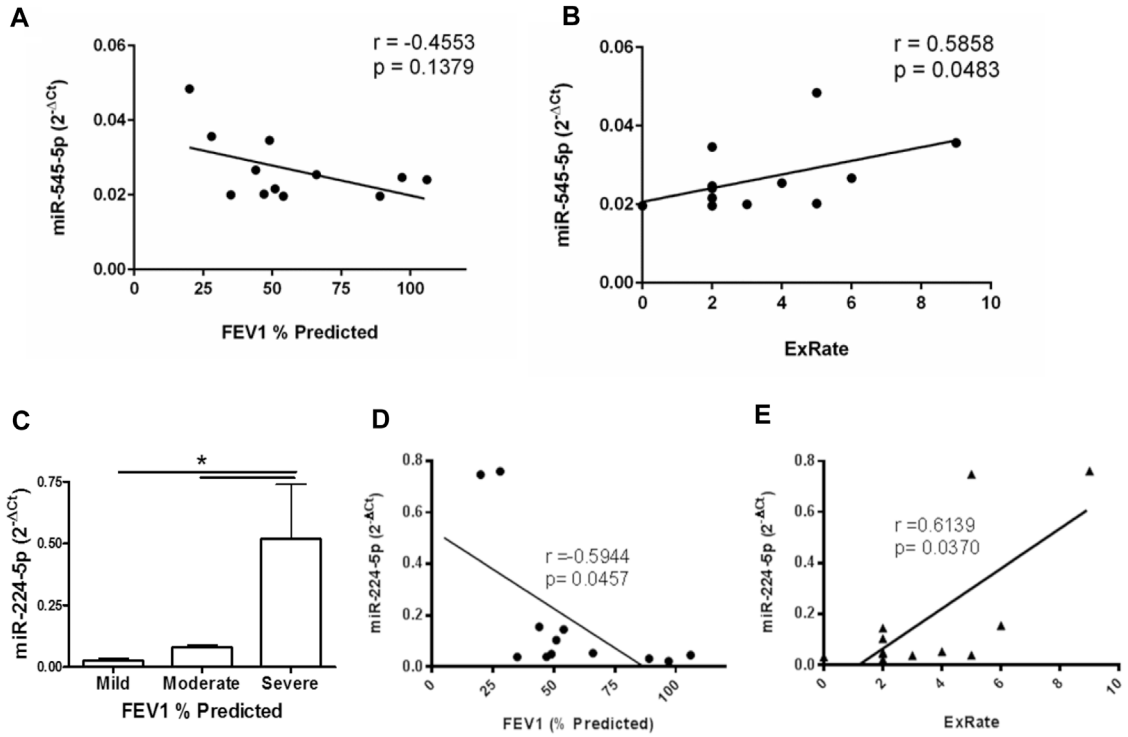

FIGURE 3 | Correlation between monocyte miR-545-5p, miR-224-5p, lung function and exacerbation rate in people with CF. $CD14^+$ monocytes were obtained from the peripheral blood of people with CF ($n = 12$). RNA was isolated from each sample and the expression of X-chromosome located miRNA were examined by qRT-PCR array. Correlation was examined between **(A)** FEV1% predicted and miR-545-5p expression, and **(B)** exacerbation rate and miR-545-5p expression. **(C)** Data depicts the normalised expression level ($2^{-\Delta Ct}$) of miR-224-5p in monocytes of people with CF, per FEV1% predicted grouping. "Mild": FEV1 ≥80 ($n = 3$), "Moderate": FEV1 40–79 ($n = 6$) and "Severe": FEV1 ≤40 ($n = 3$). *$p \leq 0.05$; one-way ANOVA followed by Tukey's multiple comparisons test, versus "Severe" group. **(D)** Correlation between FEV1% predicted and miR-224-5p expression. **(E)** Correlation between exacerbation rate and miR-224-5p expression. Correlation coefficients; *r*, were determined by Spearman's rank test and significance determined by two-tailed *t* test.

i.e., increased transcription of these miRNAs on the active X chromosome (females and males) rather than escape from X chromosome inactivation in females.

Ideal biomarkers should be detectable in easily accessed biological samples, and miR-224-5p already fulfils this criterion as it can be detected in serum (Gui et al., 2011; Li et al., 2011; Vencken et al., 2015). Future studies should examine whether this miRNA, or miR-545-5p, display a similar profile in serum of people with CF. Somewhat related to this, we have recently performed miRNA expression profiling in plasma of children with CF (Mooney et al., 2020). The focus of that study was to determine whether sex differences exist in the miRNA profile between boys and girls with CF, and its major finding was that there is a significant increase in miR-885-5p in plasma of females versus males with CF under 6 years of age. There was no non-CF group in that study with which we could retrospectively compare miR-224-5p or miR-545-5p levels.

Ideozu et al. (2019) performed miRNA profiling of plasma samples from people with CF versus healthy controls and found 11 differentially expressed miRNAs, including the X-linked miR-222-3p. This miRNA was not selected by the authors for further validation by qPCR but it would be interesting to see if its expression was significantly altered in a larger cohort, including equal numbers of male/female samples in both the CF and non-CF groups, and if there are any correlations with clinical parameters. A recent study by Stachowiak et al. (2020) reported a number of miRNAs from airway samples to show correlation with CF pulmonary exacerbation parameters, one of which was an X-linked miRNA, miR-223-3p. Increased levels of miR-223-3p were found in exhaled breath condensate and sputum during pulmonary exacerbation with concurrent *Aspergillus* infection.

SMAD4 is a validated target of miR-224-5p. Its levels have been shown to be significantly decreased in the CF subjects in our study and to weakly inversely correlate with miR-224-5p levels (McKiernan et al., 2018). Here we observed no strong or significant correlation between *SMAD4* expression in CF monocytes and FEV1% predicted or exacerbation rate.

Overall this pilot study reveals new insights into the expression of X-linked miRNA in CF peripheral blood monocytes as potential biomarkers for CF lung function decline. The number of samples used here, whilst appropriate for array-based studies, is very small therefore, we encourage others to replicate our clinical observations in larger CF cohorts which may be available to them. Furthermore, future validation studies should expand to include samples from patients with different CF genotypes e.g., homozygous p. Phe508del

and other mutations such as G551D and R117H. The effects of CFTR modulator therapy on miRNA profiles, particularly miR-224-5p and miR-545-5p, should also be evaluated. In addition it would be interesting to study the evolution of miRNA in patients before, during, and after an exacerbation. In summary, an altered X-linked miRNA profile is evident in people with CF, and some of these miRNAs; in particular miR-224-5p, correlate with clinical lung disease. Large cohort studies are warranted to confirm the utility of miR-224-5p or miR-545-5p as potential clinical biomarkers for CF disease progression.

MATERIALS AND METHODS

Isolation of CD14$^+$ Monocytes

Blood was mixed with an equal volume of 0.9% NaCl (1× saline) and layered over Lymphoprep (Axis Shield). Density gradient centrifugation was carried out at 800 × *g* for 10 min, the mononuclear cell band was aspirated, washed in Hank's Balanced Salt Solution (Lonza, BE10-543F), and monocytes were purified using the EasySep® Human CD14 Selection Cocktail (StemCell Technologies, 18058) as per the manufacturer's protocol. This kit yields up to 97% purity of monocytes. When first establishing the method in the laboratory this was tested and we routinely isolated >95% monocytes.

miScript™ Reverse Transcription for Polymerase Chain Reaction Array and Analysis

A total of 250 ng RNA extracted using the miRNeasy kit was reverse transcribed in a 1-step protocol into cDNA to use as templates for miScript PCR arrays. The reverse transcription reaction was prepared on ice then placed at 37°C for 60 min and then inactivated at 95°C for 5 min.

miRBase release 20 was utilised for identification of miRNAs located on the X chromosome. The 86 out of 118 entries in miRBase were chosen based on high confidence [sequences that have at least 10 deep sequencing reads that map to each of the 2 mature microRNA sequences (-5p and -3p)]. Each well of the custom made 96-well miScript™ PCR array plates (CMIHS02174), manufactured by SABiosciences, contained primers, reverse transcription reaction, PCR reaction and miRNA normalisation controls. Mature miRNA expression was measured with qRT-PCR using SYBR-Green based miScript PCR array according to the manufacturer's instructions on a LightCycler® 480, with pre-incubation step at 95°C for 15 min for HotStarTaq DNA Polymerase activation. The PCR cycles were i) denaturation at 95°C × 15 s, ii) annealing at 55°C × 30 s, iii) extension at 70°C × 30 s (repeated 40 times). Data analysis was performed on Ct values using SABiosciences PCR array data analysis software available at the time at http://pcrdataanalysis.sabiosciences.com/mirna. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the most stable small RNA controls. A combination of descriptive statistics and the NormFinder Excel add-in (Andersen et al., 2004) was used to identify suitable small RNAs for normalisation of data.

Gene Expression Analysis by Quantitative Real Time-Polymerase Chain Reaction

A total RNA of 200–1000 ng extracted using TRI Reagent were reverse transcribed into cDNA using the Quantitect® Reverse Transcription Kit (Qiagen, 205313).

qRT-PCR primers were designed using Primer 3 online software (http://frodo.wi.mit.edu) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and were obtained from MWG Eurofins Operon. GAPDH: (Fwd)- CAT GAG AAG TAT GAC AAC AGC CT, (Rvs)- AGT CCT TCC ACG ATA CCA AAG T; *β*-actin: (Fwd)- GGA CTT CGA GCA AGA GAT GG, (Rvs)- AGG AAG GAA GGC TGG AAG AG; SMAD4: (Fwd)- TGC ATT CCA GCC TCC CAT TT, (Rvs)- TGT GCA ACC TTG CTC TCT CA. Annealing temperatures were 57, 56 and 57°C respectively.

qRT-PCR was performed on a LightCycler® 480 (Roche) using a SYBR Green master mix (Roche, 04,707,516,001). For 20 µl reactions, optimal concentrations (200–500 nM) of each forward and reverse primer were used. Template cDNA was used at a concentration of 10% of the total reaction volume. The qRT-PCR programme was 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 57°C for 10 s (primer-dependent) and 72°C for 10 s (25 bases/second) respectively. The $2^{-\Delta\Delta Ct}$ method was used to quantify the expression of target genes relative to GAPDH and/or ACTB reference genes (Livak and Schmittgen, 2001). All qRT-PCR experiments included no-RTase and no-template controls.

Statistical Analysis

Data were analysed with GraphPad Prism 4.0 software package (GraphPad Software, San Diego, CA). All data are depicted as Mean ± SEM unless otherwise stated. Specific analyses that were performed are described for each figure. Differences were considered significant at $p \leq 0.05$.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Beaumont Hospital Ethics Committee, Dublin, Ireland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PM designed the study, carried out the experimental work, and analysed the data; KM collected the clinical samples and prepared the clinical data; AG provided intellectual input and wrote the article; NM managed the CF patients' care; CG conceived the

idea, designed the study, analysed the data, wrote the article and financed the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.739311/full#supplementary-material

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REVIEW
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Non-Coding RNA Networks in Pulmonary Hypertension

Hongbin Zang[1], *Qiongyu Zhang*[2] *and Xiaodong Li*[1]*

[1]Department of Cardiology, Shengjing Hospital of China Medical University, Shenyang, China, [2]Department of Neurology, Shengjing Hospital of China Medical University, Shenyang, China

Non-coding RNAs (ncRNAs) are involved in various cellular processes. There are several ncRNA classes, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). The detailed roles of these molecules in pulmonary hypertension (PH) remain unclear. We systematically collected and reviewed reports describing the functions of ncRNAs (miRNAs, lncRNAs, and circRNAs) in PH through database retrieval and manual literature reading. The characteristics of identified articles, especially the experimental methods, were carefully reviewed. Furthermore, regulatory networks were constructed using ncRNAs and their interacting RNAs or genes. These data were extracted from studies on pulmonary arterial smooth muscle cells, pulmonary artery endothelial cells, and pulmonary artery fibroblasts. We included 14 lncRNAs, 1 circRNA, 74 miRNAs, and 110 mRNAs in the constructed networks. Using these networks, herein, we describe the current knowledge on the role of ncRNAs in PH. Moreover, these networks actively provide an improved understanding of the roles of ncRNAs in PH. The results of this study are crucial for the clinical application of ncRNAs.

Keywords: pulmonary hypertension, long non-coding RNA, circular RNA, microRNA, network

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Shaveta Kanoria,
Wadsworth Center, United States

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Yuan Zhou,
Peking University, China
Hugo E. Verdejo,
Pontificia Universidad Católica de Chile, Chile
Hui Zhang,
University of Colorado, United States

***Correspondence:**
Xiaodong Li
Licardio@163.com

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1 INTRODUCTION

Pulmonary hypertension (PH) is a serious disease characterized by progressively increased pulmonary vascular resistance and pulmonary artery pressure; the diagnostic criterion is mean pulmonary artery pressure ≥25 mmHg (Galiè et al., 2016; Weber et al., 2018). The increased pulmonary artery pressure in PH results from changes in the structure and function of the vessel wall, which is induced by abnormal pulmonary cell proliferation, apoptosis, and migration (Bourgeois et al., 2018a). Patients with PH may experience dyspnea, fatigue, syncope, chest pain, and/or edema of the legs and ankles. The causes of PH can be broadly classified as primary and secondary causes. To date, ion channels, vasoactive substances, immune factors, and genetic factors are known to be involved in the pathogenesis of PH (Chelladurai et al., 2016; Veith et al., 2016; Bourgeois et al., 2018b).

Recently, many non-coding RNAs (ncRNAs) have been recognized as important regulators in the development of PH. Most human genes (>95%) do not produce proteins but ncRNA molecules. Among them, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) are the most widely studied. MiRNAs are small ncRNAs containing 21–22 nucleotides, which post-transcriptionally regulate gene expression (Wakiyama and Yokoyama. 2014). LncRNAs, which have more than 200 nucleotides, are transcribed from intergenic or intragenic regions. They can bind to proteins, RNA, or DNA to execute regulatory roles (Botti et al., 2017). CircRNAs are a novel class of ncRNAs with a closed loop structure, making them highly stable and capable of interacting with proteins or RNA (Di et al., 2019). NcRNAs have been identified

TABLE 1 | Query for searching articles from PubMed.

Query	Number of articles
("rna, untranslated"[MeSH Terms] or "non-coding RNA" or "ncRNA" or "noncoding RNA" or "RNA, Long Noncoding"[Mesh] or "long non-coding RNA" or "lncRNA" or "long intergenic non-coding RNA" or "lincRNA" or "RNA, Circular"[Mesh] or "circRNA" or "circular RNA" or "MicroRNAs"[Mesh] or "microRNA" OR "miRNA") and ("PAH" or "pulmonary hypertension" or "pulmonary artery hypertension")	602

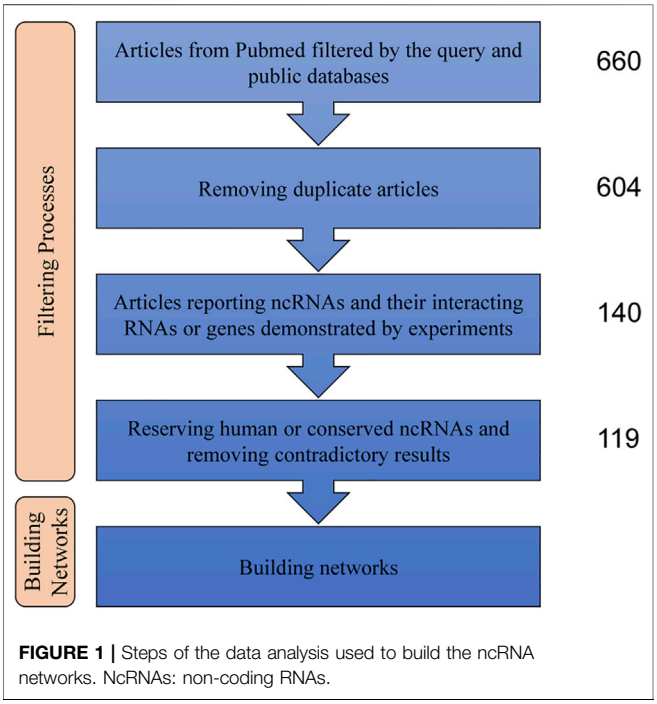

FIGURE 1 | Steps of the data analysis used to build the ncRNA networks. NcRNAs: non-coding RNAs.

to regulate multiple steps of gene expression. However, because of the large quantity and diverse mechanisms, it is difficult to comprehensively understand the roles of ncRNAs.

NcRNA-based therapeutics have emerged for several diseases, including PH. An effective ncRNA-based strategy demands a thorough understanding of the diverse and context-dependent regulatory relationships of ncRNAs. The regulation of gene expression by ncRNAs is frequently cell specific, suggesting that not only expression level, but also activity or bioavailability contribute to the biofunction of ncRNAs (Correia de Sousa et al., 2019). Thus, in this article, we reviewed the published literature to search for functional miRNAs, lncRNAs, and circRNAs in PH. Next, we constructed networks of validated ncRNAs and their interacting RNAs or genes to investigate the role of ncRNAs in PH.

2 SCREENING OF ARTICLES

2.1 Criteria for Study Selection

A literature search was performed in PubMed with the query listed in **Table 1**; we identified 602 articles. In addition, we also reviewed other public databases, including the Human microRNA Disease Database v3.2, miRWalk 2.0, and LncRNADisease v2.0, to identify validated functional ncRNAs in PH. Studies were selected when the following criteria were met: 1) the study reported pathogenic roles of miRNAs, lncRNAs, and/or circRNAs in PH; 2) mechanistic studies were performed in pulmonary arterial smooth muscle cells (PASMCs), pulmonary artery endothelial cells (PAECs), and/or pulmonary artery fibroblasts (PAFs); and 3) the relationships between ncRNAs and their interacting RNAs or genes were experimentally identified via luciferase reporter assay, western blot, and/or qPCR. Using these criteria returned 140 qualified articles (**Figure 1**).

2.2 General Characteristics of Qualified Articles

When sorted by publication date, we found that the number of eligible articles continuously increased year by year (**Figure 2A**). The impact factors (IF) of the articles ranged from 0 to 36.13; articles with $3 \leq IF < 5$ accounted for the highest proportion (**Figure 2B**). Of the 140 qualified articles, 32.14% were studies using human tissues or cells. In studies using experimental animals, rats were the most commonly used, accounting for 26.43% of the total studies (**Figure 2C**). Moreover, when classified by cell type, 78.42, 15.83, 1.44, and 4.32% of studies were performed in PASMCs, PAECs, PAFs, and both PASMCs and PAECs, respectively (**Figure 2D**).

3 NON-CODING RNA NETWORKS FOR PULMONARY HYPERTENSION

3.1 Construction of Non-coding RNA Regulatory Networks

Regulatory networks were constructed using ncRNAs and their interacting RNAs or genes in PASMCs, PAECs, and PAFs. Given ncRNA conservation among species, only human ncRNAs or ncRNAs that were conserved between human and experimental animals were included. If there were contradictory results, the results from higher-impact articles were selected. In addition, some crucial regulatory relationships between protein-coding genes and validated transcription factor–miRNA interactions from TransmiR v2.0 were also described in the networks to present an in-depth explanation on the roles of ncRNAs in PH. The nodes represented interacting molecules, and the edges represented the regulatory connections. Each edge indicated a publication supporting the connection. Square and

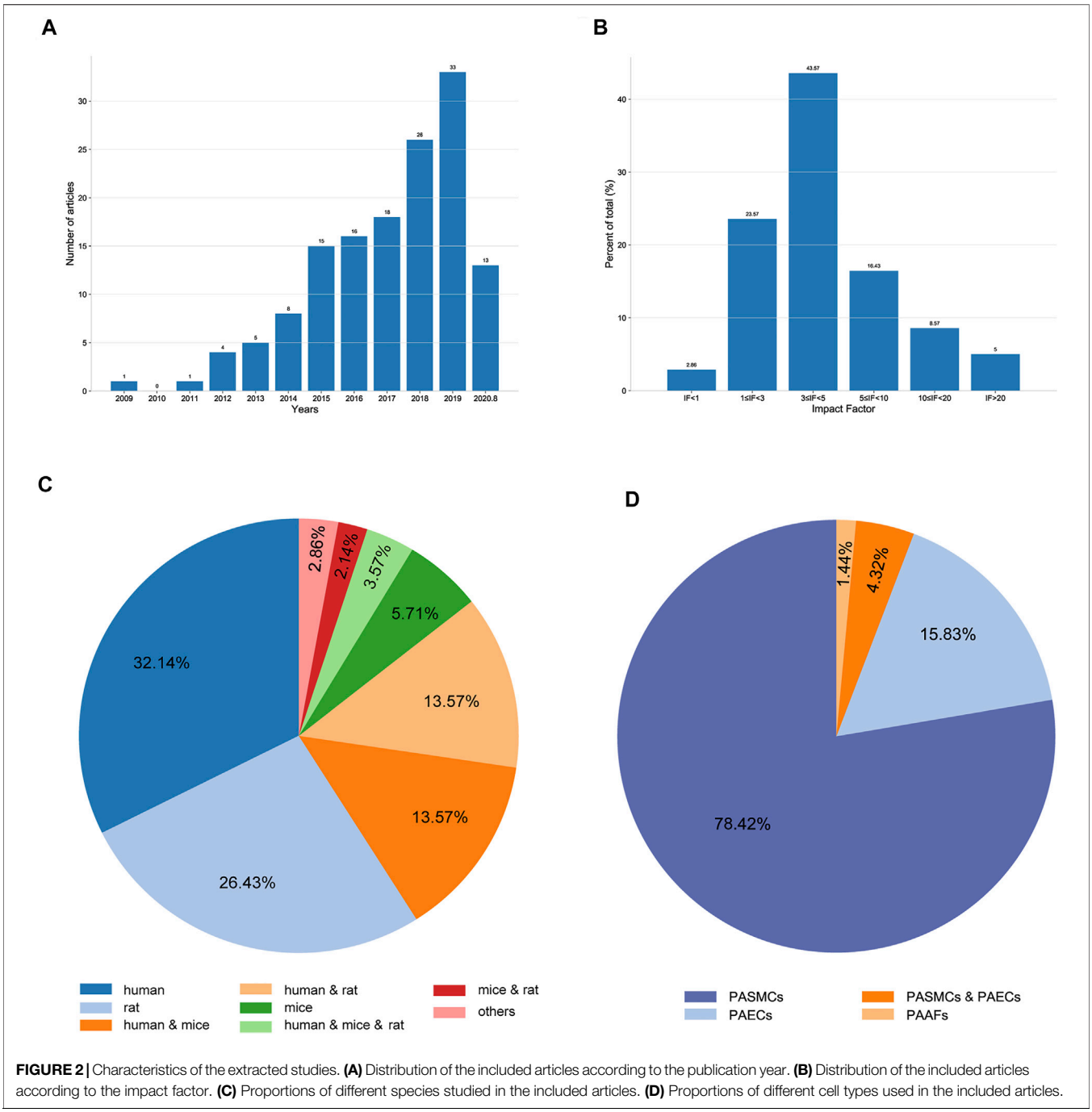

FIGURE 2 | Characteristics of the extracted studies. **(A)** Distribution of the included articles according to the publication year. **(B)** Distribution of the included articles according to the impact factor. **(C)** Proportions of different species studied in the included articles. **(D)** Proportions of different cell types used in the included articles.

circular nodes represented ncRNAs and coding RNAs or genes, respectively. Node color was based on the type of molecule (lncRNAs and circRNAs are orange, miRNAs are blue, and coding RNAs or genes are empty). Node sizes represented their degrees (number of edges that directly link to the node). Edges represented the regulatory connections: red edges depicted links indicating repressive action (semicircular arrow heads), and black edges indicated activation (traditional arrow heads). The nodes in this network were involved in cell proliferation, apoptosis, migration, metabolism, endothelial–mesenchymal transition, and extracellular matrix remodeling. The steps used in our approach are shown in **Figure 1**.

3.2 General Characteristics of the Constructed Networks

In total, 140 articles describing 14 lncRNAs, 1 circRNA, 74 miRNAs, and 110 mRNAs, were included in our networks. Considering the unique biological characteristics of different cell types, we constructed networks according to cell type. The

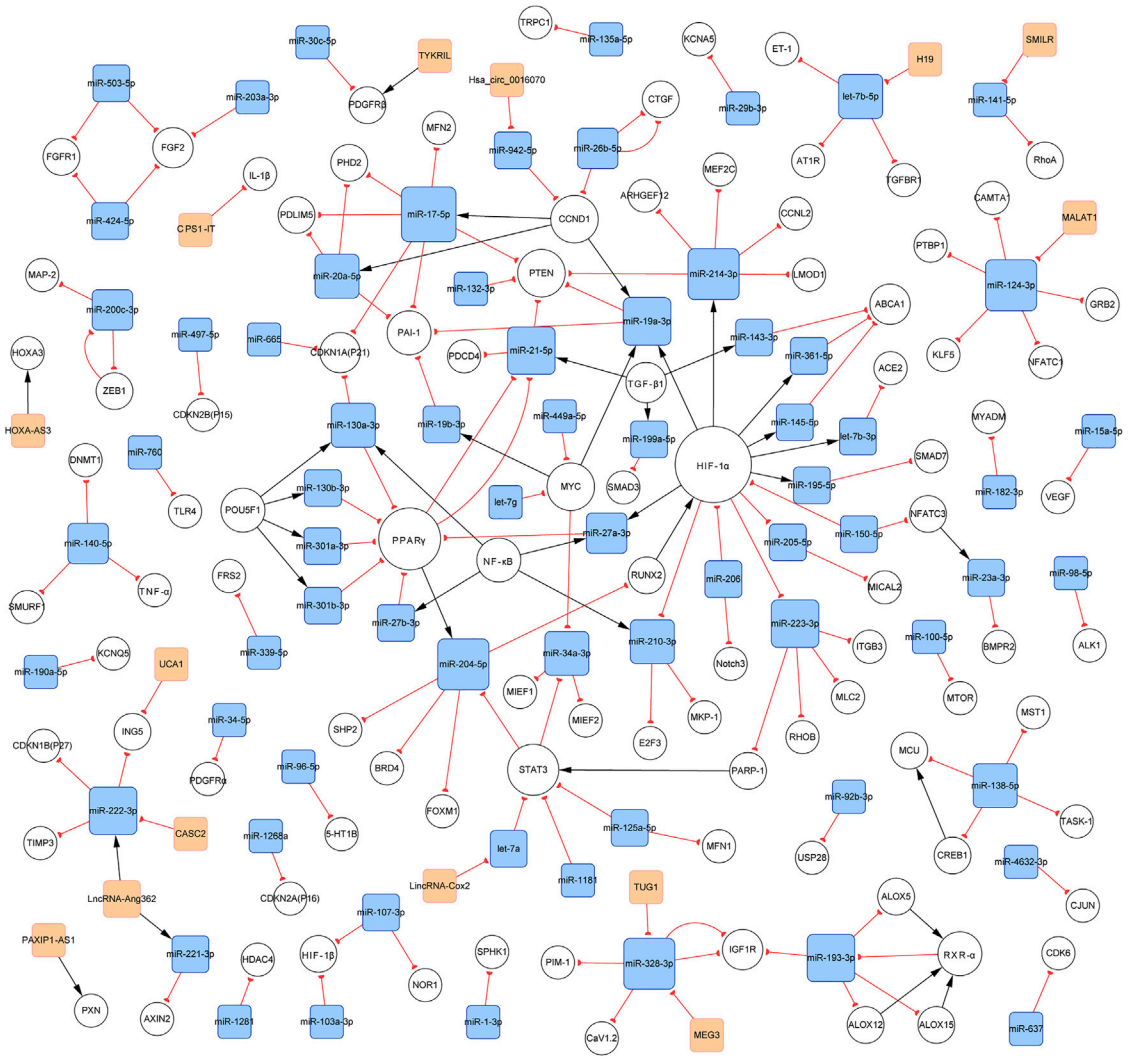

FIGURE 3 | PH-associated network of ncRNAs and their interacting RNAs or genes in PASMCs. The square and circular nodes represent ncRNAs and coding RNAs or genes, respectively. Node color is based on the type of molecule (lncRNAs and circRNAs are orange, miRNAs are blue, and coding RNAs or genes are empty). Node sizes represent the degrees (the number of edges that directly link to the node). Edges represent the regulatory connections, and each edge indicates a publication. When multiple publications describe one interaction, multiple edges connect the same two nodes. Red edges depict links indicating repressive action (semicircular arrow heads), and black edges are those indicating activation (with traditional arrow heads). The nodes in this network are involved in cell proliferation, apoptosis, migration, and metabolism. PH: pulmonary hypertension; PASMCs: pulmonary artery smooth muscle cells; lncRNAs: long non-coding RNAs; circRNA: circular RNA; miRNA: microRNA.

network of PASMCs contained 13 lncRNAs, 1 circRNA, 69 miRNAs, and 96 mRNAs. The network of PAECs contained 1 lncRNA, 25 miRNAs, and 29 mRNAs. The network of PAFs contained 6 miRNAs and four mRNAs. The networks are shown in **Figures 3–5**. Detailed network compositions are listed in **Table 2**.

3.3 Functional Enrichment Analysis

We performed gene ontology (GO) biological process term analyses and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using the database for Annotation, Visualization, and Integration Discovery (DAVID). The calculation process was dependent on a hypergeometric test, using a statistical significance threshold of $p < 0.05$ with a false discovery rate (FDR) correction. These analyses provided a general overview of the biological roles of the included ncRNAs. In addition, we performed cell type-specific functional enrichment analyses. However, owing to the lack of sufficient molecules, the enrichment analysis in PAFs could not be conducted. GO analysis and KEGG pathway enrichment in PASMCs and PAECs revealed several PH-associated terms, such as positive regulation of smooth muscle cell proliferation, positive regulation of endothelial cell proliferation, HIF-1 signaling pathway, and MAPK signaling pathway. The top 10 enriched GO biological process terms and KEGG pathways are shown in **Figure 6**.

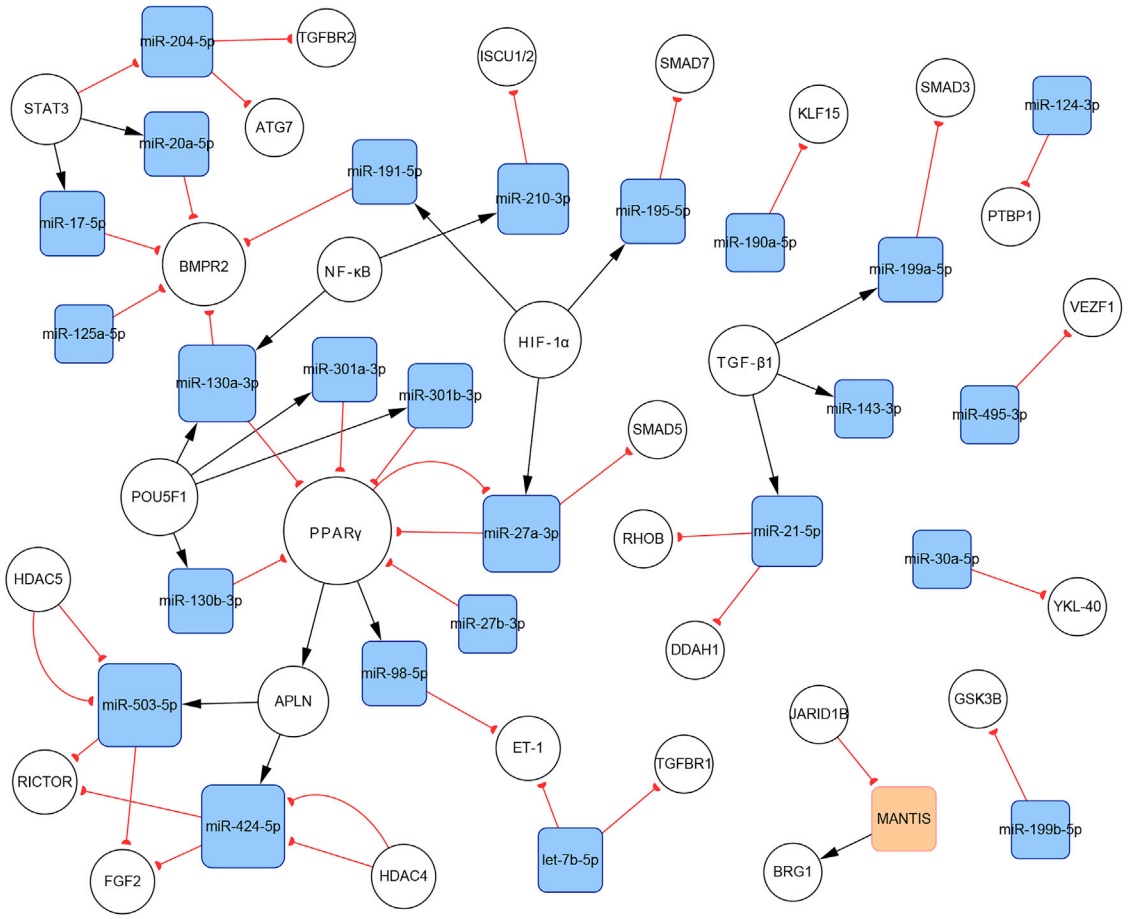

FIGURE 4 | The PH-associated network of ncRNAs and their interacting RNAs or genes in PAECs. The square and circular nodes represent ncRNAs and coding RNAs or genes, respectively. Node color is based on the type of molecule (lncRNAs are orange, miRNAs are blue, and coding RNAs or genes are empty). Node sizes represent the degrees (number of edges that directly link to the node). Edges represent regulatory connections. Each edge indicates a publication. When multiple publications describe one interaction, multiple edges connect the same two nodes. Red edges depict links indicating repressive action (semicircular arrow heads), and black edges represent those indicating activation (traditional arrow heads). The nodes in this network were primarily involved in proliferation, apoptosis resistance, migration, and endothelial–mesenchymal transition. PAECs: pulmonary artery endothelial cells.

3.4 Key Non-coding RNA Subnetworks

We built three networks according to the cell types. Here, we discuss several important subnetworks, along with their components and interactions, to improve understanding of the roles of ncRNAs in PH. Subnetworks with more than five nodes were regarded as key subnetworks.

3.4.1 The Hsa_circ_0016070/miR-942-5p/CCND1 Subnetwork

CircRNAs are associated with various cardiovascular diseases. Hsa_circ_0016070 was the only circRNA included in our networks. This circRNA is located at chr1: 203595914-203702528, strand: +, promotes cell proliferation by mediating cell cycle progression, and is increased in PH patients (Zhou et al., 2019). CCND1 is an important regulator of the cell cycle. It interacts with cyclin-dependent kinase 4 (CDK4) to form the cyclin D1–CKD4 complex, which then inactivates retinoblastoma (Rb) protein and induces G0 progression to S phase (Matsushime et al., 1991). The subnetwork showed that hsa_circ_0016070 overexpression induced CCND1 expression by buffering miR-942-5p (Zhou et al., 2019). In addition, according to our network, CCND1 could induce the expression of miR-17-5p, miR-19a-3p, and miR-20a-5p, subsequently regulating the biological activities of PASMCs (**Figure 7A**). Given the considerable number of identified circRNAs, there should be other PH-related circRNAs. A microarray expression profile in thromboembolic pulmonary hypertension patients indicated that hsa_circ_0002062 and hsa_circ_0022342 might be the key circRNAs for the development of chronic thromboembolic pulmonary hypertension (Miao et al., 2017). However, this finding has not been verified by more reliable experimental methods.

3.4.2 The TUG1/MEG3/miR-328-3p/miR-193-3p Subnetwork

Regulatory relationships are indicated by the connection lines in the subnetwork. According to this subnetwork (**Figure 7B**), both

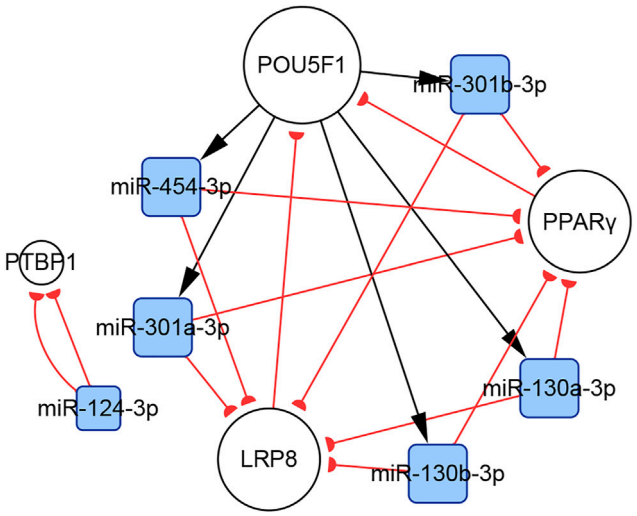

FIGURE 5 | The PH-associated network of ncRNAs and their interacting RNAs or genes in PAFs. The square and circular nodes represent ncRNAs and coding RNAs or genes, respectively. Node color is based on the type of molecule (miRNAs are blue, coding RNAs or genes are empty). Node sizes represent the degrees (number of edges that directly link to the node). Edges represent regulatory connections. Each edge indicates a publication. When multiple publications describe one interaction, multiple edges connect the same two nodes. Red edges depict links indicating repressive action (semicircular arrow heads), and black edges are those indicating activation (traditional arrow heads). The nodes in this network were primarily involved in cell proliferation and extracellular matrix remodeling. PAFs: pulmonary artery fibroblasts.

TUG1 and MEG3 can function as competing endogenous RNAs (ceRNAs) that sequester miR-328-3p. In the original studies, the TUG1/miR-328-3p and MEG3/miR-328-3p axes were identified (Wang D et al., 2019; Xing X.-Q et al., 2019). IGF1 is reported to inhibit PASMCs apoptosis and activate elastin in PASMCs. Thus, upregulating IGF1R via the TUG1/miR-328-3p and MEG3/miR-328-3p axes can induce PH by amplifying the pathogenic role of IGF1 (Wang S et al., 2019; Xing Y et al., 2019). Calcium voltage-gated channel subunit alpha1 C (CaV1.2), which contributes to vasoconstriction, is also a target gene of miR-328-3p in PASMCs (Guo et al., 2012), indicating that the TUG1/miR-328-3p and MEG3/miR-328-3p axes are involved in regulating pulmonary artery contraction and dilation. In addition, miR-328-3p can inhibit PASMC proliferation by targeting PIM-1 (Qian et al., 2016). Available data show that miR-193-3p has a shared target gene, IGF1R, with miR-328-3p, but no strong regulatory connection with miR-328-3p or TUG1 or MEG3. Thus, downregulation of miR-193-3p contributes to IGF1R overexpression as well. In addition, miR-193-3p is capable of negatively regulating multiple lipoxygenases, including ALOX5, ALOX12, and ALOX15. These lipoxygenases cause abnormal lipid metabolism, which not only directly accelerates the development of PH, but also induces the increasement of RXR-α. Moreover, miR-193-3p can be downregulated by RXR-α, which directly binds to the miR-193 promoter. Therefore, a feedback loop, which dramatically enhances abnormal miR-193-3p expression forms (Sharma et al., 2014).

3.4.3 The CASC2/UCA1/miR-222-3p Subnetwork

LncRNA CASC2 is downregulated in hypoxia-induced PASMCs. As a ceRNA of miR-222-3p, CASC2 reduces the expression of ING5, which is a target gene of miR-222-3p, ultimately promoting PASMC proliferation and migration (Han et al., 2020). P27 and TIMP3 are two additional target genes of miR-222-3p (Xu et al., 2017). P27, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, negatively regulates cell proliferation (Toyoshima and Hunter, 1994). Meanwhile, TIMP3 is a member of the TIMP family, which regulates cell proliferation, apoptosis, and migration via both MMP-dependent or MMP-independent pathways (Zhou et al., 2015). The present subnetwork links CACS2 to P27 and TIMP3 via miR-222-3p, further elaborating the mechanisms of PH (**Figure 7C**).

UCA1 is the other lncRNA in this subnetwork and is highly expressed in hypoxia-induced PASMCs. Studies indicate that UCA1 does not interact with miR-222-3p, but directly inhibits ING5 by competing with ING5 mRNA for hnRNP I, which binds to ING5 mRNA and enhances its translation. Thus, UCA1 overexpression results in the downregulation of ING5 mRNA expression (Zhu T.-T. et al., 2019). The same regulatory pattern has been found between UCA1 and P27 in breast tumor studies (Huang et al., 2014). This interaction may also work in PH and partly contributes to P27 downregulation (**Figure 7C**).

3.4.4 The MALAT1/miR-124-3p Subnetwork

LncRNA MALAT1, located at 11q13, is an 8.5-kb molecule that was identified by Ji et al. in a cancer study (Ji et al., 2003). Emerging evidence indicates that MALAT1 plays important roles in various diseases, including PH. Wang et al. reported that MALAT1 is highly expressed in pulmonary artery tissues and PASMCs from patients with PH. MALAT1 controls PASMC proliferation and migration by binding to miR-124-3p, which directly targets KLF5 (Wang D et al., 2019). Kang et al. showed that miR-124-3p also targets three regulators of the NFAT pathway, including NFATc1, CAMTA1, and PTBP1 (Kang B.-Y et al., 2013). The downregulation of miR-124-3p induces PASMC proliferation and reverses the differentiated PASMC phenotype by activating the NFAT pathway. In addition to its role in PASMCs, miR-124-3p also regulates the biological behaviors of PAH endothelial cells (PAH ECs) and PAFs. Studies have confirmed the role of the miR-124-3p/PTBP1 axis in PAH ECs and PAFs (Caruso et al., 2017; Wang et al., 2014; Zhang H et al., 2017). Downregulating miR-124-3p activates PTBP1 expression, which promotes aerobic glycolysis by increasing the PKM2/PKM1 ratio, subsequently inducing PAH EC and PAF proliferation (Anastasiou et al., 2012). Li et al. reported another target of miR-124-3p, GRB2, which enhanced the proliferation of multiple human cells (Li L et al., 2017; **Figure 7D**).

3.4.5 Subnetworks of the miR-130/301 Family

There are complicated relationships between the miR-130/301 family and other functional molecules associated with the pathogenesis of PH. In the present study, we found that

TABLE 2 | List of network interactions.

Upstream molecule	Downstream molecule	Interaction type[a]	PMID	Reference
ALOX12	RXR-α	pos	24963038	Sharma et al. (2014)
ALOX15	RXR-α	pos	24963038	Sharma et al. (2014)
ALOX5	RXR-α	pos	24963038	Sharma et al. (2014)
APLN	miR-424-5p	pos	23263626	Kim et al. (2013)
APLN	miR-503-5p	pos	23263626	Kim et al. (2013)
CASC2	miR-222-3p	neg	32206065	Han et al. (2020)
CCND1	miR-17-5p	pos	18695042	Yu et al. (2008)
CCND1	miR-19a-3p	pos	28090171	Inoue and Fry. (2015)
CCND1	miR-20a-5p	Pos	28090171	Inoue and Fry. (2015)
CPS1-IT	IL-1β	neg	30982984	Zhang et al. (2019b)
CREB1	MCU	pos	27648837	Hong et al. (2017)
H19	let-7b-5p	neg	30547791	Su et al. (2018)
HDAC4	miR-424-5p	neg	29102771	Takagi et al. (2018)
HDAC4	miR-503-5p	neg	29102771	Takagi et al. (2018)
HDAC5	miR-424-5p	neg	29102771	Takagi et al. (2018)
HDAC5	miR-503-5p	neg	29102771	Takagi et al. (2018)
HIF-1α	let-7b-3p	pos	30628484	Zhang H et al. (2019)
HIF-1α	miR-145-5p	pos	25129238	Agrawal et al. (2014)
HIF-1α	miR-191-5p	pos	25119596	Song et al. (2014)
HIF-1α	miR-195-5p	pos	28862358	Zeng et al. (2018)
HIF-1α	miR-19a-3p	pos	31682848	Zhao et al. (2019)
HIF-1α	miR-205-5p	pos	23924028	Gandellini et al. (2014)
HIF-1α	miR-210-3p	neg	22886504	Gou et al. (2012)
HIF-1α	miR-214-3p	pos	24011070	el Azzouzi et al. (2013)
HIF-1α	miR-223-3p	neg	26084306	Meloche et al. (2015a)
HIF-1α	miR-27a-3p	pos	24517586	Camps et al. (2014)
HIF-1α	miR-361-5p	pos	29339076	Zhang Y et al. (2018)
HOXA-AS3	HOXA3	pos	30304383	Zhang R et al. (2019)
Hsa_circ_0016070	miR-942-5p	neg	31593832	Zhou et al. (2019)
JARID1B	MANTIS	neg	2,8351900	Leisegang et al. (2017)
let-7a	STAT3	neg	32803651	Cheng et al. (2020)
let-7b-3p	ACE2	neg	30628484	Zhang Y et al. (2019)
let-7b-5p	AT1R	neg	30547791	Su et al. (2018)
let-7b-5p	ET-1	neg	24978044	Guo et al. (2014)
let-7b-5p	TGFBR1	neg	24978044	Guo et al. (2014)
let-7g	MYC	neg	27889560	Zhang W.-F et al. (2017)
LincRNA-Cox2	let-7a	neg	32803651	Cheng et al. (2020)
LncRNA-Ang362	miR-221-3p	pos	31313741	Wang et al. (2020)
LncRNA-Ang362	miR-222-3p	pos	31313741	Wang et al. (2020)
LRP8	POU5F1	neg	26565914	Bertero et al. (2015)
MALAT1	miR-124-3p	neg	31257528	Wang S et al. (2019)
MANTIS	BRG1	pos	2,8351900	Leisegang et al. (2017)
MEG3	miR-328-3p	neg	31477557	Xing X.-Q et al. (2019)
miR-100-5p	MTOR	neg	26409044	Wang et al. (2015)
miR-103a-3p	HIF-1β	neg	26827991	Deng et al. (2016)
miR-107-3p	HIF-1β	neg	26827991	Deng et al. (2016)
miR-107-3p	NOR1	neg	31933977	Chen et al. (2019)
miR-1181	STAT3	neg	30211651	Qian et al. (2018)
miR-124-3p	CAMTA1	neg	23853098	Kang K et al. (2013)
miR-124-3p	GRB2	neg	28496318	Li Y et al. (2017)
miR-124-3p	KLF5	neg	31257528	Wang D et al. (2019)
miR-124-3p	NFATC1	neg	23853098	Kang B.-Y et al. (2013)
miR-124-3p	PTBP1	neg	23853098	Kang K et al. (2013)
miR-124-3p	PTBP1	neg	24122720	Wang et al. (2014)
miR-124-3p	PTBP1	neg	2,8971999	Caruso et al. (2017)
miR-124-3p	PTBP1	neg	2,8972001	Zhang H et al. (2017)
miR-125a-5p	BMPR2	neg	25854878	Huber et al. (2015)
miR-125a-5p	MFN1	neg	28593577	Ma et al. (2017)
miR-125a-5p	STAT3	neg	29700287	Cai et al. (2018)
miR-1268a	CDKN2A(P16)	neg	31370272	Lee and Kang. (2019)
miR-1281	HDAC4	neg	29514810	Li et al. (2018)
miR-130a-3p	BMPR2	neg	28755990	Li L et al. (2017)
miR-130a-3p	CDKN1A(P21)	neg	25681685	Brock et al. (2015)
miR-130a-3p	LRP8	neg	26565914	Bertero et al. (2015)

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TABLE 2 | (*Continued*) List of network interactions.

Upstream molecule	Downstream molecule	Interaction type[a]	PMID	Reference
miR-130a-3p	PPARγ	neg	24960162	Bertero et al. (2014)
miR-130a-3p	PPARγ	neg	26565914	Bertero et al. (2015)
miR-130b-3p	LRP8	neg	26565914	Bertero et al. (2015)
miR-130b-3p	PPARγ	neg	24960162	Bertero et al. (2014)
miR-130b-3p	PPARγ	neg	26565914	Bertero et al. (2015)
miR-132-3p	PTEN	neg	30896881	Zeng et al. (2019)
miR-135a-5p	TRPC1	neg	30038339	Liu A et al. (2019)
miR-138-5p	CREB1	neg	27648837	Hong et al. (2017)
miR-138-5p	MCU	neg	27648837	Hong et al. (2017)
miR-138-5p	MST1	neg	23485012	Li et al. (2013)
miR-138-5p	TASK-1	neg	29257242	Liu G et al. (2018)
miR-1-3p	SPHK1	neg	29167124	Sysol et al. (2018)
miR-140-5p	DNMT1	neg	27021683	Zhang and Xu. (2016)
miR-140-5p	SMURF1	neg	27214554	Rothman et al. (2016)
miR-140-5p	TNF-α	neg	30367500	Zhu et al. (2019b)
miR-143-3p	ABCA1	neg	30195228	Yue et al. (2018)
miR-141-5p	RHOA	neg	32559140	Lei et al. (2020)
miR-145-5p	ABCA1	neg	30195228	Yue et al. (2018)
miR-150-5p	HIF-1α	neg	28715868	Chen M et al. (2017)
miR-150-5p	NFATC3	neg	30551428	Li et al. (2019)
miR-15a-5p	VEGF	neg	31894295	Zhang et al. (2020)
miR-17-5p	BMPR2	neg	19390056	Brock et al. (2009)
miR-17-5p	CDKN1A(P21)	neg	30305109	Liu J. J et al. (2018)
miR-17-5p	MFN2	neg	27640178	Lu et al. (2016)
miR-17-5p	PAI-1	neg	29644896	Chen K.-H et al. (2018)
miR-17-5p	PDLIM5	neg	25647182	Chen et al. (2015)
miR-17-5p	PHD2	neg	27919930	Chen et al. (2016)
miR-17-5p	PTEN	neg	30305109	Liu G et al. (2018)
miR-182-3p	MYADM	neg	32373233	Sun et al. (2020)
miR-190a-5p	KCNQ5	neg	24446351	Li et al. (2014)
miR-190a-5p	KLF15	neg	30538440	Jiang et al. (2018)
miR-191-5p	BMPR2	neg	31119161	Zhang Z et al. (2019)
miR-193-3p	ALOX12	neg	24963038	Sharma et al. (2014)
miR-193-3p	ALOX15	neg	24963038	Sharma et al. (2014)
miR-193-3p	ALOX5	neg	24963038	Sharma et al. (2014)
miR-193-3p	IGF1R	neg	24963038	Sharma et al. (2014)
miR-195-5p	SMAD7	neg	28862358	Zeng et al. (2018)
miR-199a-5p	SMAD3	neg	27038547	Liu H et al. (2016)
miR-199b-5p	GSK3B	neg	27188753	Wu et al. (2016)
miR-19a-3p	PAI-1	neg	29644896	Chen T et al. (2018)
miR-19a-3p	PTEN	neg	31682848	Zhao et al. (2019)
miR-19b-3p	PAI-1	neg	29644896	Chen K.-H et al. (2018)
miR-200c-3p	MAP2	neg	29044995	Yuan et al. (2017)
miR-200c-3p	ZEB1	neg	29044995	Yuan et al. (2017)
miR-203a-3p	FGF2	neg	30575929	Wang et al. (2018)
miR-204-5p	ATG7	neg	31542480	Liu H.-M et al. (2019)
miR-204-5p	BRD4	neg	26224795	Meloche et al. (2015a)
miR-204-5p	FOXM1	neg	29290032	Bourgeois et al. (2018b)
miR-204-5p	RUNX2	neg	27149112	Ruffenach et al. (2016)
miR-204-5p	SHP2	neg	21321078	Courboulin et al. (2011)
miR-204-5p	TGFBR2	neg	29196166	Yu et al. (2018)
miR-205-5p	MICAL2	neg	30853343	Tao et al. (2019)
miR-206	Notch3	neg	23071643	Jalali et al. (2012)
miR-206	HIF-1α	neg	23628900	Yue et al. (2013)
miR-20a-5p	BMPR2	neg	19390056	Brock et al. (2009)
miR-20a-5p	PAI-1	neg	29644896	Chen T et al. (2018)
miR-20a-5p	PDLIM5	neg	25647182	Chen et al. (2015)
miR-20a-5p	PHD2	neg	27919930	Chen et al. (2016)
miR-210-3p	E2F3	neg	22886504	Gou et al. (2012)
miR-210-3p	ISCU1/2	neg	25825391	White et al. (2015)
miR-210-3p	MKP-1	neg	25044272	Jin et al. (2015)
miR-214-3p	ARHGEF12	neg	31373336	Xing Y et al. (2019)
miR-214-3p	CCNL2	neg	27381447	Liu Y et al. (2016)
miR-214-3p	LMOD1	neg	27144530	Sahoo et al. (2016)

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TABLE 2 | (*Continued*) List of network interactions.

Upstream molecule	Downstream molecule	Interaction type[a]	PMID	Reference
miR-214-3p	MEF2C	neg	27144530	Sahoo et al. (2016)
miR-214-3p	PTEN	neg	28684904	Liu et al. (2017)
miR-21-5p	DDAH1	neg	24895913	Iannone et al. (2014)
miR-21-5p	PDCD4	neg	28522568	Green et al. (2017)
miR-21-5p	PTEN	neg	26208095	Green et al. (2015)
miR-21-5p	RHOB	neg	22371328	Parikh et al. (2012)
miR-221-3p	AXIN2	neg	28694128	Nie et al. (2019)
miR-222-3p	ING5	neg	32206065	Han et al. (2020)
miR-222-3p	CDKN1B(P27)	neg	28854428	Xu et al. (2017)
miR-222-3p	TIMP3	neg	28854428	Xu et al. (2017)
miR-223-3p	ITGB3	neg	30507047	Liu et al. (2019a)
miR-223-3p	MLC2	neg	27121304	Zeng et al. (2016)
miR-223-3p	PARP1	neg	26084306	Meloche et al. (2015b)
miR-223-3p	RHOB	neg	27121304	Zeng et al. (2016)
miR-23a-3p	BMPR2	neg	29864909	Zhang X et al. (2018)
miR-26b-5p	CCND1	neg	2,7322082	Wang P et al. (2016)
miR-26b-5p	CTGF	neg	2,7322082	Wang R et al. (2016)
miR-26b-5p	CTGF	neg	28816418	Zhou et al. (2018)
miR-27a-3p	PPARγ	neg	24244514	Kang B.-Y et al. (2013)
miR-27a-3p	PPARγ	neg	28484848	Xie et al. (2017)
miR-27a-3p	SMAD5	neg	31004656	Liu et al. (2019b)
miR-27b-3p	PPARγ	neg	25795136	Bi et al. (2015)
miR-27b-3p	PPARγ	neg	28484848	Xie et al. (2017)
miR-29b-3p	KCNA5	neg	31553627	Babicheva et al. (2020)
miR-301a-3p	LRP8	neg	26565914	Bertero et al. (2015)
miR-301a-3p	PPARγ	neg	24960162	Bertero et al. (2014)
miR-301a-3p	PPARγ	neg	26565914	Bertero et al. (2015)
miR-301b-3p	LRP8	neg	26565914	Bertero et al. (2015)
miR-301b-3p	PPARγ	neg	24960162	Bertero et al. (2014)
miR-301b-3p	PPARγ	neg	26565914	Bertero et al. (2015)
miR-30a-5p	YKL-40	neg	31115541	Tan et al. (2019)
miR-30c-5p	PDGFRβ	neg	25882492	Xing et al. (2015)
miR-328-3p	CaV1.2	neg	22392900	Guo et al. (2012)
miR-328-3p	IGF1R	neg	22392900	Guo et al. (2012)
miR-328-3p	IGF1R	neg	31477557	Xing X.-Q et al. (2019)
miR-328-3p	PIM-1	neg	27448984	Qian et al. (2016)
miR-339-5p	FRS2	neg	28947594	Chen J et al. (2017)
miR-34-5p	PDGFRα	neg	27302634	Wang P et al. (2016)
miR-34a-3p	MIEF1	neg	29431643	Chen K.-H et al. (2018)
miR-34a-3p	MIEF2	neg	29431643	Chen T et al. (2018)
miR-361-5p	ABCA1	neg	29339076	Zhang Y et al. (2018)
miR-424-5p	FGF2	neg	23263626	Kim et al. (2013)
miR-424-5p	FGF2	neg	24960162	Bertero et al. (2014)
miR-424-5p	FGFR1	neg	23263626	Kim et al. (2013)
miR-424-5p	RICTOR	neg	29102771	Takagi et al. (2018)
miR-449a-5p	MYC	neg	30715622	Zhang et al. (2019a)
miR-454-3p	LRP8	neg	26565914	Bertero et al. (2015)
miR-454-3p	PPARγ	neg	26565914	Bertero et al. (2015)
miR-4632-3p	CJUN	neg	28701355	Qian et al. (2017)
miR-495-3p	VEZF1	neg	31030195	Fu et al. (2019)
miR-497-5p	CDKN2B(P15)	neg	31370272	Lee and Kang. (2019)
miR-503-5p	FGF2	neg	23263626	Kim et al. (2013)
miR-503-5p	FGF2	neg	24960162	Bertero et al. (2014)
miR-503-5p	FGFR1	neg	23263626	Kim et al. (2013)
miR-503-5p	RICTOR	neg	29102771	Takagi et al. (2018)
miR-637	CDK6	neg	27794186	Sang et al. (2016)
miR-665	CDKN1A(P21)	neg	31370272	Lee and Kang. (2019)
miR-760	TLR4	neg	30226538	Yang et al. (2018)
miR-92b-3p	USP28	neg	30149918	Hao et al. (2018)
miR-942-5p	CCND1	neg	31593832	Zhou et al. (2019)
miR-96-5p	5-HT1B	neg	25871906	Wallace et al. (2015)
miR-98-5p	ALK1	neg	31322216	Li et al. (2019)
miR-98-5p	ET-1	neg	26098770	Kang et al. (2016)
MYC	miR-19a-3p	pos	17943719	Schulte et al. (2008)

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TABLE 2 | (*Continued*) List of network interactions.

Upstream molecule	Downstream molecule	Interaction type[a]	PMID	Reference
MYC	miR-19b-3p	pos	17943719	Schulte et al. (2008)
MYC	miR-34a-3p	neg	18066065	Chang et al. (2008)
NFATC3	miR-23a-3p	pos	19574461	Lin et al. (2009)
NF-κB	miR-130a-3p	pos	28755990	Li Q et al. (2017)
NF-κB	miR-210-3p	pos	25341039	Liu et al. (2014)
NF-κB	miR-27a-3p	pos	28484848	Xie et al. (2017)
NF-κB	miR-27b-3p	pos	28484848	Xie et al. (2017)
PARP-1	STAT3	pos	24270264	Meloche et al. (2014)
PAXIP1-AS1	PXN	pos	30450722	Jandl et al. (2019)
POU5F1	miR-130a-3p	pos	24960162	Bertero et al. (2014)
POU5F1	miR-130a-3p	pos	26565914	Bertero et al. (2015)
POU5F1	miR-130b-3p	pos	24960162	Bertero et al. (2014)
POU5F1	miR-130b-3p	pos	26565914	Bertero et al. (2015)
POU5F1	miR-301a-3p	pos	24960162	Bertero et al. (2014)
POU5F1	miR-301a-3p	pos	26565914	Bertero et al. (2015)
POU5F1	miR-301b-3p	pos	24960162	Bertero et al. (2014)
POU5F1	miR-301b-3p	pos	26565914	Bertero et al. (2015)
POU5F1	miR-454-3p	pos	26565914	Bertero et al. (2015)
PPARγ	APLN	pos	24960162	Bertero et al. (2014)
PPARγ	miR-204-5p	pos	24960162	Bertero et al. (2014)
PPARγ	miR-21-5p	neg	26208095	Green et al. (2015)
PPARγ	miR-21-5p	neg	28522568	Green et al. (2017)
PPARγ	miR-27a-3p	neg	24244514	Kang K et al. (2013)
PPARγ	miR-98-5p	pos	26098770	Kang et al. (2016)
PPARγ	POU5F1	neg	26565914	Bertero et al. (2015)
RUNX2	HIF-1α	pos	27149112	Ruffenach et al. (2016)
RXR-α	miR-193-3p	neg	24963038	Sharma et al. (2014)
SMILR	miR-141-5p	neg	32559140	Lei et al. (2020)
STAT3	miR-17-5p	pos	19390056	Brock et al. (2009)
STAT3	miR-204-5p	neg	23975026	Xu et al. (2013)
STAT3	miR-20a-5p	pos	19390056	Brock et al. (2009)
STAT3	miR-34a-3p	neg	24642471	Rokavec et al. (2014)
TGF-β1	miR-143-3p	pos	2,6311719	Deng et al. (2015)
TGF-β1	miR-199a-5p	pos	20705240	Davis et al. (2010)
TGF-β1	miR-21-5p	pos	20705240	Davis et al. (2010)
TUG1	miR-328-3p	neg	31679623	Wang D et al. (2019)
TYKRIL	PDGFRβ	pos	32634060	Zehendner et al. (2020)
UCA1	ING5	neg	30353369	Zhu et al. (2019a)
ZEB1	miR-200c-3p	neg	18829540	Bracken et al. (2008)

[a]*pos: positive interaction, neg: negative interaction.*

subnetworks of the miR-130/301 family were involved in multiple biological behaviors, such as proliferation, apoptosis, and migration in PASMCs, PAECs, and PAFs. In addition, these subnetworks also mediated the crosstalk of these pulmonary artery cells.

In PASMCs, the miR-130/301 family is involved in many regulatory axes. Among them, the POU5F1/miR-130/301 family/PPARγ axis, which regulates the expression of miR-204-5p and miR-21-5p, is the most explicitly elaborated axis. According to our studies, the identified target genes of the two miRNAs in PASMCs include BRD4, FOXM1, PSCD4, PTEN, RUNX2, and SHP2, which control cell proliferation, apoptosis, differentiation, and mitochondrial function (Courboulin et al., 2011; Meloche et al., 2015a; Green et al., 2015, 2017; Ruffenach et al., 2016; Liu et al., 2017; Bourgeois et al., 2018a). In addition to the miR-130/301 family, miR-27a/b-3p, which is regulated by NF-κB (Xie et al., 2017), can also act as an upstream controller of PPARγ in PASMCs. Interestingly, the subnetwork analysis indicates that the miR-130/301 family indirectly promotes HIF-1α expression by sustaining the RUNX2 level (Ruffenach et al., 2016). Conversely, HIF-1α induces the expression of miR-27a-3p, which depresses the level of PPARγ (Camps et al., 2014). Thus, a feedback loop with PPARγ and HIF-1α forms. This loop leads to a persistent pathological status. Moreover, as a crucial pathogenic molecule for PH, HIF-1α can function through several miRNAs, including miR-145-5p, miR-19a-3p, miR-195-5p, miR-210-3p, miR-223-3p, and miR-361-5p, to regulate the expression of downstream proteins, eventually causing abnormal cellular behaviors (Agrawal et al., 2014; Gou et al., 2012; Meloche et al., 2015b; Zeng et al., 2018; Zhang X et al., 2018, Zhang H et al., 2019; Zhao et al., 2019; **Figure 8A**).

In PAECs, the miR-130/301 family also plays an important role. The POU5F1/miR-130/301 family/PPARγ axis indirectly regulates the expression of ET-1 and FGF2 via miR-98-5p and

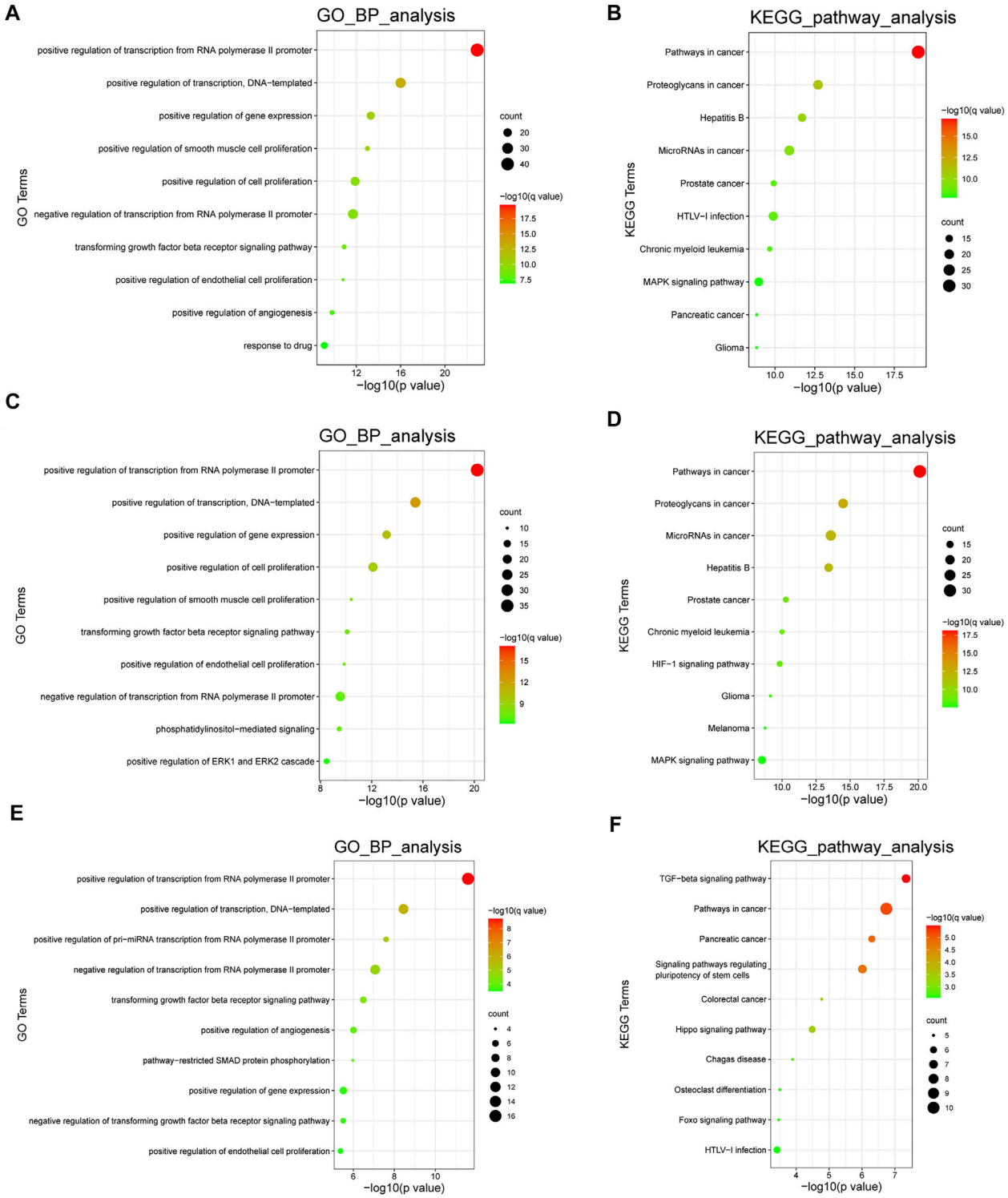

FIGURE 6 | Functional analyses of the ncRNAs. **(A,B)**. The top 10 enriched GO biological process terms and KEGG pathways in all cell types. **(C,D)** The top 10 enriched GO biological process terms and KEGG pathways in PASMCs. **(E,F)** The top 10 enriched GO biological process terms and KEGG pathways in PAECs. Node sizes indicate the number of genes enriched in functional clusters. Node colors are related to q values. GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

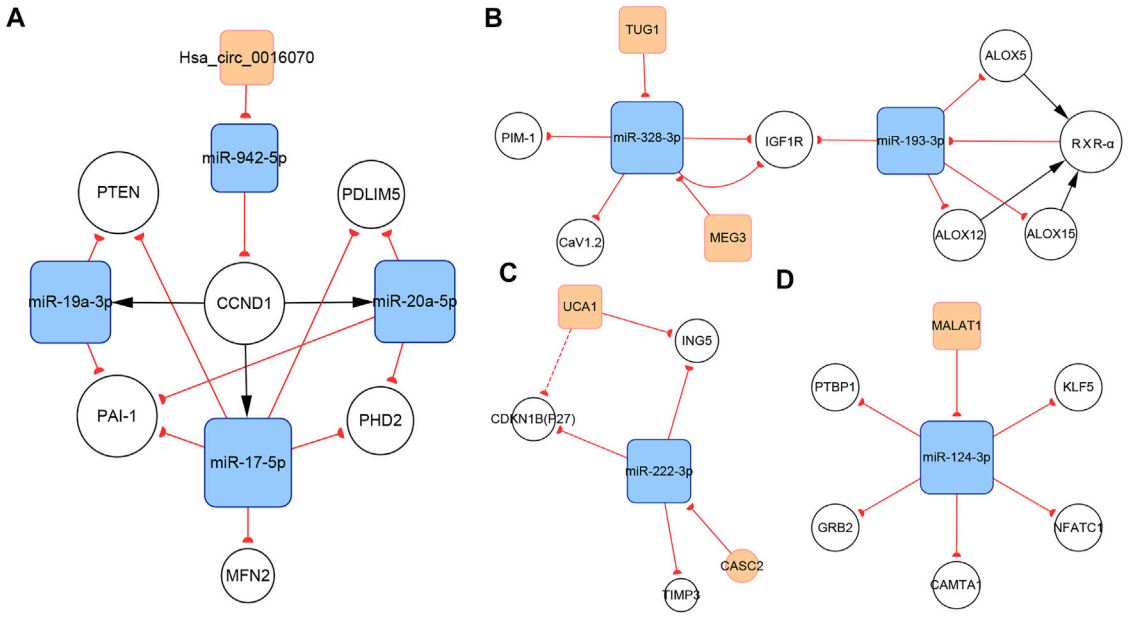

FIGURE 7 | Several key ncRNA subnetworks. **(A)** The hsa_circ_0016070/miR-942-5p/CCND1 subnetwork. **(B)** The TUG1/MEG3/miR-328-3p/miR-193-3p subnetwork. **(C)** The CASC2/UCA1/miR-222-3p subnetwork. **(D)** The MALAT1/miR-124-3p subnetwork.

miR-424/503-5p, respectively (Kim et al., 2013; Zhang Y et al., 2018). The roles of ET-1 and FGF2 in PH are well established. ET-1 is synthesized primarily in endothelial cells and mediates pulmonary artery cell proliferation, migration, and constriction through two distinct G protein-coupled receptors: ETA and ETB (Clozel, 2016). Previous studies suggest that excessive FGF2 expression promotes PAEC proliferation by activating ERK1/2 and inhibits apoptosis by inducing BCL2 and BCL-xL activity (Tu et al., 2011). Furthermore, miR-130a-3p controls the level of BMPR2, which triggers idiopathic pulmonary artery hypertension (IPAH) and is involved in the development of other types of PH (Li Q et al., 2017). Considering that miRNAs from the same family have a homologous seed region sequence, other members from the miR-130/301 family may also regulate BMPR2 expression. The transcription of miR-130a-3p is controlled by NF-κB in PAECs. Thus, NF-κB and BMPR2 are linked by miR-130a-3p. In addition, miR-17a-5p, miR-20a-5p, and miR-125a-5p also mediate BMPR2 expression. Besides, two members from the miR-17-92 family, miR-17a-5p and miR-20a-5p, link STAT3 to BMPR2 (Brock et al., 2009; Huber et al., 2015; **Figure 8B**).

In PAFs, activation of the miR-130/301 family can induce cell proliferation and extracellular matrix remodeling by inhibiting PPARγ and LRP8. Meanwhile, matrix remodeling can activate POU5F1, which subsequently promotes miR-130/301 family expression (Bertero et al., 2015). Thus, a positive feedback circuit is activated that dramatically accelerates the development of PH (**Figure 8C**).

The roles of the miR-130/301 family in different pulmonary artery cell types are not independent. Rather, the miR-130/301 family contributes to crosstalk between these cells. Extracellular matrix remodeling, which can be induced by overexpression of the miR-130/301 family, promotes proliferation and contraction of pulmonary artery cells viamiR-130/301 family-dependent and -independent pathways. The remodeled extracellular matrix can activate the POU5F1/miR-130/301 family/PPARγ axis in PASMCs, PAECs, and PAFs, subsequently regulating downstream molecules such as miR-204-5p, miR-424-5p, miR-503-5p, and FGF2 (Bertero et al., 2015). Upregulating miR-424-5p and miR-503-5p or inhibiting FGF2 in PAECs can repress PASMC and PAF proliferation induced by conditioned media from PAECs, indicating that these molecules are involved in the crosstalk among different pulmonary vascular cells. The remodeled extracellular matrix can also induce the expression of the proliferative miRNA, miR-27a/b-3p, in PACEs, and PASMCs, as well as the expression of the vasoconstrictor ET-1, and the inflammatory cytokine IL-6 in PACEs (Bertero et al., 2014; Bertero et al., 2015; **Figure 9**).

4 ENVIRONMENTAL FACTORS IN PULMONARY HYPERTENSION

Noncoding RNA interference is an important epigenetic mechanism. Recent evidence has identified the roles of epigenetic changes in the development of PH. These changes link the pathogenic genes of PH and environmental factors such as hypoxia, virus infection, and air pollution (Gamen et al., 2016). For example, BMPR2 is a transmembrane serine/threonine kinase receptor, which is essential for vascular homeostasis. Although mutations in the BMPR2 gene account for a considerable portion of patients with familial pulmonary artery hypertension (FPAH), only 20–30% of carriers with mutations in this gene suffer from PH, indicating that other

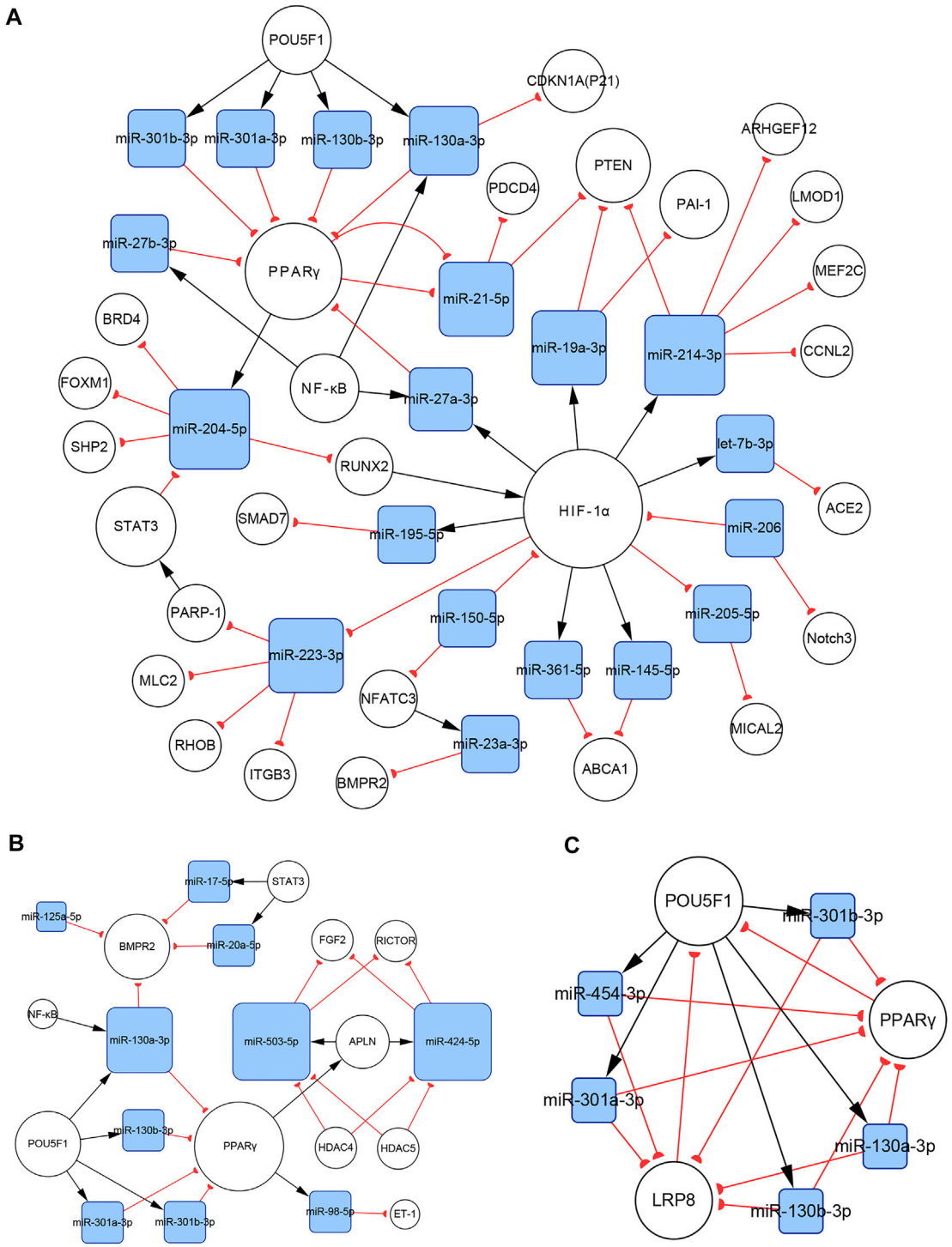

FIGURE 8 | The miR-130/301 family subnetworks in **(A)** PASMCs, **(B)** PAECs, and **(C)** PAFs. This miRNA family was involved in multiple processes, such as cell proliferation, apoptosis, migration, endothelial contraction, and matrix remodeling.

factors contribute to the onset of the disease (Orriols et al., 2017; Zhao et al., 2019). According to our network, hypoxia can induce the expression of several miRNAs by HIF-1α, such as, miR-145-5p, miR-19a-3p, miR-191-5p, miR-214-3p, and miR-27a-3p (Agrawal et al., 2014; Camps et al., 2014; el Azzouzi et al., 2013; Song et al., 2014; Zhao et al., 2019). Among them, miR-191-5p can increase cell proliferation, impair apoptosis, and induce phenotypic alteration through inhibiting BMPR2

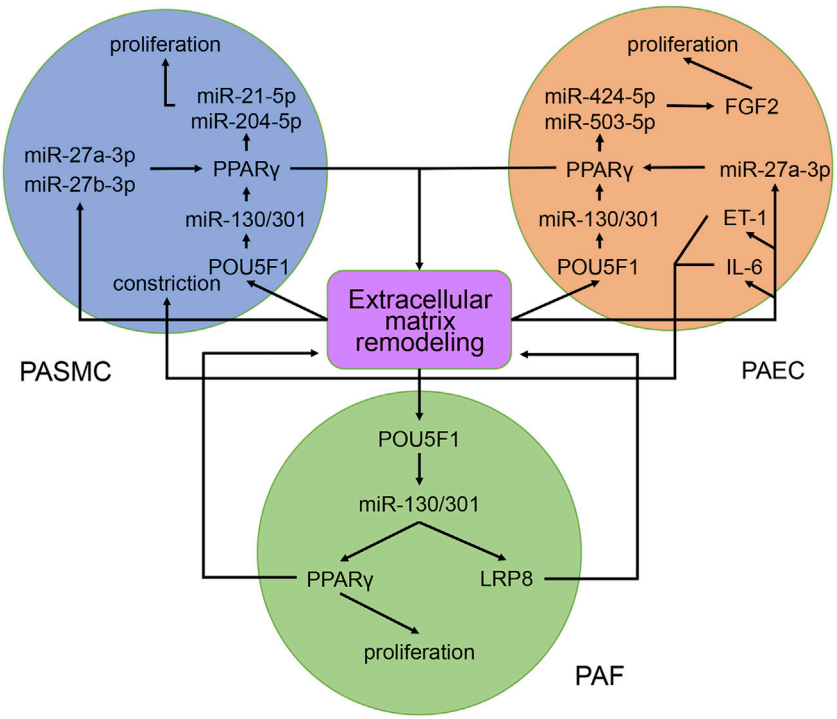

FIGURE 9 | Contribution of the miR-130/301 family to the crosstalk between various pulmonary artery cells. The remodeled extracellular matrix induces proliferation and contraction in pulmonary artery cells via miR-130/301 family-dependent and -independent pathways. Meanwhile, matrix remodeling can be induced by overexpression of the miR-130/301 family.

expression, subsequently contributing to vascular remodeling (Song et al., 2014). Therefore, the HIF-1α/miR-191-5p/BMPR2 axis reveals the connection between hypoxia and BMPR2 expression and partially explains the incomplete penetrance of BMPR2 mutations in FPAH.

5 POTENTIAL APPLICATIONS OF NON-CODING RNAS

Ultimately, studies on molecular mechanisms aim to inform clinical practices. NcRNAs are potential diagnostic biomarkers for PH. For example, circRNAs are not easily degraded, making them ideal serum biomarkers. Zhang et al. reported hsa_circ_0068481 overexpression in the serum from patients with IPAH. Furthermore, hsa_circ_0068481 expression is significantly correlated with 6-min walk distance, N-terminal pro-B-type natriuretic peptide, H2S, pulmonary hypertension risk stratification, right heart failure, and survival rate (Zhang et al., 2019a). However, because of the absence of an associated molecular mechanism, this circRNA was not included in our networks. NcRNAs may also act as potential therapeutic targets for PH. For example, Rothman et al. identified downregulation of miR-140-5p in a rat PH model. *In vitro*, miR-140-5p mimics suppressed PASMC proliferation and migration. *In vivo*, miR-140-5p mimics prevented the progression of established PH in rats (Rothman et al., 2016). The results are encouraging. However, ncRNA therapy is far from being applied in clinical settings, since a ncRNA may have diverse biofunctions. This means that when used as therapeutic agent, a ncRNA may cause adverse effects, some of which may even be life-threatening. In our opinion, carefully selected ncRNA targets and well-designed action sites can be helpful to avoid such adverse effects. These measures require a comprehensive and in-depth understanding of the mechanisms of ncRNAs in diseases. In this study, we constructed networks to demonstrate the current findings on ncRNAs from studies performed in PH patients and animal models. However, shortcomings of these studies, including the paucity of human data, sex bias, and heterogeneity of animal models, limit the translation of these findings into applications for human disease. Therefore, further studies should be performed to confirm these findings in different animal models and patient cohorts of PH. Additionally, large, well-designed, and unbiased clinical studies are required to illuminate further application of ncRNAs.

6 CONCLUSION

The roles of ncRNAs in PH remained unclear. In this study, we performed an extensive literature search and adopted uniform and strict criteria for the selection of each article to avoid biased

outcomes. The ncRNA networks were constructed by assembling ncRNAs and their interacting RNAs or genes from included articles. These networks provide a better understanding of the roles of ncRNAs in PH and can be helpful in elucidating the potential clinical applications of ncRNAs.

AUTHOR CONTRIBUTIONS

Conceptualization, HZ; writing—original draft preparation HZ and QZ; writing—review and editing, HZ and XL. All authors have read and agreed to the published version of the article.

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REVIEW
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Roles of Noncoding RNA in Reproduction

Chaofan He, Kaixian Wang, Yuanyuan Gao, Chen Wang, Leina Li, Yaping Liao, Ke Hu and Meng Liang**

School of Life Science, Bengbu Medical College, Bengbu, China

The World Health Organization predicts that infertility will be the third major health threat after cancer and cardiovascular disease, and will become a hot topic in medical research. Studies have shown that epigenetic changes are an important component of gametogenesis and related reproductive diseases. Epigenetic regulation of noncoding RNA (ncRNA) is appropriate and is a research hotspot in the biomedical field; these include long noncoding RNA (lncRNA), microRNA (miRNA), and PIWI-interacting RNA (piRNA). As vital members of the intracellular gene regulatory network, they affect various life activities of cells. LncRNA functions as a molecular bait, molecular signal and molecular scaffold in the body through molecular guidance. miRNAs are critical regulators of gene expression; they mainly control the stability or translation of their target mRNA after transcription. piRNA functions mainly through silencing genomic transposable elements and the post-transcriptional regulation of mRNAs in animal germ cells. Current studies have shown that these ncRNAs also play significant roles in the reproductive system and are involved in the regulation of essential cellular events in spermatogenesis and follicular development. The abnormal expression of ncRNA is closely linked to testicular germ cell tumors, poly cystic ovary syndrome and other diseases. This paper briefly presents the research on the reproductive process and reproductive diseases involving ncRNAs.

Keywords: miRNA, lncRNA, piRNA, spermatogenesis, follicular development, reproductive disease

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Santosh Kumar,
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Anita Gomes,
Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL), Portugal

****Correspondence:***
Meng Liang
lmhk@mail.ustc.edu.cn
Ke Hu
huke8511@163.com

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INTRODUCTION

With the development of genome-wide sequencing technology and high-throughput sequencing technology, it has been found that about 93% of the DNA sequences in the human genome can be transcribed into RNA, but only about 2% of the DNA sequences eventually encode proteins, with 90% of the DNA sequences being transcribed into ncRNAs (Consortium et al., 2007; Consortium, 2012). According to the length, a large number of noncoding RNA in these cells can be classified into lncRNA (>200 nt) and small noncoding RNAs (sncRNA, <200 nt). There are many types of sncRNAs, which can be divided into constituent and regulatory types. Regulatory sncRNAs include miRNAs and piRNAs (Chen et al., 2019a). In the past 20 years, great progress has been made in the study of the role of small noncoding RNAs in cellular life, but the study of lncRNAs has attracted people's attention in recent years. As an important member of the gene regulatory network, previous gene transcriptional "noise" plays a very important role in the physiological and pathological processes of cells (Yap et al., 2010; Wang and Chang, 2011). Studies have demonstrated that noncoding RNA participates in the regulation of spermatogenesis and oogenesis (Garcia-Lopez et al., 2015). In recent years, more and more evidence has shown that ncRNAs are regulators of many biological processes and participate in multiple levels of gene

expression regulation in the form of RNA in spermatogenesis and follicular development, such as genomic imprinting, cell proliferation, cell differentiation and meiosis (Bernard et al., 2015; Taylor et al., 2015; Robles et al., 2019).

Spermatogenesis is a process through which male animals produce male gametes continuously. That is, spermatogonia stem cells (SSCs) undergo a series of strictly regulated physiological progress to constitute spermatozoa. Spermatogenesis consists of the following stages: stem-cell mitosis produces spermatocytes which undergo two meiosis to produce haploid round sperm cells, which eventually become mature sperm (Griswold, 2016). Each stage of spermatogenesis is precisely monitored by a variety of factors. Therefore, elucidating the molecular mechanism of spermatogenesis can better elucidate the genetic regulation of male germ cell development. More significantly, it lays a solid foundation with which to diagnose and treat male infertility.

The ovary is the female reproductive organ. Its main functions consist of secreting sex hormones and producing mature eggs. The follicles, the fundamental unit of the ovary, are comprised of the oocytes located in the middle, and the surrounding granuloma cells and membrane cells. According to the different stages of development, follicles can be divided into primordial follicles, primary follicles, secondary follicles, astral follicles and preparatory follicles. In fact, only a few follicles can develop to the ovulation stage and ovulate, and most follicles move towards atresia at different stages of development. Maturation or atresia of an egg during follicular development is formally regulated by a variety of factors, including paracrine and autocrine factors in the endocrine and ovary, and cellular communication between oocytes and granulosa cells, and between granuloma cells and granulosa cells (Son et al., 2011; Emori and Sugiura, 2014). Therefore, the study of follicular development determines significance for the diagnosis and treatment of female infertility.

In recent years, abundant hallmark studies in various organisms show that non-coding RNA directly and indirectly regulate the reproductive system. For example, it interacts with proteins and regulates their functions (Statello et al., 2021), regulates histone modifications (Greco and Condorelli, 2015), promotes mRNA degradation (Beavers et al., 2015) and silence transposons (Reuter et al., 2011), etc. This review will focus on how ncRNA regulates spermatogenesis and follicular development, as well as the role of ncRNA in reproductive diseases.

MICRORNAS

microRNA Functions

MicroRNAs (miRNAs) are a class of noncoding RNAs with regulatory functions discovered in eukaryotes in recent years. They are primarily involved in the regulation of genes at the post-transcriptional level. The regulatory functions of miRNAs are indispensable, and they have recently been found to play an important regulatory role in immune diseases, cancer and reproductive diseases (Yao et al., 2010a; Fleshner and Crane, 2017; Lai et al., 2019). miRNAs are also engaged in gametogenesis during sexual reproduction.

The biosynthesis of miRNAs in animals (especially humans) has been preliminarily interpreted. First, the primary transcripts of miRNA genes (pri-miRNAs) are converted into precursor miRNAs (pre-miRNAs) in the nucleus by the RNase, Drosha. After the initial splicing, pre-miRNAs are transferred from the nucleus to the cytoplasm under the action of the transporter Exportin-5, and then further cleaved by another RNase, Dicer, to produce mature miRNAs. These mature miRNAs, together with other proteins, form the RNA-induced silencing complex (RISC), which causes the degradation of target mRNA or the inhibition of translation (Johanson et al., 2013; Wu et al., 2018). This process is shown in **Figure 1**.

The Role of microRNAs in Spermatogenesis

miRNAs are special minor molecules of RNA, with a size of approximately 22 bps. With the development of deep sequencing technology, a large number of miRNAs have been discovered in human and mouse genomes, the miRbase database (https://www.mirbase.org/) revealed 2654 mature miRNAs in the human genome and 1978 miRNAs in the mouse genome (Griffiths-Jones et al., 2008). These miRNAs are highly conserved between species and have been reported to regulate over 30% of genes in the genome (Lewis et al., 2005). The establishment of a miRNA expression profile in male germ cells is an essential prerequisite for comprehensively exploring the biological function of miRNAs in spermatogenesis. Although the role of miRNAs in the development of male germ cells has not been fully studied, high-throughput expression profiles have found that a large number of miRNAs are selectively expressed in spermatogonia, pachytene spermatocytes, spermatozoa and mature spermatozoa (Yan et al., 2007; Moritoki et al., 2014). miRNAs are specifically expressed at all stages of spermatogenesis, suggesting involvement in almost every step of male germ cell differentiation.

Some studies conducted conditional knockout of Drosha and Dicer in spermatogenic cells of mice testis after birth, and found that the knockout mice were infertile due to impaired spermatogenesis, presenting oligospermia or azoospermia, suggesting that miRNA is involved in regulating spermatogenesis and plays a crucial role (Bernard et al., 2015).

Spermatogenesis originates from SSCs, and many miRNAs are involved in the mitotic proliferation of SSCs. In recent years, with the development of analytical techniques, hundreds of miRNAs have been discovered. A large number of miRNAs were found to be highly expressed in SSCs, such as clusters of miR-17-92 and miR-290-295. miRNA-20, miRNA-21 and miRNA-106a can regulate the self-renewal of SSCs (Niu et al., 2011; He et al., 2013b). The role of miRNAs in regulating the fate of SSCs has been confirmed, but the specific regulatory mechanism remains to be further studied. Some miRNAs are associated with regulating the proliferation and apoptosis of SSCs. For example, miR-204 regulates the proliferation of SSCs by targeting Sirt1 (Niu et al., 2016), while miR-34c can affect the apoptosis of SSCs (Li et al., 2013). miR-21 is involved in SSCs

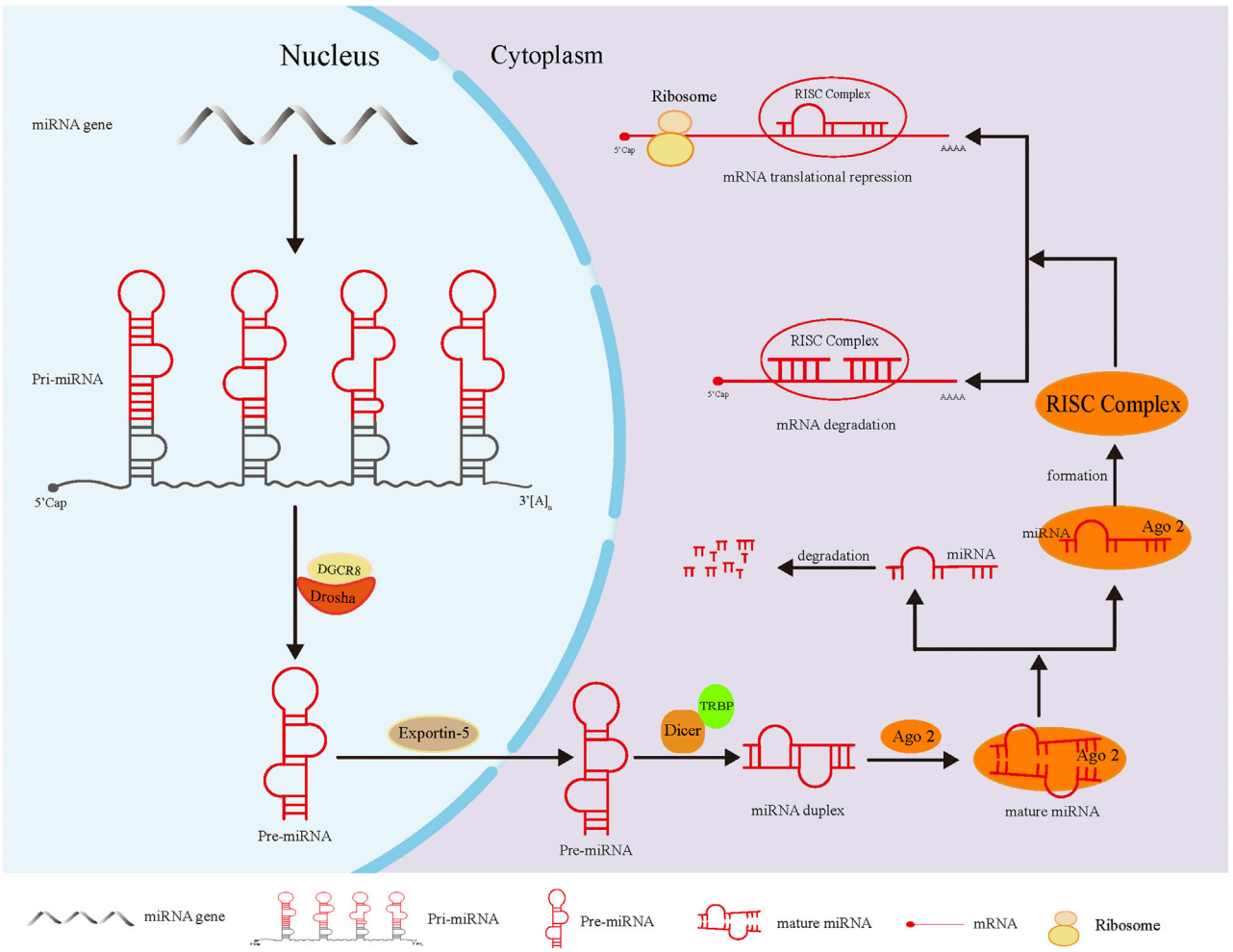

FIGURE 1 | The biosynthesis of miRNA. In the nucleus, pri-miRNA, the primary transcription product of miRNA gene, was cleaved by Drosha-DGCR8 complex into hairpin precursor miRNA. After preliminary shearing, the pre-miRNA was transferred to the cytoplasm under the action of the transporter Esportin-5. In the cytoplasm, the pre-miRNA was further cleaved by Dicer enzymes that bound to TRBP (double-stranded RNA-binding protein) to form miRNA duplex of mature length. Finally, the double-stranded miRNA was loaded into Ago2 to generate mature miRNA. Subsequently, one chain of miRNA dissociated from the complex and degrades rapidly, and the remaining chain formed the RISC complex with Ago2, which led to degradation or translation inhibition of target mRNA.

mitotic proliferation by regulating ETV5, a key gene that maintains SSCs self-renewal (Kotaja, 2014). miR-20 and miR-106a, which are preferentially expressed in SSCs, stimulate the proliferation of SSCs by targeting inhibition of STAT3 (signal transduction and transcriptional activator 3) and CCND1(cyclin D1). This is mainly because the silencing of STAT3 and CCND1 can stimulate the renewal of SSCs (He et al., 2013a). The above studies show that miRNAs have restricted expression during spermatogenesis, can regulate the proliferation of SSCs, participate in the regulation of gene transcription in the spermatogenesis and meiosis of germ cells, and play a regulatory role in maintaining the normal development of male germ cells.

There are also many miRNAs involved in meiosis, which regulate meiosis by acting on different targets and play an important role in spermatocyte differentiation. miR-10a, recently reported, also plays a critical role in mouse and human male germ cell development and spermatogenesis by regulating the meiosis process. miR-10a negatively targets RAD51, and its overexpression can lead to complete male sterility and meiosis stagnation (Gao et al., 2019). In addition, deletion of miR-871 and miR-880 also leads to meiosis stagnation in spermatogenesis, which inhibits Fzd4 gene expression by targeting the 3′-UTR of its target mRNA (Ota et al., 2019). Specific miR-34c and miR-34b-5p in pachytene spermatocytes and round spermatocytes also promote further development of spermatogenesis by inhibiting cell proliferation (Smorag et al., 2012; Wang et al., 2018b). miR-34b-5p regulates meiosis by targeting Cdk6. miR-34c affects meiosis by targeting Notch1, CDK4, MYC and other cell cycle regulators, and TGIF2 and Notch2 are direct targets of miR-34c (Bouhallier et al., 2010). In addition, miR-34c can also promote can also promote the apoptosis of male germ cells by targeting ATF1 (Liang et al., 2012).

miRNAs have become novel key factors in post-transcriptional regulation of male germ cells and are widely involved in various developmental stages of spermatogenesis (Taylor et al., 2015). Currently, although many miRNAs have been identified to play an important role in spermatogenesis and their functions and regulatory mechanisms have been annotated, the roles and potential mechanisms of many miRNAs remain unknown. In the future, it will become an important research direction to reveal the role of specific miRNAs in spermatogenesis. These results will contribute to understanding the etiology of male infertility and provide a theoretical basis for exploring the prevention and precise treatment of male infertility and other diseases.

The Role of microRNAs in Follicular Development

As embryos of mice with Dicer1 allele deletion died, Otsuka et al. used the mouse model with inefficient Dicer1 allele mutation to study and found that the Dicer1 defect impaired the growth of new capillaries in the ovaries of female mice, resulting in luteal insufficiency and infertility in female mice (Otsuka et al., 2008). Further studies showed that impaired luteal angiogenesis in Dicer1-deficient female mice was partly caused by the deletion of miR-17-5p and let-7b. miR-17-5p and let-7b can participate in angiogenesis through targeted regulation of Tissue inhibitor of metalloproteinase 1 (Timp1) expression. In addition, intra ovarian injection of miR-17-5p and let-7b in Dicer1-deficient female mice could partially normalize Timp1 expression and luteal vascular growth, but could not sustain pregnancy. This may be due to the fact that many other miRNAs are required for the maintenance of pregnancy, or that the amount of miR-17-5p and let-7b injected into the ovary is insufficient or cannot exist for a long time (Otsuka et al., 2008). MiRNAs also played an important role in ovulation in mice. Oocyte specific removal of Dicer results in spindle disorder and chromosome aggregation, leading to stagnation in the first meiosis stage (Murchison et al., 2007; Tang et al., 2007). Degradation of many maternal transcripts is critical to the completion of meiosis in oocytes, and miRNAs may be involved in this degradation process (Murchison et al., 2007).

miRNAs also play an important role in follicular granulosa cells. Specific removal of Dicer1 in mouse follicular granulosa cells leads to increased consumption of primitive follicle banks, accelerated recruitment of early follicles and increased number of degenerating follicles in ovary, and changes in many follicle-development-related genes, Cyp17a1, Cyp19a1, ZPS (Zona Pellucida Glycoproteins), GDF9 and BMP15 (Lei et al., 2010). In addition, FSH was found to regulate the expression of miR-29a and miR-30d in cultured granulosa cells in a time-specific manner (Yao et al., 2010b). The appearance of LH peak can promote the expression of miR-21, miR-132 and miR-212 in granulosa cells (Fiedler et al., 2008). TGF-β1 induced the expression of miR-224 in cultured mouse granulosa cells (Yao et al., 2010a). After 12 h treatment with FSH, progesterone expression was promoted, and 17 up-regulated miRNAs and 14 down-regulated miRNAs (including miR-29a and miR-30d) were found, suggesting that miRNAs may be involved in mediating FSH regulation of gene expression and hormone production in granulosa cells (Yao et al., 2010b). Yao et al. treated primary cultured mouse granulosa cells with TGFβ1, and screened 13 down-regulated miRNAs and three up-regulated miRNAs. Up-regulated miR-224 participated in the regulation of TGFβ1 in granulosa cell proliferation and estrogen secretion by acting on Smad4, while regulating the expansion of the cumulus oophorus by acting on Ptx3 (Yao et al., 2010a; Yao et al., 2014). Further research shows that p53 and p65 may affect female reproduction by regulating the miR-224-smad4 pathway alone or synergistically (Liang et al., 2013).

LNCRNAS

LncRNA Functions

Mammalian transcriptome analysis revealed that most lncRNAs are transcribed by RNA polymerase II, which is similar to protein-coding mRNA, with a 5 ′methylation cap and a polyadenylate tail but without a distinct open reading frame (Wilusz et al., 2009; Kopp and Mendell, 2018). Although lncRNA cannot encode protein, it can play a role in a variety of diseases through its own structural characteristics or as a signal molecule, such as neurological diseases, cardiovascular diseases, autoimmune diseases and tumors (Wapinski and Chang, 2011; Kwok and Tay, 2017; Distefano, 2018). The main biological functions of lncRNAs are shown in **Figure 2**. In the nucleus, some lncRNAs can be used as ligands of transcription factors to promote gene transcription, and can also negatively regulate the transcription process by competing transcription factors (Chen, 2016), and some lncRNAs can be used as signal molecules to regulate the transcription process (Guo et al., 2019). In addition, some specific lncRNAs can regulate gene expression by recruiting histone modification enzymes to specific sites and changing chromatin activity (Sun et al., 2016; Liu et al., 2017b). In the cytoplasm, a few lncRNAs with certain coding ability exert their functions by translating short peptides (Ruiz-Orera et al., 2014). Certain lncRNAs are precursors of miRNAs, and mature miRNAs can be generated after cleavage. Studies have also reported that lncRNAs can sponge miRNAs through the principle of base complementary pairing to inhibit the function of miRNAs. In addition, lncRNA can also combine with RBP to regulate the splicing, stability and initiation of translation of target mRNA, thus regulating gene expression in the translation stage (Yoon et al., 2014; Dykes and Emanueli, 2017).

The Role of lncRNAs in Spermatogenesis

A large number of lncRNAs have been detected in the testes of humans, rats and mice during specific developmental stages; some of the functions of lncRNAs have been characterized and annotated, for instance, Mrhl (meiotic recombination hot spot locus) (Arun et al., 2012), Tsx (testis-specific X-linked gene) (Anguera et al., 2011), Dmr (Dmrt1-related gene) (Zhang et al., 2010) and HongrES2 (Ni et al., 2011) play an important role in testicular development and spermatogenesis. For instance, Testis-specific X-linked (Tsx), as a LncRNA, was specifically expressed

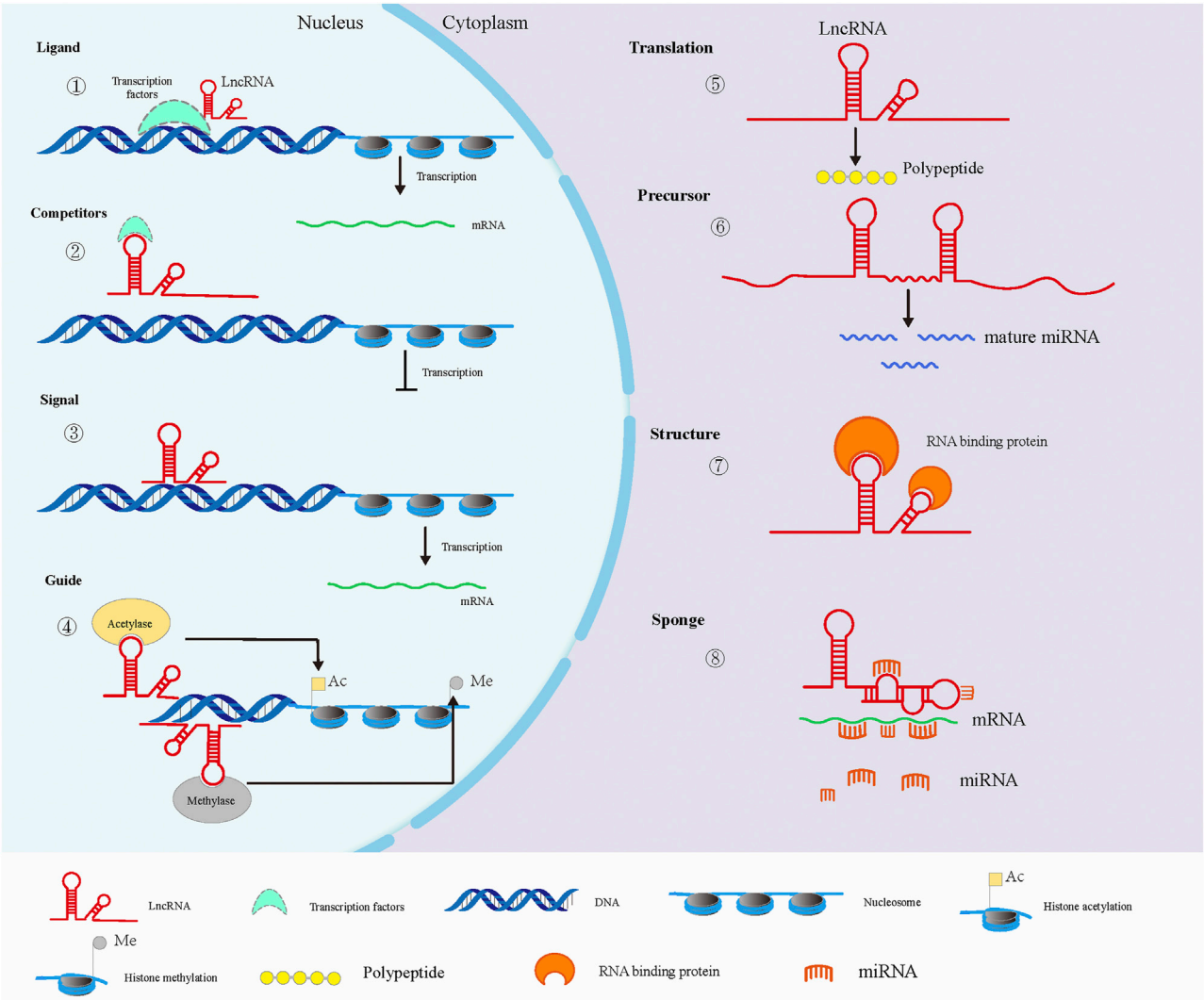

FIGURE 2 | Main biological functions of lncRNAs. In the nucleus (left), lncRNAs can interact with transcription factors to promote (1) or inhibit (2) gene transcription. In addition, lncRNA can directly regulate gene transcription as a signaling molecule(3). LncRNAs can also recruit histone modifiation-related enzymes to participate in epigenetic regulation, including histone acetylation and methylation (4). In the cytoplasm (right), minority LncRNAs have some coding ability to translate peptides (5). LncRNAs are the source of some sncRNAs in the cytoplasm (6). Some RBPs can interact with lncRNAs via specific motifs (7). Besides, lncRNAs can alleviate the inhibition of miRNA on mRNA translation via sponging miRNAs (8).

in pachytene spermatocytes, testicular weight decreased and spermatocyte apoptosis was observed in the Tsx knockout mice.

SSCs are a uniquely male germ line stem cell, which can support spermatogenesis and maintain male fertility. Liang et al. found that 241 specific lncRNAs can play an essential role in maintaining SSC survival and self-renewal through protein coding genes and miRNA (Liang et al., 2014). Weng et al. used RNA sequencing and found that 564 lncRNAs were specifically identified from 60-day-old porcine testes vs 90-day-old, it was suggested that these lncRNAs might play a potential role in porcine SSC self-renewal (Weng et al., 2017). Many studies have confirmed that lncRNAs are the key regulator of SSCs, but their role in SSC-related physiological processes has only been partially studied. Ddx5/p68 has been found to be an interaction protein of lncRNA Mrhl in spermatogonial cells, and Mrhl is involved in nuclear reservation of p68. When Mrhl or p68 is knocked down, the nuclear localization of β-catenin, a key protein in the Wnt pathway, is severely affected. Further experiments verified that Mrhl expression was down-regulated when Wnt pathway was activated, indicating that Mrhl was a negative regulator of the Wnt pathway (Arun et al., 2012). Other research has also been reported that Mrhl can directly recruit Sin3a and HDAC1 at the Sox8 promoter site to form a coinhibitory complex, thus reducing the expression level of Sox8. During spermatogonial differentiation, the Wnt pathway is activated and this inhibition disappears. These results suggest that Mrhl also plays an important role in meiosis entry and spermatogonial differentiation (Kataruka et al., 2017). Hu et al.

found that the lncRNA Gm2044 could inhibit the proliferation of GC-1 cells (mouse spermatogonia cell line) by binding to UTF-1 (Undifferentiated embryonic cell transcription factor 1). Higher Gm2044 expression levels in GC-1 cells resulted in increased cell viability and UTF1 protein inhibition. The lncRNA Gm2044 is also highly expressed in pachytene spermatocytes and inhibits Utf1 mRNA expression during spermatogenesis (Hu et al., 2018).

In view of the fact that a large number of lncRNAs have been found to be expressed in testicular development and male germ cells, but only a few have studied their functions during the development of male germ cells, the regulatory mechanism of lncRNA in spermatogenesis is worth further in-depth investigation.

The Role of lncRNAs in Follicular Development

Cumulus cells (CCs) are closer to oocytes than parietal granule cells, participate in the coordination of late follicular development and oocyte maturation, promote amino acid transport, sterol biosynthesis and glycolysis, and provide energy substrates for oocyte meiosis recovery (Hillensjo et al., 1982). Xu et al. retrospectively analyzed and compared the CC gene expression profiles of the high-quality embryo group and poor-quality embryo group according to clinical embryo development. The results showed that lncRNA Y00062, which was significantly down-regulated in CCs of the poor-quality embryo group, may be involved in regulation of the NEK7 gene (Xu et al., 2014). NEK7 is a gene that is essential for spindle assembly and mitosis (Gupta et al., 2017), indicating that the lncRNA Y00062 can play an important role in regulating the meiosis of oocytes. The lncRNA ENST00000502390 is highly expressed in CCs derived from poor-quality embryos and is related to ELOVL5 (elongation of very-long-chain fatty acids protein 5). ELOVL5 is associated with the biosynthesis of highly unsaturated fatty acids and regulates the maturation and ovulation of oocytes. That is, the lncRNA ENST00000502390 may regulate the synthesis of highly unsaturated fatty acids, and participate in oocyte maturation and ovulation (Warzych et al., 2017).

In ovarian granule cells of patients with PCOS, the lncRNA HCG26 (HLA complex group 26) is highly expressed and its expression level is linked to the number of ovarian antral follicles. The down-regulation of HCG26 expression in the human ovarian granule cell line KGN can inhibit cell proliferation and cell cycle progression, and also affect the expression of the aromatase gene and the production of 17β-estradiol (Liu et al., 2017a). It is suggested that HCG26 may be an essential regulatory gene affecting granule cell proliferation and follicular growth in patients with PCOS. It has been reported that the lncRNA Gm2044 acts as a competing endogenous RNA for miR-138-5p to rescue the inhibitory effects of miR-138-5p on Nr5a1, and then regulate 17β-estradiol synthesis (Hu et al., 2019). This serves as a new focal point for the treatment of PCOS. LncRNA Amhr2 was transcribed upstream from Amhr2 gene which regulated of anti-Mullerian hormone specific effects in ovarian granulosa cells. It was proved that LncRNA Amhr2 enhanced the promoter activity of Amhr2 and significantly inhibited the expression of Amhr2 after knocking down lncRNA Amhr2 in primary granulosa cells (Kimura et al., 2017).

PIWI-INTERACTING RNA

PIWI-Interacting RNA Functions

In 2006, four separate teams identified a new class of small RNA molecules, called PIWI-interacting RNAs, that specifically interact with PIWI proteins in the germline cells of fruit flies, mice, rats and humans. hereinafter referred to as piRNA (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006). piRNAs are an animal-specific kind of small silencing RNAs which characteristic expression in pachytene spermatocytes and sperm cells during spermatogenesis (Gou et al., 2015). piRNAs interact with the PIWI protein to play a role in spermatogenesis. An increasing number of studies have shown that subfamily PIWI proteins, including MIWI, MIWI2 and MILI, are essential factors for stem cell regeneration and male germ cell development in vertebrates (Reddien et al., 2005; Klattenhoff and Theurkauf, 2008).

The expression of three PIWI proteins in mice showed a strict temporal sequence during spermatogenesis: MILI was the first to be expressed at 12.5 d of embryo stage and continued to the spherical sperm cell stage after the completion of meiosis; The expression of MIWI2 began at 15.5 days of embryo stage and ended at 3 days after birth, which was the shortest expression time of Piwi protein. The last is MIWI, whose expression begins in the fine line phase of meiosis and ends in late sperm cells (Siomi et al., 2011). Correspondingly, piRNA expression also appeared two peaks before and after birth and at meiosis stage, piRNA of prepachytene and piRNA of pachytene, respectively (Aravin et al., 2008). Prepachytene piRNA was mainly derived from transposable element sequence and combined with MILI and MIWI2. Pachytene piRNA comes from intergene, intron and exon regions and mainly binds to MILI and MIWI. Different Piwi proteins in mice bound piRNA of different lengths: MILI bound piRNA of (~26 nt) was the shortest. MIWI2 combined with (~28 nt) piRNA; The piRNA combined with MIWI was the longest (~30 nt) (Siomi et al., 2011). On the one hand, the expression and function of Piwi/piRNA are necessary for sperm cell development and differentiation. On the other hand, the composition of Piwi/piRNA functional complex also showed space-time specific dynamic changes during spermatogenesis.

The Role of PIWI-Interacting RNA in Spermatogenesis

Previous studies have shown that Piwi/piRNA mainly silences transposition elements and regulates the expression of protein-coding genes at the epigenetic and post-transcriptional levels, thus playing an important role in the development and differentiation of animal germ cells and gametogenesis (Ozata et al., 2019). Transposon can be inserted into other sites in the genome by transcription or reverse transcription, and under the action of endonuclease, thus affecting the stability and integrity of

the genome (Onishi et al., 2021). Therefore, the activity of transposable elements needs to be strictly controlled.

Before and after mouse birth, MILI protein can bind piRNA derived from transposon and then cut transposable element RNA which is homologous and complementary to piRNA to generate secondary piRNA. The newly generated secondary piRNA can also bind to MILI and also cut the same homologous and complementary transposable element RNA with secondary piRNA to generate a new primary piRNA. If the reciprocating cycle, namely ping-pong cycle, can continuously cut the RNA transcribed from transposable element, thus effectively silencing transposable element activity (Onishi et al., 2021). Defects in cleavage activity of MIWI and MILI proteins lead to activation of transposable elements such as LINE-1, It is suggested that MIWI and MILI proteins may directly clew transposable element RNA in male germ cells of mice (De Fazio et al., 2011; Reuter et al., 2011).

More and more experimental evidence indicates that in addition to transposon silencing, piRNA can also play a biological role by regulating the expression of protein-coding genes. In mouse elongate sperm cells, pachytene piRNA, its binding protein MIWI and deadenylase CAF1 form a Pi-RISC complex, which recognizes the sequence elements of the 3′ untranslated region (3′ UTR) of target mRNA by a base incomplete pairing approach similar to miRNA. Inducing deadenylation and degradation of target mRNA, and depending on the different sequences of piRNA in millions, pi-RISC mediates degradation of thousands of different mRNA during late sperm cell development (Gou et al., 2015). In addition, pachytene piRNA can also guide MIWI protein to cleavage and degrade target mRNA in testicular tissue through a siRNA-like mechanism when it is fully or nearly fully paired with target mRNA (Zhang et al., 2015). Besides, the proper removal of MIWI/piRNA was crucial for sperm maturation in late spermatogenesis. piRNAs regulated the clearance of MIWI/piRNA machine through ubiquitin-proteasome signaling pathway, which further confirms that piRNA plays a very considerable role in the development of male germ cells (Zhao et al., 2013). Interestingly, piRNA not only targets mRNA degradation, but also promotes mRNA translation. It has been reported that MIWI/piRNA, in conjunction with eIF3f and HuR, can regulate the translation of a large number of mRNA in sperm cells, and this mechanism has been proved to be necessary for the successful acrosome assembly in mouse spermatogenesis (Dai et al., 2019). In this study, MIWI/piRNA mediated translation activation in sperm cells, providing a new clue to the important biological problem of transcription-translation uncoupling during sperm formation.

The Role of PIWI-Interacting RNA in Follicular Development

In recent years, researchers have conducted in-depth studies and found that piRNA is not only specifically expressed in testis, but also abundant piRNAs are detected in ovary (Williams et al., 2015). It has been reported that PIWIL2 was expressed in human fetal oocytes and PIWIL1 and PIWIL2 in adult ovaries. Bovine ovaries expressed PIWIL1 and high expression PIWIL3 was isolated from bovine oocytes. PiRNA is also abundant in human, bovine and rhesus monkey oocytes, but their function in oocytes has not been clearly studied (Roovers et al., 2015; Williams et al., 2015). It was found that PIWI protein was also expressed in mouse ovary oocytes, but the loss of PIWI protein did not affect female reproduction. Recent studies have reported that endo-siRNA similar to siRNA is highly expressed in mouse oocytes, while most mammalian oocytes, including human and monkey oocytes, do not express endo-siRNA (Zhang et al., 2021). Because the types and expression characteristics of small RNA in golden hamster oocytes are similar to those of most other mammals, including humans and cynomolgus monkeys, golden hamster models of PIWIL1 deletion mutants were established. Interestingly, PIWIL1-deficient females had normal ovaries and were able to produce mature oocytes, but the fertilized eggs produced by mating with wild-type males did not develop properly and remained stuck in the two-cell stage. It was found that the deletion of PIWIL1 gene resulted in abnormal accumulation of transposons in oocytes, decreased degradation of maternal mRNA and failure of zygotic gene activation during early embryonic development (Hasuwa et al., 2021; Loubalova et al., 2021). These results suggest that piRNA pathway may also play an important role in human and other mammalian female reproduction, providing an important theoretical basis and animal model for the diagnosis and treatment of piRNA pathway abnormalities and female reproductive diseases.

NON-CODING RNA IN REPRODUCTIVE DISEASES

The Role of Non-Coding RNA in Male Reproductive Diseases

Currently, infertile couples account for about 15% of married couples worldwide, and male infertility accounts for 40–50% (Dada et al., 2003). The etiology of infertility is complex, and the detection methods and related research are still relatively sparse. Research into male infertility from the molecular perspective has become important, which opens up new fields for analysis of the etiology of infertility (Robles et al., 2019).

A study using miRNA chip technology to screen the semen of infertile and healthy men found that there were 52 differentially expressed miRNAs, of which 21 were highly expressed miRNAs and 31 showed low expression in infertile patients (Liu et al., 2012). Using the same technique in normal testicular tissue and non-obstructive azoospermia patients, it was found that the expression of 173 miRNAs was altered in infertile patients, with 19 miRNAs showing up-regulated expression and 154 miRNAs being down-regulated (Zhuang et al., 2015). It was also shown that mir-34c and mir-275 were significantly and highly expressed in the testicles of NOA patients, while studies found that the highly expressed miR-34c could initiate germ cell apoptosis by targeting the amp-dependent periodic transcription factor ATF1 (Liang et al., 2012). Studies have shown that miRNA-122-5p expression is up-regulated in the testicular tissue of patients with obstructive azoospermia (OA) compared with

those with non-obstructive azoospermia (NOA). miRNA-122-5p enhanced SSC cell activity by competitively binding with the lncRNA CASC7 and targeting CBL mRNA (Zhou et al., 2020). Due to the significantly differential expression of miRNA in NOA patients and its important biological functions, miRNAs can be used as a potential molecular marker for the diagnosis and typing of male fertility, and can also provide a new solution for the clinical treatment of male infertility according to the function of miRNAs in spermatogenesis.

At the gene level, lncRNAs are not separated from DNA methylation. However, this model is commonly seen in the combination of lncRNA and methylated transferase (DNMT1, 3, etc.), and the localization of this enzyme to the promoter (CpG island) and methylation of the gene, thus inhibiting gene transcription (Somasundaram et al., 2018; Kang et al., 2019). Peng et al., analyzed DNA methylation levels in sterile and normal male sperm and found that abnormal methylation of imprinting genes H19 and SNRPN was related to abnormal sperm parameters and male infertility (Peng et al., 2018). Additional studies have found that DNA methylation mediates male reproductive organ development, spermatogenesis and male sexual behavior by affecting gene expression, indicating that lncRNAs are closely related to male infertility (Cisneros, 2004).

The Role of Non-Coding RNAs in Female Reproductive Diseases

PCOS (Polycystic ovary syndrome) is the principal element of female infertility, and is often characterized by hyperandrogenemia, oligomenorrhea and polycystic ovary syndrome, affecting 6–10% of women worldwide (Bozdag et al., 2016). In addition, about 90% of an ovulatory infertility is caused by PCOS (Misso et al., 2012). The etiology and pathogenesis of PCOS remain unclear, there is no effective cure, clinical treatment is mainly symptomatic, and long-term health management is needed. In recent years, many studies have found that non-coding RNAs play a significant role in PCOS. However, the research into the role of lncRNAs in the pathogenesis of PCOS is sparse. This paper mainly reviews the research advances of lncRNAs in PCOS.

With the development of gene sequencing technology, countless researchers have carried out the study of lncRNA sequencing in PCOS. The researchers completed microarrays or deep sequencing of different ovarian cells from PCOS patients or a rat model (Huang et al., 2016; Liu et al., 2017a; Fu et al., 2018a). These results describe hundreds of thousands of differentially expressed lncRNAs. TERRA is a lncRNA composed of different numbers of UUAGGG repetitive sequences, (Azzalin et al., 2007). TERRA is associated with the regulation of telomere length, telomerase activity and heterochromatin (Wang et al., 2015). Some studies have suggested that abnormalities of telomeres may be involved in the pathogenesis of PCOS (Wei et al., 2017). Wang et al. performed a prospective control study and found that the relative telomere length of PCOS patients was higher than that of the control group, but the expression level of TERRA was lower than that of the control group. In the PCOS group, the expression level of TERRA was negatively correlated with the concentration of testosterone, while the telomere length of leukocytes was positively correlated with the concentration of testosterone (Wang et al., 2017a), suggesting that TERRA and testosterone regulate the telomere length of leukocytes, resulting in PCOS.

In addition, many lncRNAs are involved in the occurrence and development of PCOS, lnc-MAP3K13-7:1 was highly expressed and accompanied by DNA hypommethylation in granulosa cells of PCOS patients. The study demonstrated that Lnc-MAP3K13-7:1 bound DNMT1 as a recruitment agent and mediated its ubiquitination degradation. The CDKN1A promoter was demethylated and its expression level increased, finally, GCs proliferation was inhibited (Geng et al., 2021). CTBP1-AS is a lncRNA associated with the androgen receptor signaling pathway. Liu et al. found that after adjusting for age and body mass index (BMI), the expression of CTBP1-AS in peripheral blood leukocytes of women with PCOS was higher than that of women in the control group, and showed that individuals with a high expression of CTBP1-AS had an increased risk of PCOS. After adjusting for age, BMI and IR to evaluate the steady-state model, the expression level of CTBP1-AS was positively correlated with the concentration of serum total testosterone (Liu et al., 2015).

Research into the role of lncRNAs in PCOS has just begun. Although the sequencing results show that there are numerous differentially expressed lncRNAs, research into these differentially expressed lncRNAs is not prolific enough, and only a few studies have discussed the function and mechanism of lncRNAs. Current studies mainly focus on the expression and mechanism of lncRNAs in peripheral blood leukocytes, cumulus cells, granulosa cells and follicular fluid. According to the current research, lncRNAs may participate in the production of steroids, enhance the activity of steroid receptors, obesity and IR and other metabolic processes, affect the development, proliferation and apoptosis of ovarian cells, and then lead to the development of PCOS (Chen et al., 2019b; Mai et al., 2019). The study of lncRNAs in PCOS has a broad prospect, and more extensive and in-depth studies are needed to explore the mechanism of action of lncRNAs in PCOS and the relationship between lncRNAs and miRNAs, in order to help us to better understand the pathogenesis of PCOS; this is of great significance for the clinical diagnosis and treatment of this syndrome.

In recent years, more and more studies have investigated miRNA expression in women with PCOS (Santamaria and Taylor, 2014; Butler et al., 2020; Gebremedhn et al., 2021). It has been reported that there are a variety of miRNAs in human follicular fluid, including miR-483-5p, miR-132, miR-320, miR-520c-3p, miR-24 and miR-222, which are related to estrogen secretion. miR-24, miR-193b and miR-483-5p regulate progesterone secretion, and the expression of miR-132 and miR-320 in follicular fluid of polycystic follicles is significantly lower than that in normal follicular fluid (Sang et al., 2013; Sorensen et al., 2016). These results suggest that miRNA in follicular fluid plays an important role in regulating steroid hormone synthesis. Changes in their expression are associated with polycystic ovaries. Roth et al. compared miRNAs in follicular fluid of patients with normal

TABLE 1 | Function of lncRNAs in reproductive system.

Gene name	Species and samples	Biological functions	Mechanism	Reference
LncRNA HOTTIP	Mouse spermatogonial cell line GC-1 cells	Promote cells G2/M phase arrest and early apoptosis	The expression of γ -H2Ax and p53 was up-regulated by adjacent gene Hoxa13	Liang and Hu (2019)
NONMMUT074098.2	Mouse spermatogonial cell line GC-1 cells	Apoptosis	Knocking down NONMMUT074098.2 activated the p38 MAPK pathway	Li et al. (2019b)
LncRNA AK015322	Mouse SSC line C18-4 cells	Promote cells proliferation	LncRNA AK015322 sponged miR-19b-3p and alleviated the inhibition of ETV5	Hu et al. (2017)
LncRNA ZFAS1	Human ovarian granulosa cells(PCOS patients)	Endocrine, proliferation and apoptosis	LncRNA ZFAS1 bound miR-129 to promote the expression level of HMGB1	Zhu et al. (2020)
LncRNA NEAT1	Ovarian granulosa cells (PCOS rats)	Proliferation and apoptosis	Knockdown LncRNA NEAT1 promoted IGF1 expression via miR-381	Zhen et al. (2021)
LncRNA ROR	Human epithelial ovarian carcinoma cell lines SKOV3 and 3AO cells	Epithelial-mesenchymal transition (EMT)	LncRNA ROR promoted EMT via mir-145/FLNB axis	Li et al. (2019a)
LINC00339	Human epithelial ovarian carcinoma cell lines SKOV3 and HO-8910 cells	Proliferation, migration, and invasion	LINC00339 bound miR-148a-3p to promote the expression level of ROCK1	Pan et al. (2019)
LncRNA SLC7A11-AS1	Human testicular embryonic carcinoma cells line NCCIT cells	Increased ROS levels	The expression of SLC7A11 was negatively regulated and the oxidative stress level of cells was increased	Sanei-Ataabadi et al. (2020)
miR-18b	Human follicular fluid with PCOS	Promoted progesterone release, inhibited testosterone and estradiol release	miR-18b targeted the mRNAs of IL8, SYT1 and IRS2	Roth et al. (2014)
miR-224	Mouse granulosa cells	Induced GCs proliferation and increased estrogen release	miR-224-mediated target gene Smad4 pathway	Yao et al. (2010a)
miR-16	Human ovarian granulosa cells with PCOS	Proliferation cycle and apoptosis	miR-16 promoted the proliferation and inhibited apoptosis of ovarian GCs by directly targeting PDCD4	Fu et al. (2018b)
miR-141, miR-429 and miR-7-1-3p	Human semen with non-obstructive azoospermia patients	Potential markers of NOA	miR-141 reduced the expression levels of Cbl and Tgfβ2, and miR-7-1-3p down-regulated Rb1 and Pik3r3.	Wu et al. (2013)

follicles and polycystic ovary, and identified 5 miRNAs that were up-regulated in follicular fluid of polycystic ovary. They are miR-9, miR-18b, miR-32, miR-34c and miR-135a, respectively, and their potential target genes interleukin-8 (IL-8), Synaptogamin1 and insulin receptor substrate2 (IRS2) are down-regulated in polycystic ovaries (Roth et al., 2014). The abnormal expression of these genes is closely associated with abnormal follicular development and polycystic ovaries. In addition, up-regulated miR-27a-3p inhibits granulosa cell proliferation by targeting Smad5 and Creb1 genes, promotes apoptosis of ovarian granulosa cells and transformation of androgen and estrogen, thus participating in the occurrence of abnormal PCOS follicles (Wang et al., 2017b; Wang et al., 2018a). Besides, some miRNAs that function in the reproductive system are summarized in **Table 1**.

Ovarian cancer constitutes one of the most common cancers of the female reproductive system. However, the molecular mechanism of ovarian cancer remained unclear, so it is necessary to further study the biological behavior of ovarian cancer at present. NcRNAs provided a suitable cut-off point in the diagnosis and treatment of ovarian cancer. Previous studies have revealed that microRNA-224 inhibits the expression of cyclin A by targeting KLLN, thereby promoting the proliferation of epithelial ovarian cancer cells (Hu and Liang, 2017). In addition, we identified some lncRNAs that regulate reproductive system functions, which are detailed in **Table 1**.

OUTLOOK

Non-coding RNAs are important regulatory molecules in the human body and are essential for the development of a pathophysiological state. The formation of gametes is extremely composite, and spermatogenesis can be simply summarized as the proliferation and meiosis of primitive germ cells, with the formation of mature sperm occurring due to the deformation of sperm cells. The development of follicles requires the passage of primordial follicles, primary follicles, secondary follicles, and mature follicles, and the eventual discharge of the cumulus oocyte complex. These processes are strictly and precisely regulated, and the failure of any one of them can intervene in the continuation of life. Non-coding RNAs are an important element in these processes.

Despite the fact that current high-throughput experimental techniques and open ncRNA database resources have given rise to the discovery of many ncRNAs, the function of these ncRNAs in gametogenesis and reproductive disease remains unclear. Therefore, it can be inferred that there are still innumerable reproductive ncRNAs with important functions to be studied. This is also suggested that we have great potential to study the regulatory mechanism of ncRNAs in gametogenesis and reproductive disease. MiRNA binding to mRNAs is reversible, enabling the regulation of gene expression. Competitive endogenous RNA (ceRNA) competitively binds miRNA to regulate gene expression. LncRNAs can act as a sponge of

miRNAs, adsorbing miRNAs and interacting with each other to constitute a huge and complex ceRNA network which is able to regulate the function of target genes.

Research into the role and mechanism of ncRNAs in the generative process is a difficult and persistent task. Understanding the role of post-transcriptional gene regulation in reproduction will help to clarify the causes of reproductive failure and hopefully provide methods and targets for the treatment of infertility, as well as additional contraceptive methods. Scientists need to continue to clarify the functions of lncRNA in spermatogenesis and oogenesis, and strive for the diagnosis and treatment of infertility.

AUTHOR CONTRIBUTIONS

ML and KH directed this work and co-reviewed the manuscript. CH wrote the manuscript. KW and YG helped to collect relevant information. CW, LL, and YL modified the figures. All authors read and approved the final manuscript.

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Screening and Comprehensive Analysis of Cancer-Associated tRNA-Derived Fragments

Yiran Zhou[1,2], *Qinghua Cui*[1,2] *and Yuan Zhou*[1,2]*

[1]*MOE Key Lab of Cardiovascular Sciences, Department of Biomedical Informatics, Center for Noncoding RNA Medicine, School of Basic Medical Sciences, Peking University, Beijing, China,* [2]*MOE Key Lab of Cardiovascular Sciences, Department of Physiology and Pathophysiology, Center for Noncoding RNA Medicine, School of Basic Medical Sciences, Peking University, Beijing, China*

tRNA-derived fragments (tRFs) constitute a novel class of small non-coding RNA cleaved from tRNAs. In recent years, researches have shown the regulatory roles of a few tRFs in cancers, illuminating a new direction for tRF-centric cancer researches. Nonetheless, more specific screening of tRFs related to oncogenesis pathways, cancer progression stages and cancer prognosis is continuously demanded to reveal the landscape of the cancer-associated tRFs. In this work, by combining the clinical information recorded in The Cancer Genome Atlas (TCGA) and the tRF expression profiles curated by MINTbase v2.0, we systematically screened 1,516 cancer-associated tRFs (ca-tRFs) across seven cancer types. The ca-tRF set collectively combined the differentially expressed tRFs between cancer samples and control samples, the tRFs significantly correlated with tumor stage and the tRFs significantly correlated with patient survival. By incorporating our previous tRF-target dataset, we found the ca-tRFs tend to target cancer-associated genes and onco-pathways like ATF6-mediated unfolded protein response, angiogenesis, cell cycle process regulation, focal adhesion, PI3K-Akt signaling pathway, cellular senescence and FoxO signaling pathway across multiple cancer types. And cell composition analysis implies that the expressions of ca-tRFs are more likely to be correlated with T-cell infiltration. We also found the ca-tRF expression pattern is informative to prognosis, suggesting plausible tRF-based cancer subtypes. Together, our systematic analysis demonstrates the potentially extensive involvements of tRFs in cancers, and provides a reasonable list of cancer-associated tRFs for further investigations.

Keywords: tRNA-derived fragments, cancer prognosis, cancer subtype, bioinformactics analysis, cancer immunity, small non coding RNAs

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***Correspondence:**
Yuan Zhou
zhouyuanbioinfo@hsc.pku.edu.cn

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INTRODUCTION

As its name implied, tRNA-derived fragment (tRF), is a novel class of non-coding RNA (ncRNA) cleaved from mature transfer RNAs (tRNA) (Lee et al., 2009; Thompson and Parker, 2009). In early days, tRFs were widely misunderstood as tRNA degeneration byproducts. However, in recent years, extending scope of tRFs' biological functions had been uncovered, bringing tRFs back to researchers' view (Li et al., 2018). For example, tRFs can be loaded onto Argonaute (AGO) family proteins to perform microRNA-like post-transcriptional regulations on target RNAs (Li et al., 2012; Shao et al., 2017). Some tRFs are also found capable of facilitating ribosome biogenesis by interacting with Twi12

to enhance pre-rRNA processing (Couvillion et al., 2012) or accelerating the mRNA translation of ribosomal proteins (Kim et al., 2017). Besides, some other tRFs were also reported to be capable to reduce global translation efficiency (Yamasaki et al., 2009; Ivanov et al., 2011), regulate immuno-functions (Wang et al., 2006) and serve as epigenetic regulators (Chen et al., 2016).

Comparable to tRFs' functions, their dysregulation could be associated with various diseases such as nonalcoholic fatty liver disease (Zhu et al., 2020), Alzheimer's disease (Wu et al., 2021), arterial injury (Zhu et al., 2021) and especially cancers (Balatti et al., 2017; Shao et al., 2017; Falconi et al., 2019; Zhu et al., 2019; Gu et al., 2020; Zhang et al., 2021). Especially, a series of investigations have uncovered novel associations between tRFs and cancers. For instance, Falconi et al. found that a new tRF derived from the 3′-end of tRNA-Glu is significantly down-regulated in breast cancer, and finally validated its tumor repressive role (Falconi et al., 2019). Zhu et al. compared the plasma tRNA levels between liver cancer patients and healthy donors, and rationally determined four tRFs as diagnostic biomarkers (Zhu et al., 2019). However, in most of the above experimental researches on tRF-cancer associations, only few tRFs and cancer types were considered due to the limitation of low-throughput approaches or sample size. Therefore, specific computational screening of cancer-associated tRFs based on high-throughput datasets is continuously in demand to better understand the roles of tRFs in cancer, including but not limited to tRF dysregulation in various cancer types, key oncogenesis pathways targeted by tRFs and the associations between tRF expression pattern and cancer progression and prognosis.

Recently, Rigoutsos lab established a comprehensive database for human tRFs termed MINTbase v2.0 (Pliatsika et al., 2018), which provides detailed annotations of 26,744 tRFs. More importantly, by re-analyzing the small RNA-sequencing library from TCGA project (Cancer Genome Atlas Research et al., 2013), MINTbase v2.0 also provides 10,814 tRF expression profiles. This dataset provides an unprecedented chance for extensive investigating the characteristics of tRFs in cancer. Indeed, based on this dataset, Rigoutsos lab has revealed a lot of biological characteristics of tRFs in various cancer types. For example, in 2015 they firstly investigated the tRF length distribution in the breast cancer dataset and revealed the tRF expression dependence on race (Telonis et al., 2015). Subsequently in 2018, The tRF profiles of prostate cancer were also uncovered (Magee et al., 2018). In the same year, they constructed a complex tRF-miRNA-mRNA co-expression network for triple-negative breast cancer and then discovered many altered mRNA-mRNA co-expression associations depending on disease state and race. More importantly, this heterogeneity could be largely explained by differential tRF-mRNA co-expression, demonstrating tRFs' important biological functions (Telonis and Rigoutsos, 2018). The investigation of tRF-mRNA co-expression network was soon extended to a pan-cancer scale (32 cancer types) (Telonis et al., 2019), where they identified more tRF-involved pathways and additionally found some pathways are regulated by tRFs in a sex-dependent manner, further highlighting the regulatory roles of tRFs. However, on the other hand, their researches tended to depict the general biological characteristics of tRFs (for example, tRF length variation, sex- and race-dependent disparity and tRF-mRNA co-expression pattern) on the whole set of widely-expressed tRFs, but not focused on the specific screening about tRFs that are associated with oncogenesis, cancer progression and prognosis. Therefore, it is still necessary to investigate the roles of tRFs in cancer by a more specifically designed computational pipeline that combines tRF expression with available clinical data of cancer patients. In this work, based on the annotations from MINTbase v2.0 and the clinical information of TCGA samples, we firstly screened cancer-associated tRFs (ca-tRFs) across seven TCGA cancer types, including breast invasive carcinoma (BRCA), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung squamous cell carcinoma (LUSC) and thyroid carcinoma (THCA). Then, a series of analyses were conducted to define the functional characteristics of ca-tRFs. In the following text, we will firstly describe our integrative analysis pipeline, and then the analysis results and discussion thereof.

MATERIALS AND METHODS

Screening of ca-tRFs

The overall computational pipeline of this work is depicted in **Figure 1**. Datasets and key source code used in this work were also uploaded to the GitHub (https://github.com/Load-Star/ca-tRF/). In this work, we screened ca-tRFs by integrating the tRF expression profiles and the clinical information of the TCGA samples, which were obtained from the MINTbase V2.0 (Pliatsika et al., 2018) database and the TCGA official portal (Cancer Genome Atlas Research et al., 2013), respectively. To adapt downstream analyses, the tRF expression data was log2-transformed and processed with batch effect correction by R package *limma* (Ritchie et al., 2015), where the tissue source site and plate ID of each sample were modeled as the correction covariates. In the following step for ca-tRF screening, we considered three candidate tRF sources: 1) the differentially expressed tRFs between cancer samples and normal samples (referred as cde-tRF hereafter) identified by multivariate limma-trend approach (by R package *limma*), where the tissue type (cancer or normal), patient sex and race were modeled as dummy variables while patient age was modeled as numeric variable; 2) the tRFs significantly correlated with clinical tumor stage (referred as tsca-tRF hereafter) identified by Spearman's rank correlation test (by python package *Scipy*); 3) the tRFs significantly correlated with patient survival (referred as psca-tRF hereafter) detected by multivariate Cox proportional hazards model (by python package *lifelines*), where the patient sex and race were modeled as dummy variables while tRF expression abundance and patient age were modeled as numeric variables. The *p*-values were corrected by Storey's q-value approach. To ensure the robustness of the results, here we only considered the cancer types with sufficient samples and clinical information about tumor stage and survival, including BRCA, HNSC, KIRC, KIRP, LIHC, LUSC, and THCA. Besides, only the tRFs detectable (with an expression threshold of no less than 1 RPM) in no less than 10% samples were retained. In addition, we applied a "two out of three" strategy for further false positive control. More specifically, only if one tRF passed at least two of the total three tests (namely the above-mentioned multivariate limma-trend

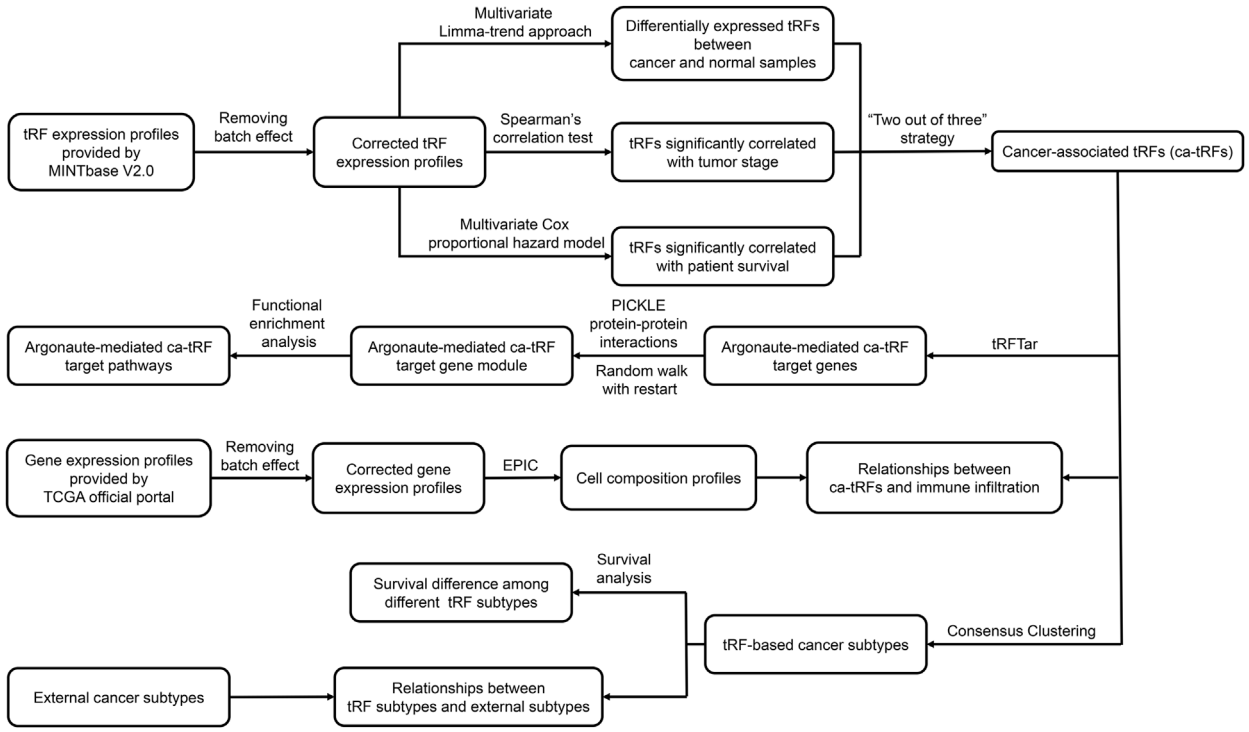


FIGURE 1 | Analysis pipeline of the work. In this work, based on the tRF expression profiles of TCGA samples provided by MINTbase V2.0, we screened cancer-associated tRFs (ca-tRF) for seven cancer types, including BRCA, HNSC, KIRC, KIRP, LIHC, LUSC, and THCA, by considering three candidate tRF sets including the differentially expressed tRFs between cancer samples and normal samples (cde-tRF), the tRFs significantly correlated with clinical tumor stage (tsca-tRF) and the tRFs significantly correlated with patient survival (psca-tRF). The ca-tRFs were further determined by a "two out of three strategy," which means a ca-tRF must belong to at least two of the total three sets. Then we performed extensive analyses on the screened ca-tRF set. First, by incorporating the dataset of Argonaute-mediated tRF-target gene interactions from the tRFTar database, we located the ca-tRF target genes and further the target gene modules. Then by performing functional enrichment analysis on the gene modules, the carcinogenic and cancer-promoting roles of ca-tRFs were inferred. Subsequently, we deconvoluted the gene profiles of TCGA samples *via* the EPIC tool to measure the immune infiltration degree and further demonstrated the immuno-regulatory roles of ca-tRFs. Next, by means of consensus clustering, we identified several ca-tRF-based cancer subtypes. Survival curves exhibit significant survival differences among these subtypes, indicating the prognostic values of ca-tRFs. Finally, to reveal the dependence or independence of ca-tRFs on the other molecular signatures, the relations between ca-tRF subtypes and various existing external subtypes were investigated.

approach, Spearman's rank correlation test and multivariate Cox proportional hazards model), it could be listed as a ca-tRF of the corresponding cancer type. According to our observations, the amounts of tRFs overrepresented by Spearman's correlation test and Cox regression model are much smaller than those overrepresented by limma-trend approach. Therefore, to screen enough ca-tRFs for downstream analyses, a relaxed but acceptable q-value cutoff 0.1 was used during the ca-tRF screening. The integrated list of screened ca-tRFs were shown in **Supplementary Data S1**.

Identification and Functional Investigation of Gene Modules Targeted by ca-tRFs

Like microRNAs, some tRFs are also capable of binding AGO-family proteins and regulate target genes. Recently, we identified a considerable number of tRFs and genes from AGO-associated crosslinking-immunoprecipitation and high-throughput sequencing (AGO-CLIP) libraries and strictly screened AGO-mediated tRF-gene interactions by computationally simulating the base pairing (more specifically, annealing processes) between tRFs and the regions in mRNAs/lncRNAs covered by AGO-CLIP. The interactions satisfying tight base pairing (normalized free energy less than −1.25 kcal/mol) had been deposited into a database named tRFTar (Zhou et al., 2021). Here in order to understand the biological functions that the ca-tRFs may participate in, ca-tRF target genes were retrieved from tRFTar. To control the false positive rate, only the interactions supported by tRF-gene co-expression in TCGA samples were retained. Our hypothesis is that tRFs that 1) show tight base-pairing with potential target genes on Argonaute-covered target regions and 2) show significant expression correlation with potential target genes potentially act as regulatory targeting factors. It is also likely that tRFs themselves could be served as the targets of other regulatory molecules like miRNAs but we here only focused on tRF-gene interactions to avoid much complicated reasoning that involves tRF-miRNA-gene triplexes. Then for each cancer type, taking the ca-tRF target genes as seed genes, an extended gene module targeted by ca-tRFs was detected by the algorithm of random walk with restart (RWR) on the protein-protein

interaction (PPI) network. The PPI network was constructed from 214,666 cross-validated interactions in the PICKLE 3.0 database (Dimitrakopoulos et al., 2020). The RWR algorithm could be described as the following steps:

(1) Construct an adjacent square matrix A to represent whether the gene i (the i-th row) and gene j (the j-th column) are directly adjacent in the network:

$$A_{i,j} = A_{j,i} = \begin{cases} 1, \ gene\, i \in Neighbor\left(gene\ j\right) \\ 0, else \end{cases} \quad (1)$$

where *Neighbor(gene j)* represents the gene set directly adjacent to gene j in the network, including gene j itself.

(2) Construct a transition matrix T to represent the probabilities from gene i (the i-th row) to gene j (the j-th column) in an iteration:

$$T_{i,j} = \frac{A_{i,j}}{\sum_{k}^{n_{gene}} A_{i,k}} \quad (2)$$

where n_{gene} represents the total number of genes in the network.

(3) Construct a column vector e as the input to represent the initial probabilities of seed genes (ca-tRF target genes here):

$$e_i = \begin{cases} 1/n_{seed} & , \quad gene\, i \in \{seed\ genes\} \\ 0 & , \quad else \end{cases} \quad (3)$$

where n_{seed} represents the total number of input seed genes.

(4) Perform iterations according to the following formula until the s achieves convergent:

$$s^{n+1} = \begin{cases} e & , \quad n = 1 \\ c \cdot e + (1-c) \cdot \left(T^T s^n\right) & , \quad n \neq 1 \end{cases} \quad (4)$$

where n represents the iteration number and c is exactly the restart probability. If $c = 1$, then the s will be constantly equal to e; if $c = 0$, then the RWR model will reduce to traditional random walk model. In this work, a moderate $c = 0.5$ was selected to balance the local properties of the input and the global properties of the network.

(5) Determine the genes with top probabilities in the convergent s for downstream analyses, here we selected the top genes whose cumulative probability reach at 0.25 as the resulting gene module.

Next, we conducted gene functional enrichment analyses (by Fisher's exact test) for the gene modules so that potential functional roles of ca-tRFs playing in cancers could be inferred. Firstly, according to a list of 711 cancer-associated genes provided by the NCG v6.0 database (Repana et al., 2019), we evaluated the enrichment degree of cancer-associated genes in the modules. Then, the R software package *clusterProfiler* (Yu et al., 2012) was used to perform enrichment analyses of Gene Ontology Biological Process (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) to investigate the biological processes and pathways that the gene modules are involved in. After semantic deduplication, the terms with q-value no more than 0.05 were deemed as the statistically significant results.

Estimation of Immune Infiltration by Gene Expression Deconvolution

We downloaded tumor samples' gene expression data from the TCGA official portal and then deconvolute them into quantitative cell composition lists by the EPIC tool (Racle et al., 2017). EPIC supposes different cell types prominently express their own cell type marker genes. Given the built-in marker genes' abundance of several pre-defined cell types (T cell, B cell, macrophage, natural killer cell, cancer-associated fibroblast and endothelial cell), the absolute proportions of these cell types inherent in the inputted TCGA bulk gene expression profiles can be solved by constrained linear model. Here the immune infiltration degree was quantified by the proportions of T cells.

Identification and Analysis of ca-tRF-Based Cancer Subtypes

By using the consensus clustering computational framework provided by the R package *ConsensusClusterPlus* (Wilkerson and Hayes, 2010), we clustered tumor samples' ca-tRF expression profiles and assigned subtype labels to each tumor sample according to the clustering result (**Supplementary Data S2**). Consensus clustering is a widely used algorithm in biological clustering problems (Niu et al., 2016; Lu and Leong, 2018; Zhang et al., 2018). For a consensus clustering task, a basic clustering method needs to be assigned and here the PAM clustering algorithm, an improved version of K-mean clustering which is more robust to noises and outliers, was adopted. The principle of consensus clustering is measuring the distances within every sample-sample pairs by their probabilities of being clustered into the same group (called "consensus index" below) in several times of sub-sampling processes. Specifically, a sub-sampling proportion p (0.8 here) and times T (100 here) are required to be pre-defined. Then for each time, a subset of samples will be randomly selected according to p and then clustered by the PAM clustering algorithm. The sub-sampling process disturbs the original data structure to an extent, thus if two samples are objectively similar, they will resist this disturbance and present a high consensus index. On the contrary, two dissimilar samples will exhibit low consensus indexes. Therefore, if the clustering performed well, the overall consensus indexes of sample-sample pairs will be concentrated at 0 (for samples belonging to different groups) or 1 (for samples belonging to the same group). According to this property, we rationally determined the cluster number k for each cancer, namely the number of ca-tRF-based subtypes, where a smaller slope of the cumulative distribution curve between the interval of (0.2, 0.8) was preferred. In case the slopes are comparable among different k choices, the area under the cumulative distribution curve, which is usually approximating to the maximum when the k saturated, was considered as the secondary metric for the optimization of k.

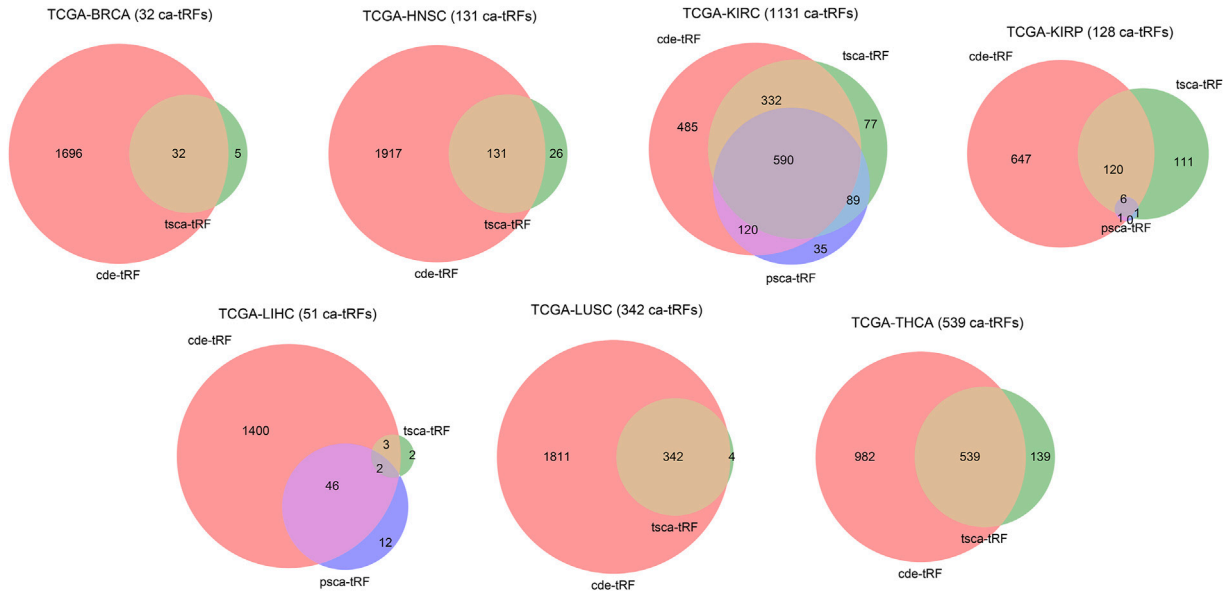


FIGURE 2 | Venn diagram illustrating the intersections of three candidate ca-tRF sources. Three candidate ca-tRF sources, including the differentially expressed tRFs between cancer samples and normal samples (cde-tRF), the tRFs significantly correlated with clinical tumor stage (tsca-tRF) and the tRFs significantly correlated with patient survival (psca-tRF) were considered. For each cancer, the tRF numbers of these three sets and their intersections are shown by Venn diagram. If one tRF belongs to at least two sets (i.e., appears in any intersection area), then it was listed as a ca-tRF of the corresponding cancer. The finally determined numbers of ca-tRFs are shown following the cancer names.

After clustering, the timeline-dependent survival rates of distinct subtypes were visualized by Kaplan-Meier plot for each cancer type using the R packages *survival* and *survminer*. Meanwhile the overall and specific inter-subtype survival differences were evaluated by log-rank test (**Supplementary Data S3**). For KIRC, KIRP, and LIHC, we also collected previous subtyping results based on the other indicators such as mRNA, microRNA, methylation level and so on (**Supplementary Data S4**) (Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research, 2017; Ricketts et al., 2018) to check their accordance and discrepancy with ca-tRF-based subtypes. The overlapping significance between ca-tRF subtypes and the other subtypes were measured by Fisher's exact test. We further calculated enrichment scores (ES) for LIHC samples in 236 biological pathways from KEGG and Molecular Signatures Database Hallmark (MSigDB Hallmark) (**Supplementary Data S5**), by single sample gene set enrichment analysis (ssGSEA) (Barbie et al., 2009). The ESs among different cancer subtypes were compared by Kruskal-Wallis test (for multi-group comparison) or Mann-Whitney test (for two-group comparison) to measure the differences of pathway activities. For all above statistical tests, a p-value cutoff 0.05 was used.

RESULTS

Overview of ca-tRFs Across Seven TCGA Cancer Types

In this work, we screened ca-tRFs for seven TCGA cancer types with sufficient samples and clinical information, including BRCA, HNSC, KIRC, KIRP, LIHC, LUSC, and THCA. Three tRF sets, including the differentially expressed tRFs between cancer samples and normal samples (cde-tRF), the tRFs significantly correlated with clinical tumor stage (tsca-tRF) and the tRFs significantly correlated with patient survival (psca-tRF), were firstly constructed as candidate sources of ca-tRFs (See Materials and Methods). If one tRF is detectable (no less than 1 RPM) in no less than 10% samples and exists in at least two sets, then it was determined as a ca-tRF (**Figure 2**; **Supplementary Data S1**). We firstly investigated the major parental tRNA isoacceptors from which these ca-tRFs were derived by using Fisher's exact test. As the result, the ca-tRFs are found widely originated from the tRNA Val (TAC) and Arg (CCT) while the tRNA Leu (CAG) and Gly (CCC) could serve as secondary sources of ca-tRFs (**Figure 3A**). Then we defined cancer-specific tRFs and cancer-general tRFs as the ca-tRFs identified in only one cancer and those identified in no less than two cancers, respectively (**Figure 3B**). For all cancer types, a considerable proportion of cancer-general tRFs were observed. For comparison purpose, we also defined the tRFs staggered no more than 2 nt with the general ca-tRFs in their parental tRNAs as the tRFs near cancer-general tRFs. In the same way, tRFs staggered more than 2 nt with the general ca-tRFs in their parental tRNAs were defined as the tRFs far away from the cancer-general tRFs. By procedure, we found that for most cancers the cancer-specific tRFs significantly prone to be derived near the general ones in comparison with background (Fisher's exact test; **Figure 3C**), outlining the sequence similarity and therefore the potential functional similarity between cancer-specific and cancer-general tRFs. Inspired by this point, we visualized the cleavage site distribution of tRFs in mature

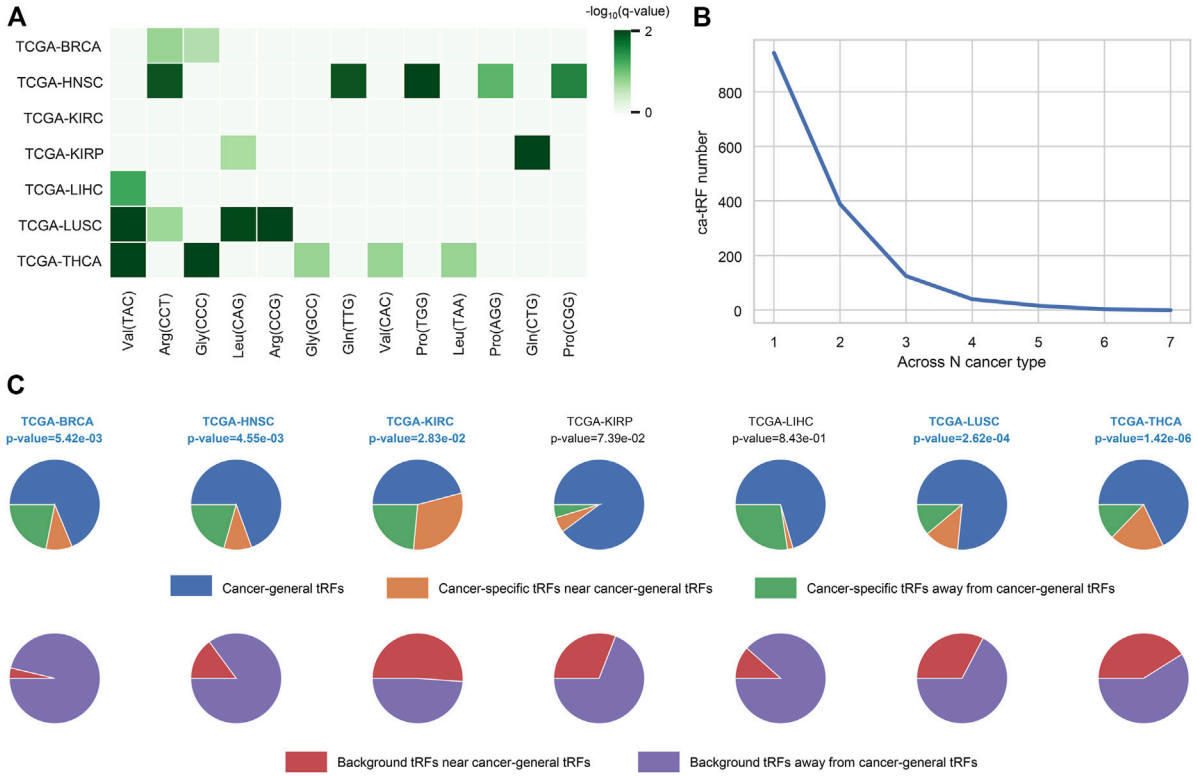


FIGURE 3 | Overview of ca-tRF distribution. **(A)** The major parental tRNA isoacceptors of ca-tRFs of each cancer type. **(B)** The distribution of associated cancer type number among ca-tRFs. **(C)** The proportions of the cancer-general ca-tRFs (blue), the cancer-specific ca-tRFs near the general ca-tRFs (yellow) and the cancer-specific ca-tRFs away from the general ca-tRFs (blue). For comparison, the pie charts depicting the proportions of background tRFs near (red) and far away from (purple) the general ca-tRFs are also shown below. Here the "near" and "away from" are defined by whether the two compared tRFs are staggered with each other no more than or more than 2 nt in their parental tRNA, respectively. The statistical significance about whether the cancer-specific ca-tRFs are tending near to the general ca-tRFs than the background was measured by Fisher's exact test and the significant cancer types were highlighted with blue and bold font.

tRNAs (**Supplementary Figure S1**). Firstly, the background tRFs sourced from the middle or middle-latter sections of tRNAs are relatively less than those from the other sections, which is accordant with previous reports (Telonis et al., 2019). Further, in comparison with the background, the ca-tRFs of BRCA, HNSC, KIRP are found more likely from the 5′ half parts of mature tRNAs whereas the ca-tRFs of LUSC and LIHC prone to come from the 3′ half parts. That is to say, the cancer-associated tRFs may exhibit more prominent location bias in comparison with background tRFs for several cancer types.

We also noticed 19 most widely identified general ca-tRFs that are presented in at least five cancer types (**Supplementary Data S1**). As an instance, the statistical results of tRF-28-RS9NS334L2DB, a ca-tRF across six cancer types, are depicted in detail in **Figure 4**. Specifically, tRF-28-RS9NS334L2DB is significantly differential expressed in all considered seven cancer types when comparing cancer samples with normal control, and in most cancers (i.e., BRCA, HNSC, LIHC, LUSC, and THCA), an up-regulation was observed (bar plots in **Figure 4**). tRF-28-RS9NS334L2DB expression abundance was also found significantly positively correlated with clinical tumor stage in 5 cancers (HNSC, KIRC, KIRP, LUSC and THCA; correlograms in **Figure 4**) and poor prognosis in 2 cancers (KIRC and LIHC; Kaplan-Meier plots in **Figure 4**). Therefore, tRF-28-RS9NS334L2DB may be positively indicative in a noticeable range of cancer onset and development.

ca-tRFs Tend to Target Cancer-Related Pathways and Associate With Tumor Immune Infiltration

After the ca-tRF screening, we further investigated their potential functions. In consideration of tRFs' microRNA-like, AGO-dependent mRNA targeting ability, we firstly asked whether the target genes of ca-tRFs could participate in important biological processes related to cancer. According to the AGO-mediated tRF-gene interactions recorded in the tRFTar database (Zhou et al., 2021), for each cancer type we detected a significant gene module targeted by ca-tRFs by means of the RWR model on the PPI network (See Materials and Methods). We first noted that the ca-tRF target modules are enriched with the curated cancer-associated genes (**Figure 5A**), demonstrating the dysregulation of ca-tRFs is likely to contribute to oncogenesis and cancer development. Subsequently, referring to the gene sets of GO-BP and KEGG, we performed functional enrichment analysis for each ca-tRF target module (**Figure 5B–C**). Extensive terms well-

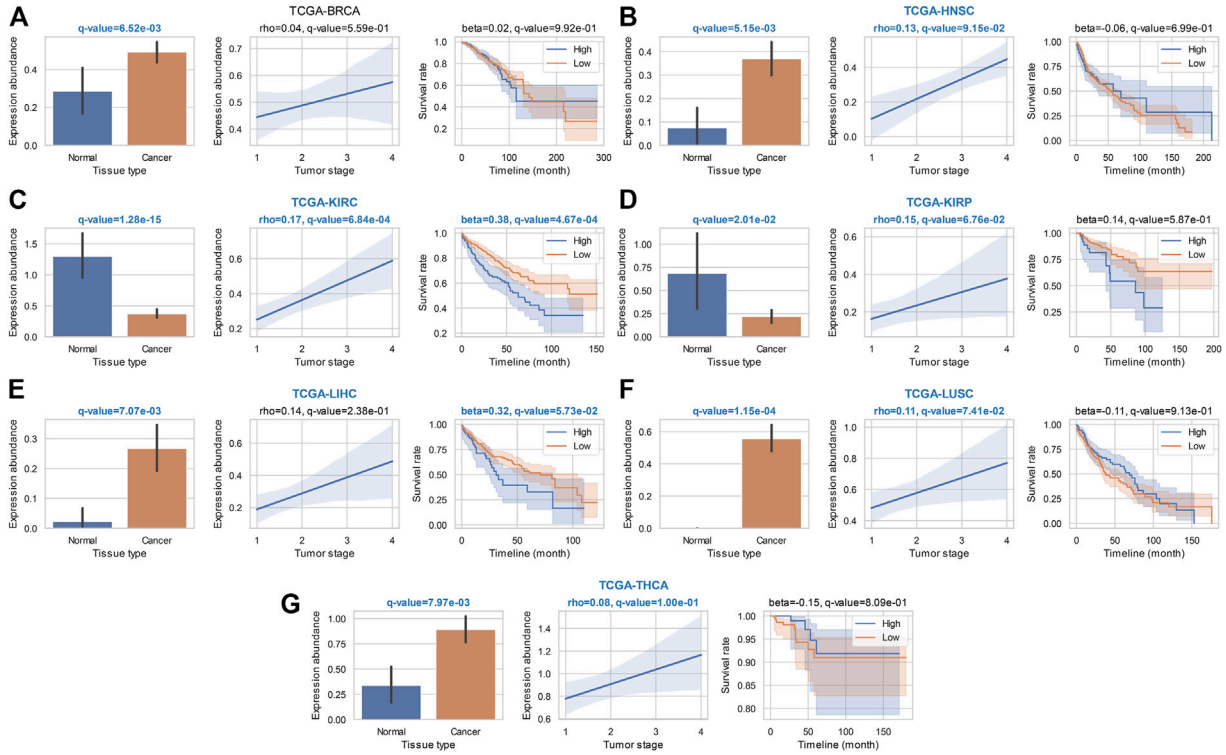


FIGURE 4 | Statistical results of a widely identified ca-tRF (tRF-28-RS9NS334L2DB) in ca-tRF screening. Bar plot (with error bars), correlogram, Kaplan-Meier plot for tRF-28-RS9NS334L2DB in the statistical tests of cde-tRF, tsca-tRF, and psca-tRF screening are respectively depicted and q-values as well as key indicators (i.e., rho of Spearman's correlation and beta of Cox regression) are shown above the plots. The significant q-values (<0.1) are highlighted with blue and bold font. Meanwhile, if tRF-28-RS9NS334L2DB is listed as ca-tRF in one cancer type (two out of three sets), the cancer type name is also highlighted. The results in **(A)** BRCA; **(B)** HNSC; **(C)** KIRC; **(D)** KIRP; **(E)** LIHC; **(F)** LUSC, and **(G)** THCA are shown accordingly.

known to be associated with cancer such as ATF6-mediated unfolded protein response (Lin et al., 2021), blood vessel development (Carmeliet and Jain, 2011), regulation of cell cycle process (Evan and Vousden, 2001), focal adhesion (Eke and Cordes, 2015), PI3K-Akt signaling pathway (Martini et al., 2014), cellular senescence (Campisi, 2013) and FoxO signaling pathway (Farhan et al., 2017) are presented across multi-cancer types, further supporting the associations of ca-tRFs with the cancer-related functions. It should be noted that among these terms, blood vessel development, regulation of cell cycle process, focal adhesion and PI3K-Akt signaling pathway are also identified by Telonis et al. as the pathways universally regulated by tRFs (Telonis et al., 2019). But on the other hand, there are still novel tRF target pathways that are not previously overrepresented such as ATF6-mediated unfolded protein response, cellular senescence and FoxO signaling pathway. This result also highlights the importance of specific screening the pathways that are likely disturbed by tRFs through the extensive tRF-target analysis.

The functional enrichment results also revealed a few immuno-pathways and infectious processes like myeloid leukocyte activation, hepatitis, human T-cell leukemia virus 1 infection and bacterial invasion of epithelial cells, implying plausible immuno-regulatory roles of ca-tRFs. To further explore this point, we estimated the T-cell infiltration degree of tumor samples by gene expression deconvolution (See Materials and Methods) and further studied its correlation with ca-tRF expression abundance for each cancer type. As expected, ca-tRFs exhibit significantly higher correlations with T-cell infiltration than other tRFs (**Figure 6**). Considering the significance of T-cell infiltration in cancer prognosis (Gu-Trantien et al., 2013; Ge et al., 2019; Zhang et al., 2019), this result indicates the closer link between ca-tRFs and tumor immunity.

ca-tRF-Based Cancer Subtypes are Informative to Prognosis

Given the above-mentioned associations of ca-tRFs with cancer-related pathways and tumor immune infiltration, we further tested whether the ca-tRFs, if collectively investigated, could serve as an informative indicator of cancer prognosis. According to the ca-tRF expression profiles, we clustered the tumor samples under the consensus clustering framework (See Materials and Methods). Consequently, we identified five subtypes for HNSC, LIHC, and LUSC, six subtypes for KIRC, seven subtypes for BRCA and THCA and nine subtypes for KIRP (panels A–B of **Figures 7**, **8** and **Supplementary Figures S2–S6**; **Supplementary Data S2**). Among these cancers, HNSC, KIRC, KIRP, and LIHC exhibit significant survival differences among

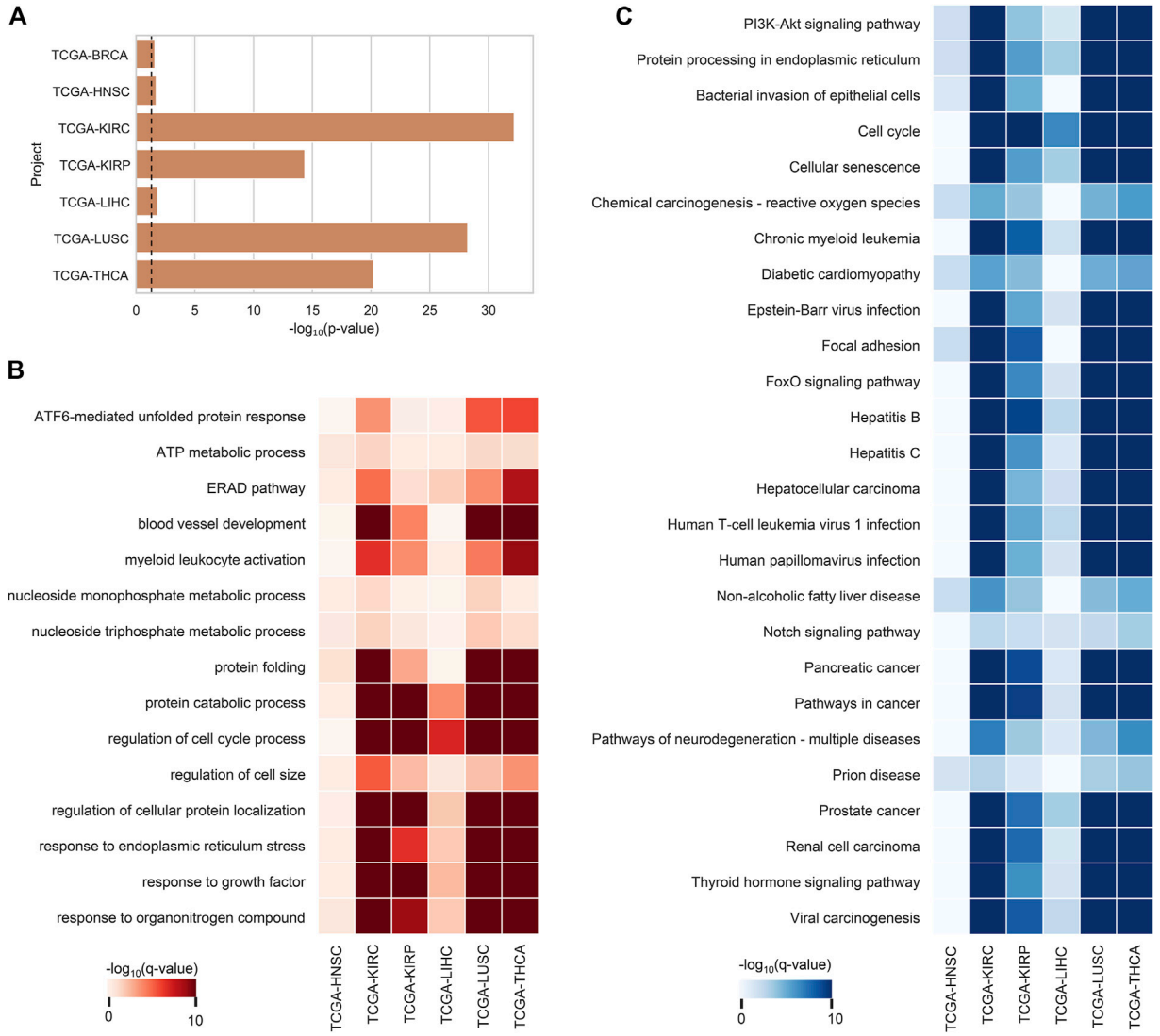


FIGURE 5 | Enrichment analysis results of ca-tRF target gene modules. **(A)** The enrichment degree of cancer-associated genes (curated by NCG v6.0 database) in ca-tRF target gene modules. The dashed line represents the statistically significant cutoff (p-value < 0.05). **(B)** The GO-BP enrichment results of tRF target gene modules. Only the significant results (q-value < 0.05) are colored and only the terms presented in no less than 6 cancer types are depicted. **(C)** The KEGG enrichment results of tRF target gene modules. Only the significant results (q-value < 0.05) are colored and only the terms presented in no less than 5 cancer types are depicted. BRCA is not shown in subplot B and C because there are no significant terms.

subtypes in overall, and the differences are especially obvious in KIRC and LIHC (p-value less than 0.0001) (panel C of **Figures 7**, **8** and **Supplementary Figures S2–S6**), suggesting the indicative role of ca-tRF-based subtyping in cancer prognosis. By contrast, the prognostic divergence is not significant in BRCA, LUSC, and THCA. Previous researches had reported the phenomenon of tRF expression disparity depending on sex and race (Telonis et al., 2015; Magee et al., 2018; Telonis and Rigoutsos, 2018; Magee and Rigoutsos, 2020). Inspired by this point, we further grouped the ca-tRF subtypes by sex and race (only the sex and race with sufficient samples considered) to investigate whether the disparity is also presented in survival rate (panel D of **Figures 7**, **8** and **Supplementary Figures S3–S4**). BRCA, LUSC, and THCA were not included in this analysis because their original ca-tRF subtypes are failed to show decent survival differences. As a result, the patterns of survival curves among different subtypes are still maintained when considering specific sex and race, indicating the robustness of ca-tRF subtyping.

Comparison of ca-tRF-Based Subtypes With the External Subtypes

Next, we further surveyed whether the ca-tRF-based subtypes could be linked or independent with the previous subtyping methods. In this comparison, only cancer types with sufficient external subtype data and showing significant prognostic

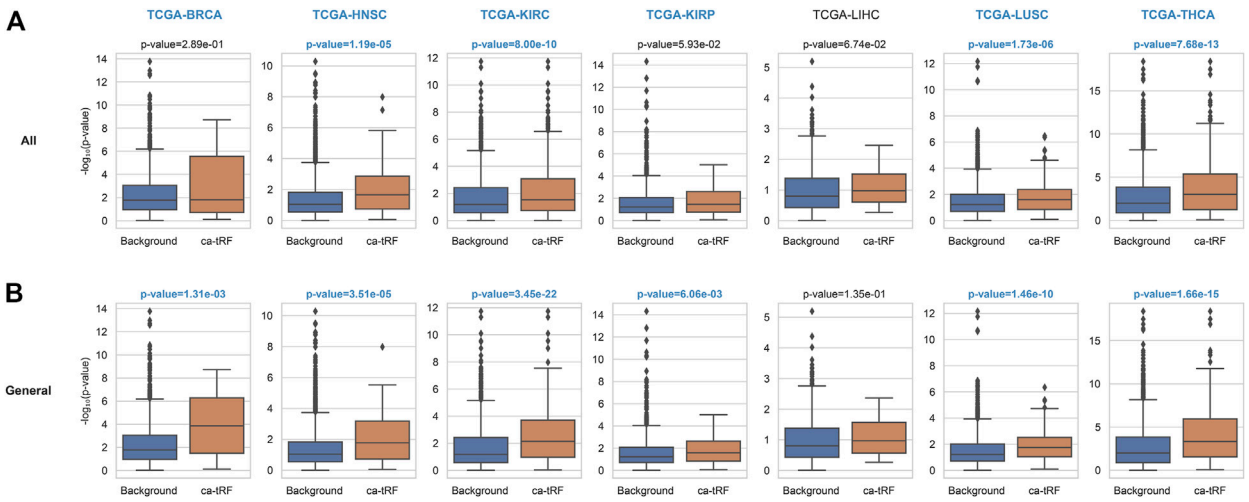


FIGURE 6 | ca-tRFs are more likely to be correlated with T-cell infiltration. The correlation of tRF expression and T-cell infiltration degree are measured by Spearman's correlation test. And the -$\log_{10}$ (*p*-value) distribution of **(A)** all ca-tRFs and **(B)** cancer-general ca-tRFs are respectively compared with background by Mann-Whitney test. The significant *p*-values (<0.05) and cancer names are shown with blue and bold font.

divergence in ca-tRF-based subtyping were considered, containing LIHC (**Figure 9**), KIRC (**Supplementary Figure S7**) and KIRP (**Supplementary Figure S8**). For these three cancer types, various existing external subtypes based on mRNA, miRNA, methylation level and so on were covered in this comparison (**Supplementary Data S4**), including but not limited to the subtypes delineated by TCGA Consortia and identified by non-negative matrix factorization (Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research, 2017; Ricketts et al., 2018). We found most ca-tRF-based subtypes could decently overlap with at least one external subtype. However, as for subtype 2 of KIRC as well as subtype 2 and 6 of KIRP, no matched external subtype was found. Moreover, for all the surveyed cancers, although there are wide overlaps between ca-tRF-based subtypes and external subtypes, few ca-tRF-based subtypes could be fully represented by another external subtype. In other words, there are also detectable independence between ca-tRF subtypes and external subtypes, indicating tRFs' potential to serve as novel prognostic factors that could supplement known subtyping.

To better demonstrate the associations and independence between the ca-tRF-based subtypes and the external subtypes, we further investigated LIHC subtypes in details because 1) ca-tRF-based subtypes show prominent survival differences in LIHC and 2) there are the largest number of external subtypes for reference in LIHC (8 kinds of subtypes in total), which could maximally reflect the relationships between the subtypes based on ca-tRFs and the other indicators. As depicted in **Figure 8C**, in LIHC, ca-tRF subtype 2 is of the best survival (low-risk group), ca-tRF subtypes 1 and 3 are with medium risks (medium-risk group) and ca-tRF subtypes 4 and 5 are the most malignant (high-risk group). Besides, the ca-tRF subtype 2, 4, and 5 significantly overlap with five, four, and six external subtypes, respectively. More importantly, the subtype 2, 4, and 5 were found significantly overlapped with iCluster subtypes (**Figure 9H**), which comprehensively integrate multi-dimensional data including transcriptome, miRNA expression pattern, methylome, proteome and genomic variation. Unlike ca-tRF subtype 2, 4, and 5 that show decent overlap with external subtypes, ca-tRF subtype 1 and 3 only match three and two external subtypes, respectively, and show no significant overlap with any iCluster subtypes, indicating they are relatively independent to the external subtypes (**Figure 9**).

We further noted that samples assigned to different ca-tRF-based subtypes would be associated with different pathways, even in the same iCluster subtype group. To compare the molecular signatures of different ca-tRF subtypes, we evaluated the activities of 236 biological pathways for LIHC samples (**Supplementary Data S5**) by ssGSEA enrichment scores (ES) and further screened 50 most variable pathways among ca-tRF subtypes by Kruskal-Wallis test (see Materials and Methods for more details). The screened pathways could be clustered into four pathway sets: the set 1 involves cell cycle, DNA repair, RNA process and apoptosis; the set 2 involves glycometabolism, mTOR signaling, ROS signaling and unfolded protein response; the set 3 includes many metabolism- and degradation-associated pathways; and the few other pathways were assigned to the pathway set 4 (**Figure 10A**). As expected, further pairwise comparisons demonstrate the pathway activities among ca-tRF subtypes are significantly different and meanwhile the low- and high-risk group exhibit more distinctive signatures than the medium-risk group (**Figure 10A**). More interestingly, we noted that the ES differences in pathway set 1 and 2 are still maintained when specifically considering iCluster subtype 1 (**Figure 10B**) and 3 (**Figure 10C**). That is to say, although the samples within the iCluster subtype 1 or 3 are of similar multi-omics characteristics, they will be distinguishable in many important pathway activities when further considering ca-tRF expression patterns. Such observation indicates that ca-tRF-based subtyping would

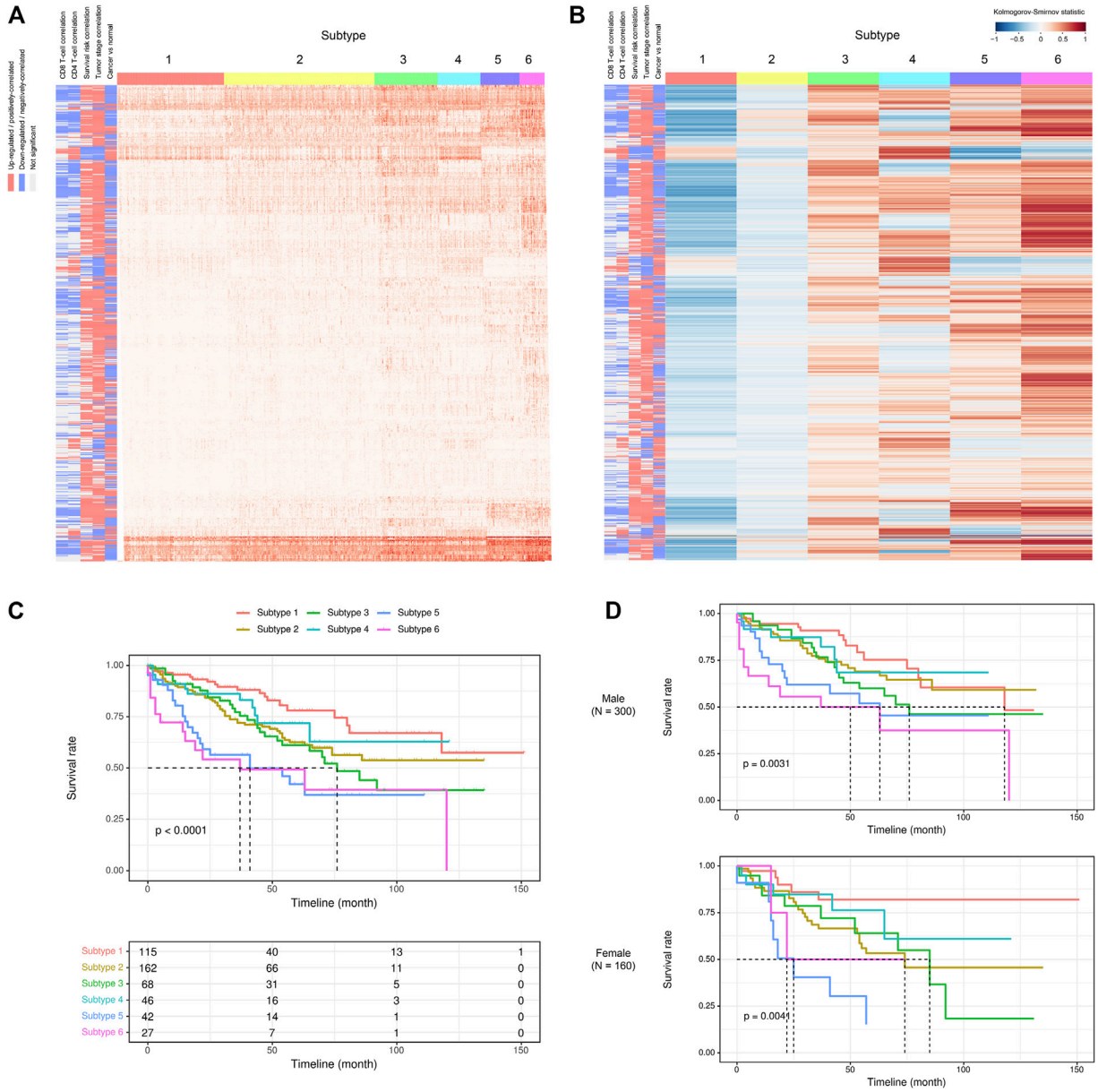


FIGURE 7 | ca-tRF-based subtypes and corresponding survival distinction in KIRC. **(A)** The subtypes suggested by ca-tRF expression pattern. Columns and rows represent samples and ca-tRFs, respectively, and the color blocks along columns and rows represent subtypes and various ca-tRF-related biological features, respectively. **(B)** Heatmap of Kolmogorov-Smirnov statistics showing relative ca-tRF expression abundance across subtypes. **(C)** Kaplan-Meier plots showing the distinction of survival among different subtypes. The risk table showing the sample number at risk is also shown below for reference. **(D)** Sex-specific Kaplan-Meier plots showing the distinction of survival among different subtypes.

specifically reveal the functional characteristics between different sample groups that are independent to the external multi-omics characteristics-based subtyping.

DISCUSSION

As a novel class of non-coding RNA, tRF has been getting increasing attention in recent years. Experimental researches have revealed that some tRFs could serve as biomarkers in some cancers (Falconi et al., 2019; Zhu et al., 2019). However, these low throughput experimental researches are not sufficient to explore the landscape of dysregulated tRFs at the pan-cancer level. In this work, according to TCGA samples' tRF expression data provided by MINTbase v2.0, we screened ca-tRFs for seven TCGA cancer types with a "two out of three" strategy. From the pan-cancer perspective, the ca-tRFs are found significantly derived from the tRNA Val (TAC) and Arg (CCT). Moreover,

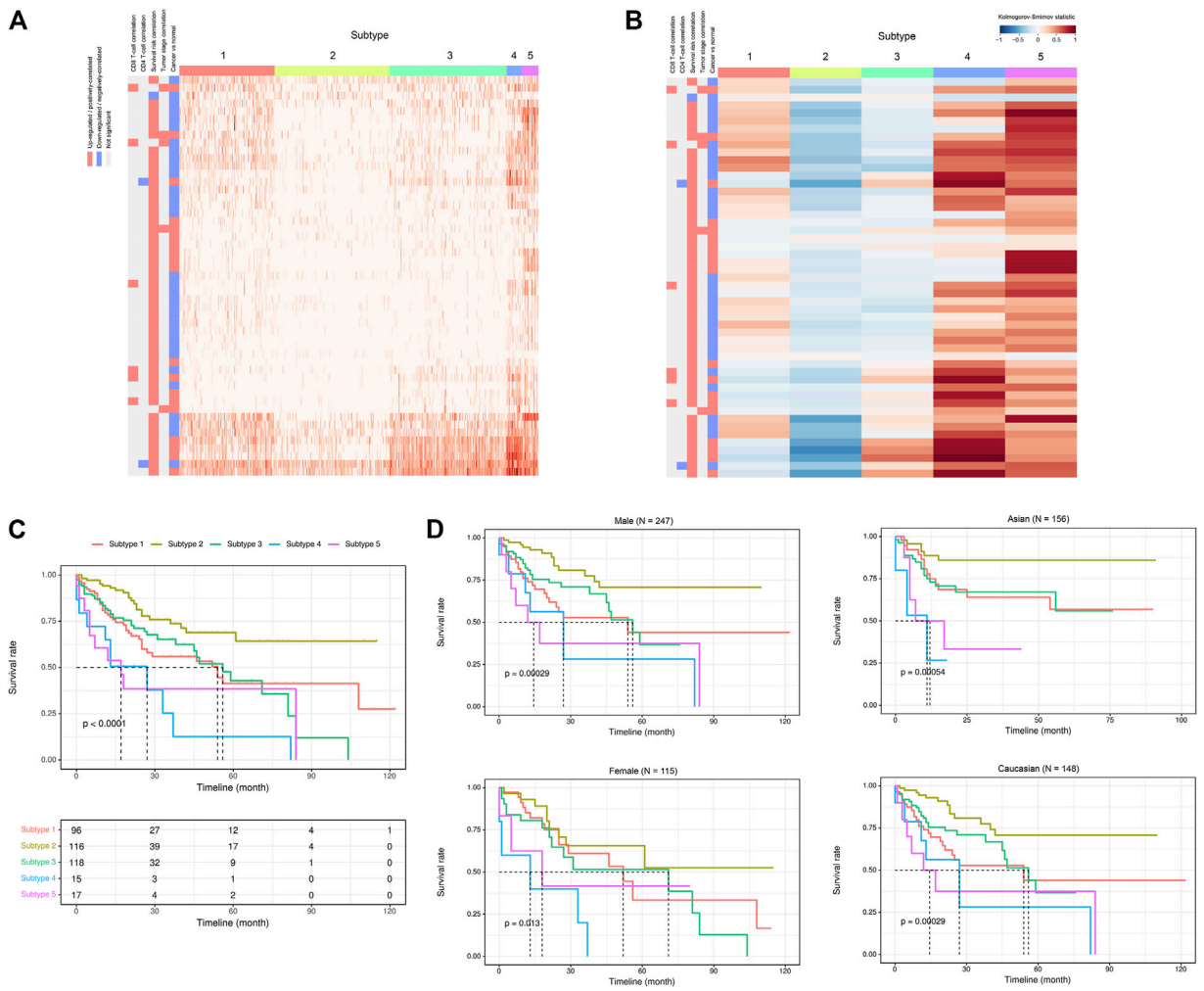
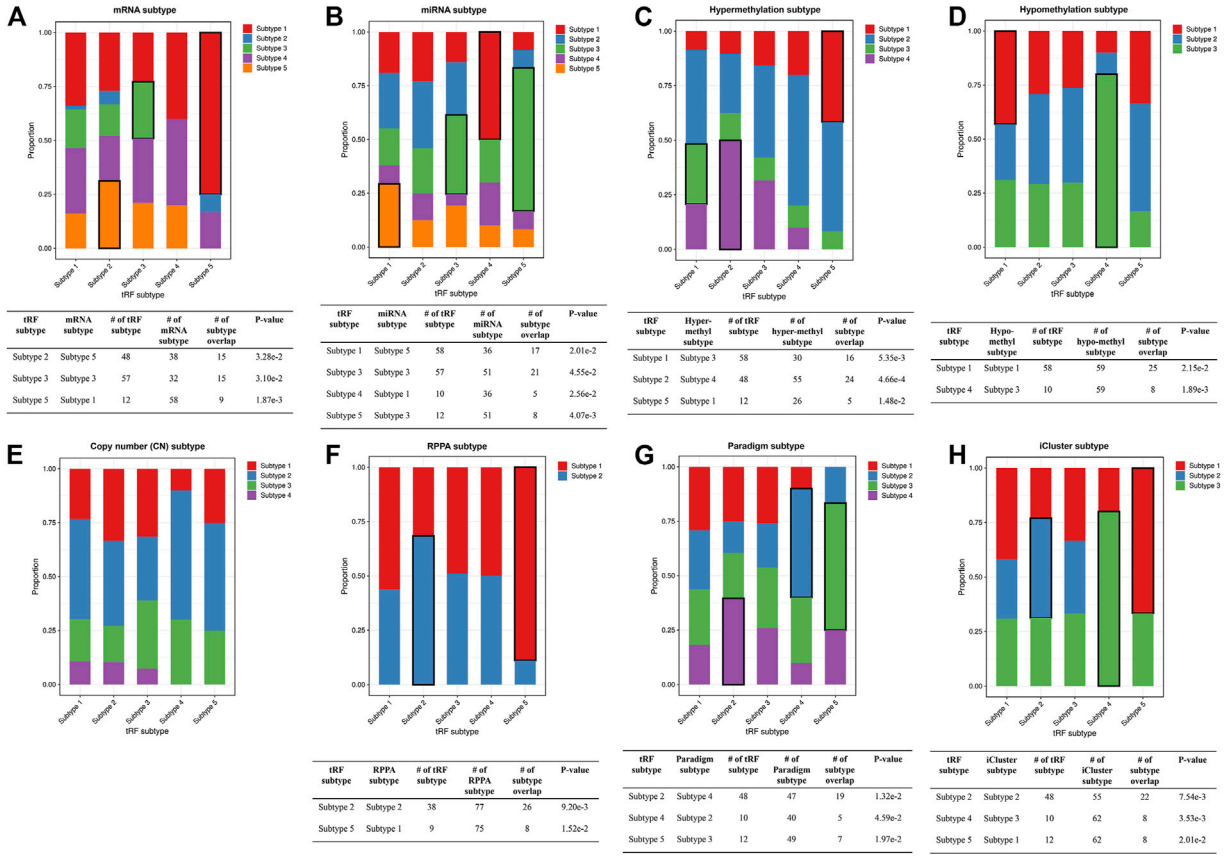


FIGURE 8 | ca-tRF-based subtypes and corresponding survival distinction in LIHC. **(A)** The subtypes suggested by ca-tRF expression pattern. Columns and rows represent samples and ca-tRFs, respectively, and the color blocks along columns and rows represent subtypes and various ca-tRF-related biological features, respectively. **(B)** Heatmap of Kolmogorov-Smirnov statistics showing relative ca-tRF expression abundance across subtypes. **(C)** Kaplan-Meier plots showing the distinction of survival among different subtypes. The risk table showing the sample number at risk is also shown below for reference. **(D)** Sex- and race-specific Kaplan-Meier plots showing the distinction of survival among different subtypes.

we observed that most identified ca-tRFs are presented in multiple cancer types, and more interestingly, a considerable fraction of cancer-specific ca-tRFs are actually derived from the proximal region of cancer-general ca-tRFs on tRNAs. Unlike the biogenesis of canonical RNAs which are directly transcribed from the genome, tRFs are originated from the cleavage of mature tRNAs. Our results indicate the distribution of ca-tRFs on tRNAs are not random, and there are likely "hotspots" in tRNAs to produce ca-tRFs in tumors.

We also noticed that the variation tendency of ca-tRFs in three candidate tRF sets (i.e., cde-tRFs, tsca-tRFs, and psca-tRFs) are not always unidirectional. For example, a tRF may be significantly down-regulated in cancer samples in comparison with normal control but positively correlated with clinical tumor stage (**Supplementary Data S1**). This phenomenon demonstrates some tRFs may play opposite roles in oncogenesis and cancer progression. Meanwhile, it also reflects the advantages of our multi-view screening to capture comprehensive features of ca-tRFs.

Subsequent functional analysis reveals that the ca-tRF target gene modules participate in many oncogenesis and tumor progression-related processes such as ATF6-mediated unfolded protein response, angiogenesis, cell cycle process regulation, focal adhesion, PI3K-Akt signaling pathway, cellular senescence and FoxO signaling pathway. Meanwhile, the ca-tRFs also tend to be correlated with T-cell infiltration in comparison with other tRFs. Both results imply that ca-tRFs could play critical roles in cancer development and thus be of prognostic values. Indeed, cancer subtyping based on the ca-tRF expression pattern exhibit significant differences in survival and the differences are especially obvious in KIRC and LIHC. In addition, the survival patterns are still robust when considering specific sex



tRF subtype	mRNA subtype	# of tRF subtype	# of mRNA subtype	# of subtype overlap	P-value
Subtype 2	Subtype 5	48	38	15	3.28e-2
Subtype 3	Subtype 3	57	32	15	3.10e-2
Subtype 5	Subtype 1	12	58	9	1.87e-3

tRF subtype	miRNA subtype	# of tRF subtype	# of miRNA subtype	# of subtype overlap	P-value
Subtype 1	Subtype 5	58	36	17	2.01e-2
Subtype 3	Subtype 3	57	51	21	4.55e-2
Subtype 4	Subtype 1	10	36	5	2.56e-2
Subtype 5	Subtype 3	12	51	8	4.07e-3

tRF subtype	Hyper-methyl subtype	# of tRF subtype	# of hyper-methyl subtype	# of subtype overlap	P-value
Subtype 1	Subtype 3	58	30	16	5.35e-3
Subtype 2	Subtype 4	48	55	24	4.66e-4
Subtype 5	Subtype 1	12	26	5	1.48e-2

tRF subtype	Hypo-methyl subtype	# of tRF subtype	# of hypo-methyl subtype	# of subtype overlap	P-value
Subtype 1	Subtype 1	58	59	25	2.15e-2
Subtype 4	Subtype 3	10	59	8	1.89e-3

tRF subtype	RPPA subtype	# of tRF subtype	# of RPPA subtype	# of subtype overlap	P-value
Subtype 2	Subtype 2	38	77	26	9.20e-3
Subtype 5	Subtype 1	9	75	8	1.52e-2

tRF subtype	Paradigm subtype	# of tRF subtype	# of Paradigm subtype	# of subtype overlap	P-value
Subtype 2	Subtype 4	48	47	19	1.32e-2
Subtype 4	Subtype 2	10	40	5	4.59e-2
Subtype 5	Subtype 3	12	49	7	1.97e-2

tRF subtype	iCluster subtype	# of tRF subtype	# of iCluster subtype	# of subtype overlap	P-value
Subtype 2	Subtype 2	48	55	22	7.54e-3
Subtype 4	Subtype 3	10	62	8	3.53e-3
Subtype 5	Subtype 1	12	62	8	2.01e-2

FIGURE 9 | Stacked bar plots showing the relations between ca-tRF subtypes and external subtypes in LIHC. Stacked bar plots showing the enrichment of external subtypes (clustered by TCGA Consortia) in ca-tRF subtypes, including **(A)** mRNA subtypes identified by consensus clustering, **(B)** miRNA subtypes identified by non-negative matrix factorization, **(C–E)** hyper-methylation, hypo-methylation and SNP-based copy number subtypes identified by hierarchical clustering, **(F)** protein subtypes (namely, RPPA subtypes) identified by consensus clustering, **(G)** Paradigm subtypes and **(H)** multi-omics subtypes identified by iCluster. The statistical significance was measured by Fisher's exact test with the *p*-value cutoff 0.05 and the significant relations are highlighted by black frames. The sample numbers of subtypes and subtype overlaps presented in the significant relations are also shown in the tables below.

and race, therefore ca-tRFs are potential to serve as universal prognostic factors. We also found most ca-tRF subtypes could decently link with external subtypes. But on the hand, the decent but not prominent overlaps also imply detectable independence between ca-tRF subtypes and external subtypes, indicating ca-tRFs would provide novel predictive clues for cancer prognosis. As a demonstration, we deeply investigated the relationships between ca-tRF subtypes and iCluster subtypes in LIHC, by means of ssGSEA. We found the ca-tRF expression patterns could associate with many pivotal biological pathways such as cell cycle, apoptosis, mTOR signaling pathway and so on regardless of the multi-omics characteristics of iCluster subtype 1 and 3, underlining the important regulatory roles of ca-tRFs.

Our analysis has systematically uncovered the potential roles of tRFs in oncogenesis and development and provided a reasonable ca-tRF list for future researches. However, there are still limitations in current work. The first limitation is in the step of investigating ca-tRF functions by the tRF-target interaction analysis, where we only considered the AGO-mediated tRF-gene interactions based on AGO-CLIP datasets. However, as we describe before, beyond binding with AGO-family proteins, tRFs also have various other functions which may involve some other RNA-binding proteins (RBP) (Couvillion et al., 2012; Kim et al., 2017). But to our best knowledge, so far there has not been tRF dataset focusing on the other RBPs because high-throughput techniques for profiling RBP interactions like CLIP-seq are mainly designed for mRNAs rather than tRFs. Suffering from this limitation, our results could only reveal partial ca-tRF functions in cancer. On the other hand, although there are evidences that the functions of some tRFs are indeed AGO-dependent, AGO-association presented in CLIP data do not necessarily indicate AGO-dependent mechanism-of-action. Therefore, in comparison with some technologies which can directly capture AGO-mediated small RNA-gene interactions (for example, CLASH) (Helwak et al., 2013), CLIP-derived interactions should contain more false positives. However, in the current stage, such CLASH-based datasets like are insufficient (usually identifying ~1,000 tRF-gene pairs) to profile large-scale tRF-gene

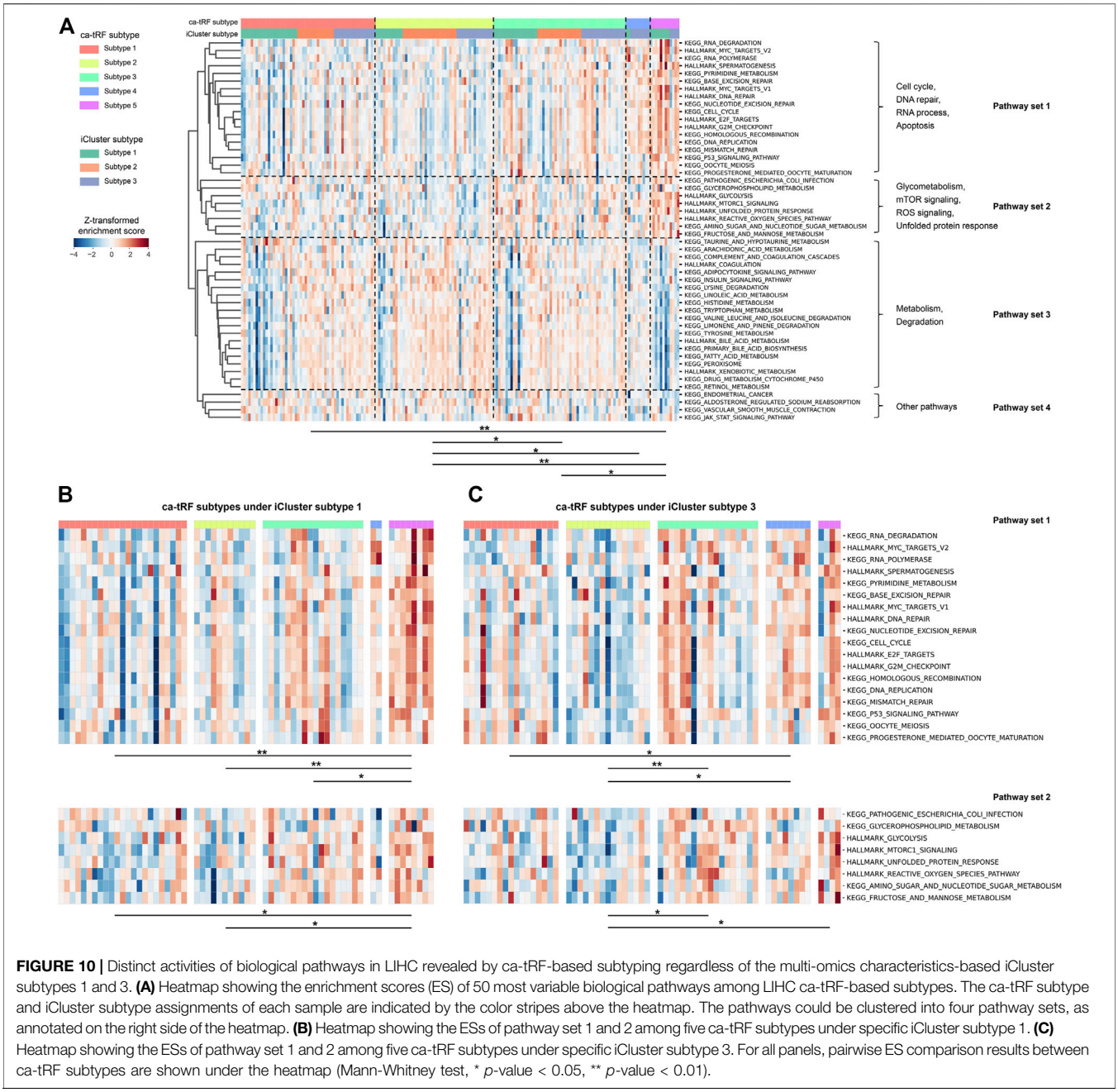


FIGURE 10 | Distinct activities of biological pathways in LIHC revealed by ca-tRF-based subtyping regardless of the multi-omics characteristics-based iCluster subtypes 1 and 3. **(A)** Heatmap showing the enrichment scores (ES) of 50 most variable biological pathways among LIHC ca-tRF-based subtypes. The ca-tRF subtype and iCluster subtype assignments of each sample are indicated by the color stripes above the heatmap. The pathways could be clustered into four pathway sets, as annotated on the right side of the heatmap. **(B)** Heatmap showing the ESs of pathway set 1 and 2 among five ca-tRF subtypes under specific iCluster subtype 1. **(C)** Heatmap showing the ESs of pathway set 1 and 2 among five ca-tRF subtypes under specific iCluster subtype 3. For all panels, pairwise ES comparison results between ca-tRF subtypes are shown under the heatmap (Mann-Whitney test, * p-value < 0.05, ** p-value < 0.01).

interactions. Therefore, we still adopted the more widely-used CLIP datasets, and to reduce false positives, we constrained the results with tight base pairing. In the previous work (Zhou et al., 2021), we validated the tRFs presented in the screened tRF-gene duplexes exhibit much better complementarily pairing abilities with CLIP-peaks than randomly generated small RNAs. Besides, the screened tRF-gene pairs are more prone to be co-expressed than background in TCGA samples, indicating the regulatory roles of these tRFs. Moreover, in accordance with the fact that AGO proteins are prone to bind smaller RNAs, ~89% of the screened interactions involve tRFs less than 24 nt. When filtering the interactions with consistent co-expression in TCGA samples (i.e., the tRF-gene interaction set we used in this work), the ratio of interactions involving smaller tRFs will be dominating (~98%). Overall, it could be rationally inferred that these interactions are with decent reliability. Another thing should be noted that Kumar et al. found the abundance of tRFs loaded on AGO2 are much lower than AGO1, AGO3 and AGO4 (Kumar et al., 2014), based on Hafner el al.'s HEK293 datasets (Hafner et al., 2010), plausibly indicating tRFs' poor AGO2-binding ability. However, we found this phenomenon is not very repeatable in some other datasets such as Vongrad et al.'s human macrophage datasets (Vongrad et al., 2015), Benway et al.'s HK-2 datasets (Benway and Iacomini, 2018) and Hamilton et al.'s DU145 datasets (Hamilton et al.,

2016). For example, in the AGO2-specific PAR-CLIP data of human macrophages generated by Vongrad et al., many tRFs are of hundreds of RPMs (median 150.13 RPMs of top 30 tRFs) and more surprisingly, much more abundant than miRNAs (median 5.80 RPMs of top 30 miRNAs). Higher abundances of tRFs relative to miRNAs were also observed in Benway et al.'s AGO2 PAR-CLIP data (median 32.86 RPMs of top 30 tRFs versus median 1.08 RPMs of top 30 miRNAs) and Hamilton et al.'s AGO2 PAR-CLIP data of DU145 cell line. What's more, some publications have experimentally validated some tRFs should rely on AGO2 protein to perform downstream regulation (Li et al., 2012; Luo et al., 2018; Green et al., 2020). Therefore, the AGO2 protein is still considered in our pipeline.

The second limitation is due to the extreme scarcity of small RNA-sequencing data in public databases, we could not validate the ca-tRF-based subtyping in a totally independent dataset. Therefore, we could only perform validation on the original TCGA sample set. Specifically, for each cancer type, we randomly split the original set into training set and testing set with the ratio of 1:1, and then re-screened ca-tRFs on the training set and re-clustered tumor samples on both training and testing set based on the re-screened ca-tRFs. Subsequently, we measured the set similarity between the original ca-tRF subtypes and the re-clustered ca-tRF subtypes by Ochiai coefficient. As a result, the re-clustered subtypes exhibit decent consistency with the original subtypes, where most cancer types can reach at an Ochiai coefficient of 0.5 in average (**Supplementary Figure S9**). In other words, each original subtype could expectedly match with a highly overlapped re-clustered subtype, reflecting the robustness of ca-tRF-based subtyping. Nevertheless, the ca-tRF subtypes are still required to be further validated when the external resource of small RNA-sequencing is abundant enough in the future.

The third limitation worth discussing is, in this work we mainly surveyed tRF-cancer associations from the perspective of expression pattern. However, in recent years, many computational approaches based on machine learning and graphic theory are proposed to predict the associations between non-coding RNAs and diseases (Chen et al., 2018; Zeng et al., 2018), which provides another approach to discover novel tRF-cancer associations. However, an obligate perquisite of these algorithms is a sizable training dataset derived from prior experimental knowledge. And to our best knowledge, existing publications about tRF and cancer are extremely scarce (actually less than 200, by querying the keywords "tsRNA cancer" and "tRNA-derived fragment cancer" in PubMed). Therefore, in current stage, existing data have not been able to support these algorithms, but we believe it would be feasible with the accumulation of experimental data in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YuZ, YiZ, and QC contributed to conception and design of the study. YiZ performed the analysis and drafted the manuscript. YuZ supervised the study and revised the manuscript. All authors approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.747931/full#supplementary-material

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