STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF CIRCULAR RNAs

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STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF CIRCULAR RNAs

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EDITORIAL
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Editorial: Structural and Functional Characterization of Circular RNAs

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Editorial on the Research Topic

Structural and Functional Characterization of Circular RNAs

Circular RNAs (circRNAs) are a class of covalently closed RNA molecules without any free ends. The existence of circRNAs was first discovered in plant viroids, followed by their discovery in eukaryotic cells by electron microscopy more than 40 years ago (Sanger et al., 1976; Hsu and Coca-Prados, 1979). However, they were mostly considered non-functional splicing byproducts or intronic lariats. Their identification and functional characterization progressed slowly until the development of high-throughput RNA-sequencing methods and novel computational tools (Salzman et al., 2012; Jeck et al., 2013). The last few years have seen an exploding number of studies elucidating the molecular mechanisms of biogenesis and function of circRNAs. Recent discoveries established that circRNAs are generated by the backsplicing of pre-mRNA, mediated by RNA-binding proteins (RBPs) and inverted repeat sequences in the flanking introns (Jeck et al., 2013; Chen and Yang, 2015). Interestingly, circRNAs have been identified in all eukaryotic model organisms and are believed to be ubiquitously expressed, and some of the circRNAs are conserved across species (Jeck et al., 2013). Moreover, the altered expression of circRNAs during development and diseases has been reported, and their extraordinary stability and presence in biofluids make them promising biomarkers for disease diagnosis (Zhang et al., 2018). Increasing evidence suggests that circRNAs regulate the activity of microRNAs and RNA-binding proteins, and some are translated into peptides (Panda et al., 2017; Panda, 2018; Sinha et al., 2021). Recent studies have shown that circRNAs play critical roles in various pathophysiological processes (Lee et al., 2019). Although circRNAs have been established as crucial regulators of gene expression and disease development, many of the molecular details of circRNA biogenesis and function remain to be explored. In addition, only a tiny subset of circRNAs has been functionally characterized among more than a million circRNAs identified thus far (Vromman et al., 2021). Based on the above, this research topic aimed at contributing towards the elucidation of the role of circRNAs in various physiological and pathological conditions.

The review articles in this topic were all directed towards discussing the recent progress in the field of circRNA biology and their relevance in human health. Guria et al. provide an extensive overview of the mechanisms of circRNA biogenesis, classification and nomenclature, methods of detection, and their role in animal and plant physiology. Zhang et al. summarize the expression and regulatory role of circRNAs in plant growth, development, and stress responses. Qin et al. highlight the current knowledge of structure, biogenesis, and function of linear lncRNAs and circRNAs. They also highlight the role of circRNAs as biomarkers for disease diagnosis. Another article by Li et al. discusses the current understanding of circRNA expression and function in the central nervous system. Acha et al. review the current knowledge of circRNAs in blood malignancies and their potential value as diagnostic and therapeutic targets. Rajappa et al. discuss the emerging potential of circRNAs in cancer diagnosis and therapy. Another article by Liu et al. reviews the current knowledge of circRNAs in cervical cancer and the strategies for the use of

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circRNAs in future clinical diagnosis, prognosis, and treatment of cervical cancer. Zucko and Boris-Lawrie highlight the current understanding of circRNAs and the use of translational outcomes of circRNA research to improve the health and productivity of food animals.

The original research articles in this topic expand our understanding of the physiological significance of circRNAs in disease development. Using computational analyses, Khan et al. provide a comprehensive mechanism of gene regulation through circRNA–miRNA–mRNA regulatory networks in various cancers. Li et al. present data demonstrating that *circTLK1* promotes glioma progression by activating JAK/STAT signaling through the miR-452-5p/SSR1 pathway. Liu et al. demonstrate that circGNB1 regulates cell proliferation, migration, and tumor growth in triple-negative breast cancer by regulating the miR-141-5p-IGF1R axis. Han et al. identified differentially expressed circRNAs in the peripheral blood samples of patients with heart failure compared to healthy humans. They also show that hsa_circ_0097435 could act as a sponge for various miRNAs and regulate myocardial cell injury. Zhang et al. identified thousands of circRNAs in the liver of Whitespotted Bamboo Shark and constructed the mRNA–miRNA–circRNA regulatory network for the Glutathione S-transferase P1 gene. Another interesting article by Sun et al. describes the presence of internal complementary base-pairing sequences in extremely long circRNAs, allowing the circRNAs to present in double-stranded or pseudoknot structures. They hypothesize the "open-close effect" which may be a novel molecular function of circRNAs.

In summary, this Research Topic expands our knowledge on the relevance of circRNAs in animal and plant physiology regulation. Also, it discusses the future use of current knowledge for the diagnosis and therapy of human diseases. We hereby thank all the authors for contributing to this exciting Research Topic.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Circular RNAs—The Road Less Traveled

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Circular RNAs are the most recent addition in the non-coding RNA family, which has started to gain recognition after a decade of obscurity. The first couple of reports that emerged at the beginning of this decade and the amount of evidence that has accumulated thereafter has, however, encouraged RNA researchers to navigate further in the quest for the exploration of circular RNAs. The joining of 5′ and 3′ ends of RNA molecules through backsplicing forms circular RNAs during co-transcriptional or post-transcriptional processes. These molecules are capable of effectively sponging microRNAs, thereby regulating the cellular processes, as evidenced by numerous animal and plant systems. Preliminary studies have shown that circular RNA has an imperative role in transcriptional regulation and protein translation, and it also has significant therapeutic potential. The high stability of circular RNA is rendered by its closed ends; they are nevertheless prone to degradation by circulating endonucleases in serum or exosomes or by microRNA-mediated cleavage due to their high complementarity. However, the identification of circular RNAs involves diverse methodologies and the delineation of its possible role and mechanism in the regulation of cellular and molecular architecture has provided a new direction for the continuous research into circular RNA. In this review, we discuss the possible mechanism of circular RNA biogenesis, its structure, properties, degradation, and the growing amount of evidence regarding the detection methods and its role in animal and plant systems.

Keywords: circRNA, biogenesis, long non-coding RNA, miRNA sponging, backsplicing

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INTRODUCTION

Circular RNAs (CircRNAs) have recently spread into the non-coding RNA world. The circRNAs are formed by the covalent circularization of a 3′ downstream donor and the 5′ upstream acceptor in an alternate form of pre-mRNA splicing by a process called backsplicing (Szabo and Salzman, 2016). However, the mechanisms of biogenesis, nuclear export, degradation, and the functional significance of circRNAs, remain unclear or exist as proposed theories. Mounting evidence on the presence of circRNAs in all the organisms tested so far shows that the circRNAs are an integral part of living systems (Salzman et al., 2012, 2013; Memczak et al., 2013; Zhang et al., 2013; Zhang X.-O. et al., 2016; Zhang Y. et al., 2016; Ashwal-Fluss et al., 2014; Starke et al., 2015; Pamudurti et al., 2017; Tan et al., 2017; Yang et al., 2017). Despite this, our understanding of their structural and functional aspects is limited. In this review, we have made an attempt to highlight the promising discoveries that have been made in the field of circRNAs in the recent past.

HISTORY

The first circRNA ever seen by an electron microscope was a plant viroid, and it was subsequently proven to be so due to its circular nature and through the use of various biochemical analyses, such as analyzing its resistance to degradation by snake venom phosphodiesterase and 5′-phosphorylation (Sanger et al., 1976). Similarly, the first ever animal virus reported to contain circRNA was the Hepatitis delta virus (HDV) (Kos et al., 1986). However, the animal kingdom contributed immensely to the understanding of a plethora of diverse avenues within circRNA biology. The 1990s witnessed a few endogenous circRNAs originating from deleted in colorectal carcinoma (DCC) (Nigro et al., 1991), Sex-determining region Y (SRY) (Capel et al., 1993), proto-oncogene ETS-1 (Cocquerelle et al., 1992), Cytochrome P450 2C24 (Zaphiropoulos, 1996, 1997), and Sodium/Calcium exchanger (NCX1) (Li and Lytton, 1999) genes in humans, mice, rats, and monkeys. These were discovered by sequencing the PCR products containing the backsplice junctions with the 5′ exon present downstream of the 3′ exon (Jeck and Sharpless, 2014). Similarly, in the beginning of the 21st century, a muscle blind (MBL) gene from *Drosophila* (Houseley et al., 2006), human antisense non-coding RNA in the INK4 locus (ANRIL) (Burd et al., 2010), and cerebellar degeneration-related autoantigen 1 antisense (CDR1as) (Hansen et al., 2011) genes were reported to express circRNAs. These findings suggested that the formation of circRNAs was an odd and irregular phenomenon during splicing, and this was termed to be a mis-splicing process (Cocquerelle et al., 1993).

The concept of mis-splicing on the formation of circRNAs was changed entirely when thousands of circRNAs were reported to exist independently of different human and mouse cell lines (Salzman et al., 2012; Jeck et al., 2013; Memczak et al., 2013). Since then, circRNAs have been discovered in a wide range of organisms, including Zebrafish (Shen et al., 2017; Liu H. et al., 2019; Sharma et al., 2019), *Caenorhabditis elegans* (Cortés-López et al., 2018), *Saccharamyces cerevisiae*, *Schizosaccararomyces pombe*, *Plasmodium falciparum*, and *Dictyostelium discoideum*. Similarly, the presence of circRNAs was also reportedly found in *Arabidopsis thaliana* (Wang P. L et al., 2014), *Oryza sativa* ssp. *Japonica, Oryza sativa* ssp. *Indica, Nicotiana benthamiana* (Guria et al., 2019), and in 12 other plant species (Chu et al., 2017). However, their identification and characterization reveal that the biogenesis of circRNAs originates through the act of backsplicing and not through mis-splicing, as was reported previously. This growing amount of evidence suggests circRNAs have an important role in the eukaryotic tree of life (Wang P. L et al., 2014).

BIOGENESIS

Numerous circRNAs were serendipitously discovered from scrambled exons in different human cell types (Salzman et al., 2012). These reports have suggested that circRNA may significantly contribute to exon scrambling, but all scrambled exons need not necessarily be circRNAs. The two most pivotal models of circRNA biogenesis are by direct backsplicing and exon skipping or by lariat intermediate formation (Chen and Yang, 2015) (**Figure 1**). Both models give rise to circRNAs and linear RNAs from the flanking regions, which raises further questions regarding the frequency of occurrence of one model over another. The exon-skipped linear RNA is either degraded (Egecioglu et al., 2012; Bitton et al., 2015) or results in a truncated protein that is different from the native protein.

Recent studies have led to the discovery of many essential *cis* and *trans* factors that have a positive or negative regulatory effect on circRNA biogenesis (**Figure 1**). CircRNA production requires the joint involvement of spliceosomal machinery and the natural splice sites (Starke et al., 2015) through a co-transcriptional mechanism (Ashwal-Fluss et al., 2014; Huang and Shan, 2015). Hence, competition might occur between the canonical splicing and backsplicing mechanisms in the same sequence to form linear mRNA or circRNA, respectively (Ashwal-Fluss et al., 2014; Chen and Yang, 2015). The presence of roughly 1% of circRNAs among mRNAs reveals that canonical splicing is more prominent than backsplicing (Salzman et al., 2013). However, post-transcriptional regulation of circRNA biogenesis is also reported in Fused in Sarcoma (FUS) gene-depleted motor neurons *in-vitro* (Errichelli et al., 2017). Mutations in natural splice sites from 5′GU to 5′CA decreases circRNA production (Ashwal-Fluss et al., 2014). *In-vitro* studies using single exon minigenes show that, when both the 5′ and 3′ splice sites are mutated, the spliceosomal machinery is inclined toward the next cryptic splice site, which leads to an increase or decrease in the circumference of the circle (**Figure 2**). It may ultimately result in weakening of the circularization efficiency. On the other hand, it has also been validated that any sequence can be circularized if the last three nucleotides in the 5′ and 3′ spliceosomal recognition sites remain unchanged (Starke et al., 2015). Conversely, most of the plant circRNAs are joined by non-canonical splice sites (Ye et al., 2017; Chu et al., 2018a,b; Guria et al., 2019); the probable reason for this could be the flexibility in binding of the spliceosome machinery. Due to high complementarity, the microRNA (miRNA)-mediated cleavage of circRNAs is possibly another striking reason for the lower number of circRNAs in plants, as shown in *Vitis vinifera* L. (Gao et al., 2019). Moreover, the identification of miRNA binding and cleavage sites in circRNA, either by rapid amplification of cDNA ends (RACE) or degradome sequencing, is difficult due to lack of a 5′ cap and 3′ poly-A tail. This is compelling evidence, and there might yet be other unidentified mechanisms involved in the biogenesis of circRNA in plants (Chu et al., 2018a,b). Overall, the biogenesis of circRNA is regulated by spliceosomes and the recognition of both the canonical and non-canonical splice junctions. This eventually results in the biogenesis of different types of circRNAs, such as exon–intron circRNA, exon-intergenic circRNA, etc. (**Table 1**), which are all backspliced from different genomic regions.

The role of *cis*-flanking sequences cannot be avoided in the circularization of intervening sequence(s). Flanking introns or exons are found to be much longer (∼3 fold) in circularizing the intervening exonic or intronic sequences, respectively, than

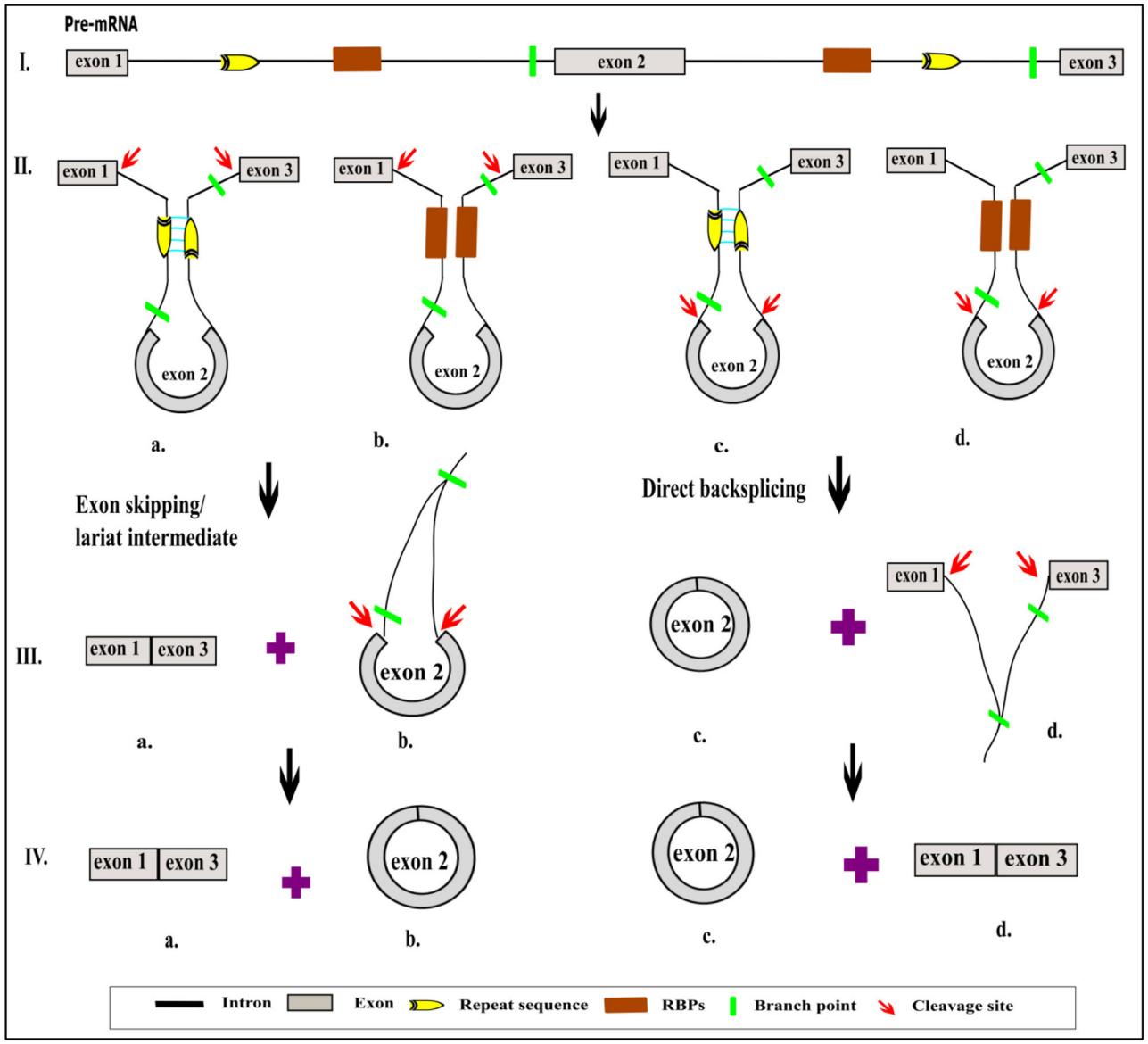

FIGURE 1 | Schematic representation of role of *cis* sequence and *trans* factor in different models of circRNA biogenesis. Normally, the pre-mRNA is enriched with the exonic and intronic region (I). The non-coding intronic region harbors the highly conserved sequence in the 5′ and 3′, which is essential for splicing by spliceosomal machinery (I). In addition to the conserved sequence, flanking introns consist of a repeat sequence or RBP site, which help to bring the 5′ and 3′ ends of intervening exon closer together due to either the base pairing (IIa, IIc) or by the binding of RBP (IIb, IId). Due to this proximity, circRNAs are generated by exon skipping or direct backsplicing (IIIa, IVa). Exon skipping produces linear RNA first, followed by the circularization of an intervening exon along with the formation of a lariat containing flanking introns (IIIb). Subsequent splicing yields a circular exonic RNA (IVb). In contrast, in direct backsplicing, exonic circRNA is generated first (IIIc, IVc) and is then followed by an exon–intron lariat (IIId). The latter is processed further to convert it into linear RNA (IVd). The pictorial representation is not to scale.

the normal corresponding sequences, which generally undergo linear splicing (Jeck et al., 2013; Barrett and Salzman, 2016; Bolha et al., 2017). Indeed, the presence of complementary, reverse complementary, direct repeats (DR), and inverted repeats (IR) indicates that they are enriched in longer flanking introns, which bring the 5′ and 3′ sequences close enough for circularization. The presence of Alu and IRAlu repeats in the ZWILCH locus yields circRNA formation in mice but not in humans, thereby implying that circRNA expression is species specific in nature (Hansen et al., 2011). In plants, however, the miniature inverted-repeat transposable elements (MITES) found in flanking introns of rice exonic circRNAs (Lu et al., 2015), and reverse complementary pairs of LINE1-like elements (LLECRPs) in *Zea mays* (Chen et al., 2018) play a critical role in circRNA biogenesis. Understanding the role of repetitive sequences in flanking regions of circRNAs in polyploid species will be of great

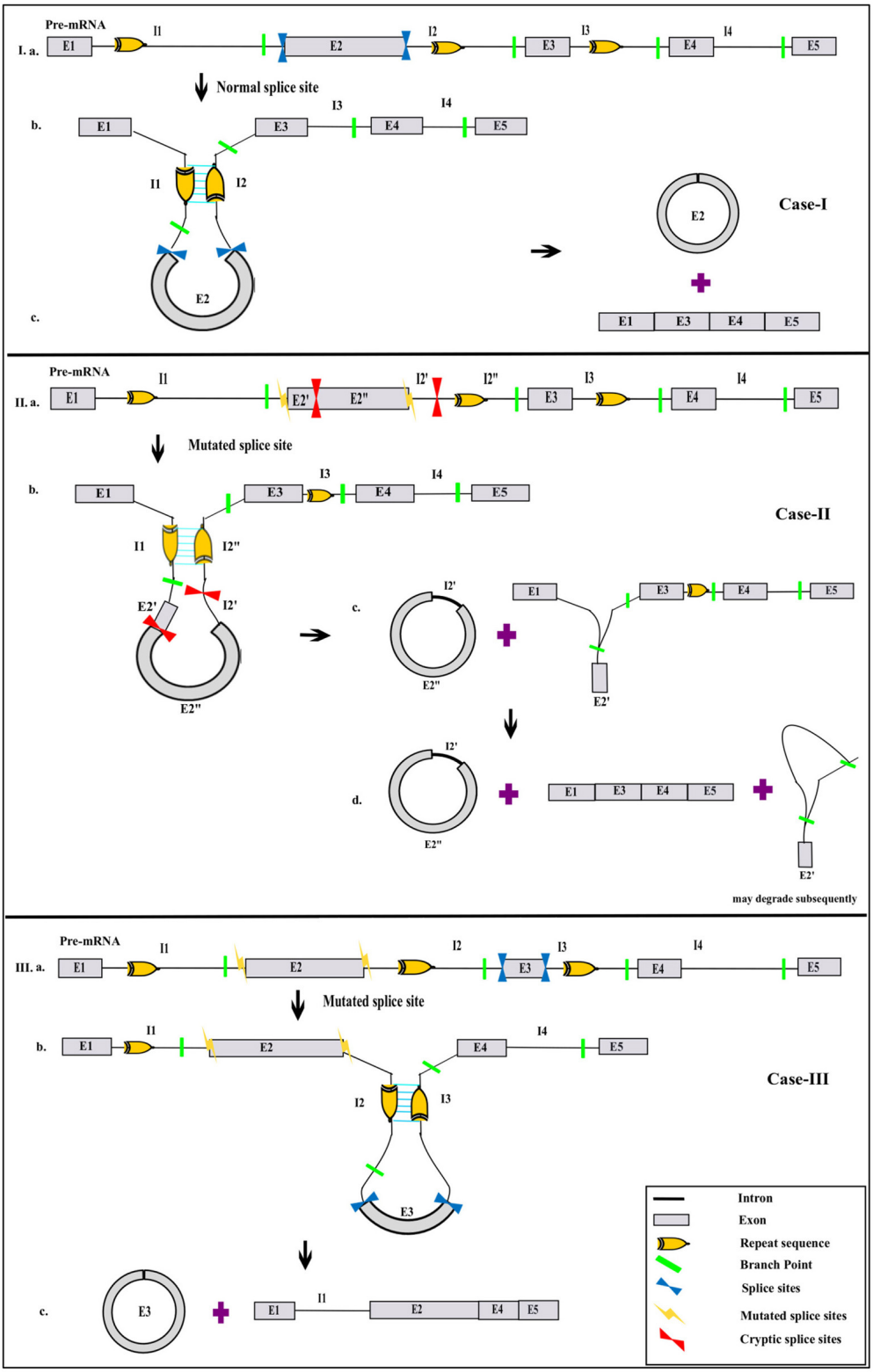

FIGURE 2 | Possibilities of circRNA biogenesis during splice site mutation. Canonical splice sites in exon(s) of pre-mRNA (Ia) surrounded by a repeat sequence in flanking introns brings the 5′ and 3′ ends of intervening exons closer (Ib), thereby resulting in the formation of a linear RNA and a circRNA (Ic). However, mutation in the

(Continued)

FIGURE 2 | natural splice site of pre-mRNA (IIa, IIIa) allows the spliceosome to access the next cryptic splice site possible, present within either an exon or intron (IIb). Cleavage at the cryptic site results in production of circRNA with a shortened exon and an extended intron before the cryptic site, whereas the leftover exon may remain, along with the exon–intron lariat (IIc). Subsequent splicing of the latter leads to the formation of an exon-skipped linear RNA, followed by the possible degradation of a remnant exon (IId). In another scenario of natural splice-site mutation where the immediate cryptic sites are unavailable, the spliceosome may be more inclined toward the next possible splice site of the exon that is flanked by a repeat sequence (IIIb). This may result in the generation of a new exonic circRNA and an exon-skipped linear RNA with intron retention (IIIc). The pictorial representation is not to scale.

TABLE 1 | Types of circRNAs.

CircRNA types	Abbreviation	1st Splice site on	2nd splice site on	Remarks on splice sites
Exonic circRNA	e-circRNA	Exon	exon	It can be on the same or multiple exons
Intronic circRNA	i-circRNA	Intron	intron	Both sites on the single intron
Exon–intron circRNA	ei-circRNA	exon	Intron	1st splice site on exon and 2nd splice site on intron, may or may not be spanned by the intervening exon or/and intron.
Intron–exon circRNA	ie-circRNA	Intron	Intron	On different introns spanned by one or multiple exons
UTR circRNA	u-circRNA	UTR	UTR	Both can be on 5′ or 3′ or there can be one at 5′ and one on 3′
UTR-exon circRNA	ue-circRNA	UTR	Exon	circRNA formed at 5′ or 3′ end
UTR-intron circRNA	ui-circRNA	UTR	Intron	circRNA formed at 5′ or 3′ end with single or multiple exons in between
Intergenic circRNA	ig-circRNA	Intergenic	Intergenic	Both sites on same intergenic region
Intergenic-genic circRNA	igg-circRNA	Intergenic	Genic	Genic could be from exon or intron
Across genic circRNA	Ag-circRNA	Genic	genic	Sites will be spanned by intergenic region

interest to the assessment of genome complexity (Chu et al., 2018b). This is because the repetitive or reverse complementary sequences in longer flanking introns cover only for a limited amount of exonic circRNA production in plants (Ye et al., 2015; Chu et al., 2018a). However, Starke et al. (2015) proved that reverse complementary repeats (RCR) of 103 nucleotides (nt) caused a 1.3-fold increase in the efficiency of circularization, suggesting that the presence of longer flanking introns is not always mandatory. To prove the above observation, a minimum of 30–40 nt RCR in the flanking sequence has been shown to be enough for circularization (Liang and Wilusz, 2014; Ivanov et al., 2015). The length of single exon has also been found to be longer to produce single exonic circRNA than normal exons, which undergo canonical splicing (Bolha et al., 2017). Biogenesis of circular intronic RNAs (ciRNA) requires a motif having 7 nt GU-rich element near the 5′ splice site and 11 C-rich nucleotides closer to the branch point. The presence of these elements may help to escape from cleavage (2′-5′ linkage) by debranching enzyme thereby favoring the circRNA biogenesis (Zhang et al., 2013).

Apart from the *cis* sequences, the *trans* factors, such as the RNA-binding proteins (RBPs) are found to be important for circularization of exonic circRNAs, which have a conserved binding site in the flanking introns. Excess Muscleblind-like splicing regulator 1 (MBNL1) proteins bind to the conserved motifs present in the flanking introns of the second exon of their own gene and regulate circMBNL1 biogenesis (Ashwal-Fluss et al., 2014). The RBP Quaking (QKI), an alternative splicing factor, binds to the intronic conserved motifs to form circRNA during human epithelial–mesenchymal transition (EMT). QKI also has the ability to circularize any linear RNA provided its ends are connected to quaking binding sites (Conn et al., 2015). Similarly, FUS controls the regulation of circRNA biogenesis in mouse motor neurons by binding to ∼1,500 nt long flanking introns conserved in human pluoripotent stem cell-derived motor neurons (Errichelli et al., 2017). Apart from the cytoplasmic exonic circRNAs, nuclear exonic circRNAs were also reported (Jeck and Sharpless, 2014; Errichelli et al., 2017), which reveals a new direction toward finding novel functions of these nuclear circRNAs. *C. elegans* are not rich in repeat sequences but contain reverse complementary sequences. Owing to this, Adenosine (A) to Inosine (I) editing by adenosine deaminase acting on RNA1 (ADAR1) is frequent in the intronic sequences, which are responsible for circularizing intervening exons (Ivanov et al., 2015). The knocking down of ADAR1 and ADAR2 in human and mouse cell lines and in *Drosophila* increases circRNA expression without perturbing the linear RNA transcript (Ivanov et al., 2015; Rybak-Wolf et al., 2015). Thus, the biogenesis of circRNAs is regulated by divergent pathways, and understanding this detailed mechanism would require further research.

PROPERTIES

Stability

One of the most attractive features of circRNA is its stability. The circular nature of 2′-5′ linked or 5′-3′ backspliced RNA confers its existence for more than 48 h (Jeck and Sharpless, 2014), as evidenced by its resistance to exonuclease degradation when

compared to linear RNA (half-life <10 h). This could be one of the major reasons behind its detection, even if it constitutes only 1% of poly-A RNA (Salzman et al., 2013). However, the stability of circRNAs in serum is around 15 s, and the probable reason behind this could be the presence of circulating endonucleases (Jeck and Sharpless, 2014).

Conserved Nature

The compilation of various RNA-seq data sets so far show the conserved nature of circRNA across different species in both animals and plants; for example, circRNA originating from genes like Imprinted in Prader-Willi syndrome (IPW), Plasmacytoma Variant Translocation 1 (PVT1) (Salzman et al., 2013), Homeodomain Interacting Protein Kinase 2 (HIPK2), HIPK3, and KIAA0182 (Jeck et al., 2013) are reported to be present in both mice and humans. Similarly, the expression of circMBNL1 was reported in human and *Drosophila* heads (Ashwal-Fluss et al., 2014). More than 700 common exonic circRNAs, shared between *O. sativa* and *A. thaliana* (Ye et al., 2015), were reported, and a similar observation was also observed between *O. sativa Indica* and other plants (Guria et al., 2019). These examples depict the conservation of circRNA that originated from the genomic locus, which evolved with a paralogous or orthologous nature.

Expression Specificity

The expression of circRNA is tissue specific (Memczak et al., 2013; Salzman et al., 2013; Gao et al., 2015; Zhao W. et al., 2017), isoform specific, and development specific, as seen in *O. sativa*, *A. thaliana* (Ye et al., 2015, Gao et al., 2015), and in WI-38 fibroblast cells (Panda et al., 2016). The differential expression of circbHLH93 has been shown in eight developmental stages of *Phyllostachys edulis* (Wang Y. et al., 2019). Stress-specific circRNA expression was also reported in *O. sativa* during phosphate imbalance (Ye et al., 2015), drought stress in *T. aestivum* (Wang Y. et al., 2017), and cold tolerance in *V. vinifera* (Gao et al., 2019). However, experimental validations are yet to be established mechanistically. Tissue/cell-specific circRNA expression has been shown. CircSRY, for example, is expressed in adult mouse testes (Capel et al., 1993) but present as linear *Sry* mRNA in the developing genital ridge (Barrett and Salzman, 2016). CircZFAND6 was absent, and linear *Zfand6* was present in the NHLF cell line. However, A549 cells expressed a single circular isoform of ZFAND6, whereas dual circular isoforms are found in other cell lines. Longer circAMBRA1 were highly expressed in MCF-7 cells, whereas a higher expression of short circAMBRA1 was observed in HepG2 cells (Salzman et al., 2013). The competitive edge of canonical splicing over backsplicing yields more linear RNA than circRNA, and the opposite is also found to be true. For example, circCAMSAP1 expression was 20 times more abundant than its linear counterpart in many of cell lines tested. Overexpression of circRNA, as compared to its linear counterpart, was also observed in 50 other genes examined in A549, AG04450, and HeLa cell lines (Salzman et al., 2013). Similarly, circRNA coming from the KIAA0182 gene locus in the human fibroblast (Hs68) cell line expressed a 10-fold increase over its linear mRNA (Jeck et al., 2013). Recently, we have also reported that ∼20% of circRNAs are highly expressed than linear RNAs, which are spliced out from the same locus in *O. sativa* ssp. *Indica* (Guria et al., 2019).

Types

CircRNAs are classified based on the location of the splice junction in the genome from which they originate. The three basic types of circRNAs are exonic, intronic, and exonic–intronic. Chu et al. (2018a), however, have recently summarized 10 different types of circRNA (**Table 1**).

Previously, the presence of antisense circRNA, overlapping circRNA, and sense overlapping circRNA was also reported in *T. aestivum* (Wang Y. et al., 2017). Since then, the types of circRNAs have been accumulating; International Nomenclature is strictly required to maintain uniformity and to avoid confusion in the identification of the circRNAs by the global circRNA research community.

CircRNA–RBP Interaction

Emerging evidence has proven that circRNA–RBP interaction is fundamental to various dimensions of cellular processes. The interaction of RBP with circRNA may cause the RBP to be sequestered away from its action or circRNA to act as an RBP sponge. HuR, a translational activator, binds to PABPN1 mRNA and enhances its translation. Recently, HuR has been found to interact with circPABPN1, curtailing HuR binding with PABPN1 mRNA and curtailing its translation—a classic example of an RBP getting sponged by a circRNA (Abdelmohsen et al., 2017). Similarly, circFOXO3 sequesters cell cycle proteins, such as CDK2 and p21, and it reduces their interaction with cyclin A and cyclin E, resulting in G1- or S-phase cell cycle arrest (Du et al., 2016). Likewise, an excess MBL protein can bind and help to circularize a portion of its own pre-mRNA, resulting in the decreased production of its cognate mRNA (Ashwal-Fluss et al., 2014). CircRNA can also act as a positive regulator. CircPAIP2, circEIF3, and circANKRD52 bind to Pol II transcription machinery of their corresponding genes, and they augment the expression of linear transcript (Zhang et al., 2013). To further strengthen the circRNA–RBP interaction process, Circinteractome has been found to analyze the presence of such networks in humans. The pipeline has identified 117,000 circRNA that interact with EIF4A3. Similarly, it also detects hsa_circ_0024707 harboring 85 AGO2 binding sites and is thus called an RBP super sponge (Dudekula et al., 2016).

Structure

An RNA molecule that is circular, is without any free ends, and contains an ejected arm comprising complementary regions capable of forming a double strand (**Figure 3**) can also be called a circRNA (Liu C.-X et al., 2019). The circRNA first discovered, the potato spindle tuber viroid (PSTVd), is monomeric and contains rod-shaped structures with multiple distinct loops (López-Carrasco and Flores, 2017). The structure is stable with minimum free energy in the absence of any protein interaction,

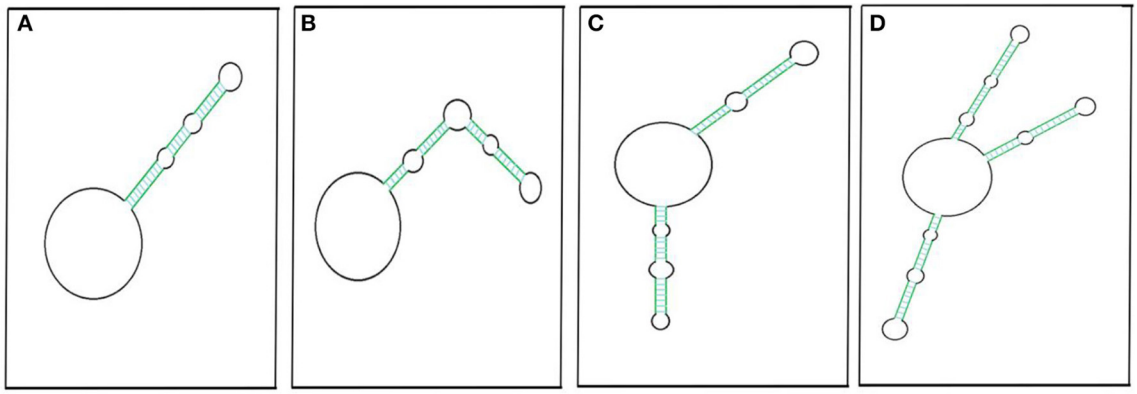

FIGURE 3 | Pictorial representation of circRNAs with putative secondary imperfect duplex(es). CircRNA with a double-stranded RNA (dsRNA) foldback structure can be made possible by having single **(A,B)**, double **(C)**, or multiple projections **(D)**. Colored parallel lines represent different sizes of complementary regions (dsRNA regions), and black loops represent different sizes of non-complementary regions. The pictorial representation is not to scale.

leaving PSTVd as a naked form (López-Carrasco and Flores, 2017).

Liu C.-X et al. (2019) have likewise reported 26 circRNAs from multiple cell lines that have been found to have ≥1 intra-molecular dsRNA duplex secondary structure. Most of these structures are 16–26 bp long, though there are two exceptions: circDHX34 has one 29 bp-long dsRNA projection and circPPP1CB has two projections, of which one is 32 bp long. These intra dsRNA duplexes are reported to have an inhibitory function. The dsRNA binding protein PKR masks the phosphorylation sites and inhibits its activation, highlighting the functional importance of the secondary structure within circRNA. Besides this, these duplexes almost resemble the dsRNA foldback loops, which have shown to be imperative for miRNA biogenesis (Czech and Hannon, 2010; Kumar et al., 2017). The endoribonuclease Dicer may recognize similar secondary structures in circRNAs for miRNA biogenesis. However, the possible mechanism of miRNA generation from circRNAs is yet to be determined. Recently, we reported extremely lengthy circRNAs from *O. sativa* ssp. *Indica* that encompass sequences for different miRNAs (Guria et al., 2019). Hence, the processivity of these mature miRNAs may be linked to circRNA sequences. Along with this, internal modification, such as N6-methyladenosine (m6A) of an miRNA precursor (Alarcón et al., 2015), which is also frequent in circRNAs (Zhou C. et al., 2017), may facilitate miRNA processing. Based on this evidence, we hypothesized the possibility of miRNA biogenesis resulting from circRNAs.

Furthermore, a DNA–RNA hybrid loop, or R-loop, from exon 6-skipped circRNA of *SEP3* has been identified in *A. thaliana*. This hybrid loop decreased its own transcription elongation, favoring enhanced exon skipping and leading to a phenotypically defective SEP3.3 variant (Conn et al., 2017). On the other hand, the non-naturally occurring exon 5-skipped circRNA, having a high secondary complementary intramolecular structure, had a reduced probability of R-loop formation which was increased upon heat denaturation. This further highlights how, if exon 6-skipped circRNA had a secondary complementary structure, the probability of R-loop formation would have been inadequate to produce the SEP3.3-generated phenotype (Conn et al., 2017). Thus, the role of the secondary structure of circRNA emphasized the transcriptional control of its own and, possibly, other target genes with high complementarity. In addition, temperature might play a role in the formation of circRNA variants, as shown in the case of *SEP3*. It has been hypothesized that circRNA generation is favored under low temperature condition, resulting in more variants of circRNA (Conn et al., 2017). Due to the prevalence of frequent alternative backsplicing, circRNA isoforms with varied internal sequence compositions are expected. Based on this, Gao et al. (2016) designed CIRI_AS using forward splice junction (FSJ) reads and backward splice junction (BSJ) reads to explore the internal structures of circRNAs, such as intramolecular dsRNA loops (Liu C.-X et al., 2019).

Localization

Several reports have confirmed the presence of exonic circRNA in cytoplasm (Jeck et al., 2013; Memczak et al., 2013; Jeck and Sharpless, 2014), whereas the intron-retained circRNAs, like exon–intron circRNA and intronic circRNA, are found exclusively in the nucleus (Zhang et al., 2013; Barrett and Salzman, 2016; Ebbesen et al., 2017). Similarly, it is believed that circRNAs containing retained introns, like intron–intergenic circRNA, may be present in the nucleus. Surprisingly, in the Neuro2a (N2a) cell line, it has been shown that the exonic circRNAs are also localized in the nucleus (Errichelli et al., 2017). However, the nuclear export mechanism of exonic circRNAs remains unknown. One possibility could be the escape of circRNAs when the nuclear envelope disintegrates during mitosis (Jeck and Sharpless, 2014). Recently, Huang et al. (2018) correlated the export mechanism of circRNAs in relation to its length. The *Drosophila* nuclear export protein Hel25E and its human homolog UAP56 (DDX39B) have a conserved K-K/S-L-N motif that is responsible for the export of long circRNA

(>1,200 nt) to the cytoplasm through the nuclear pore complex. Similarly, another member of the human exportin family, URH49 (DDX39A) is accountable for the export of <400 nt short circRNA into the cytoplasm. URH49 is dependent on the R-S-F-S motif, but swapping with the K-K/S-L-N motif in the URH49 results in the alteration of its property and it begins to behave like Hel25E/UAP56. However, the nuclear export protein involved in short circRNA export in *Drosophila* is yet to be deciphered. Moreover, it has been hypothesized that long and short circRNAs may follow the NXF1–NXT1 and PHAX–CRM1 pathway, respectively, for cytoplasmic export; this is similar to mRNA, and this needs to be determined experimentally (Huang et al., 2018). Another study has highlighted the nuclear export of circRNA by the direct binding of IGF2BP1 to circRNA followed by exportin2 (XPO2) attachment in an RAN-GTP-dependent manner (Ngo et al., 2019, 24th RNA Society Annual Meet, Krakow).

METHODS OF DETECTION AND VALIDATION

As mentioned above, sporadic evidence concerning the presence of circRNA before the end of the millennium hypothesized it as being a result of splicing noise. However, reports on the identification of circRNAs in the current decade (Salzman et al., 2012, 2013, Memczak et al., 2013) and other studies thereafter have led researchers to develop methodologies to characterize the circRNAs. Numerous computational tools have been developed that have been further improvised to detect circRNAs by setting out various criteria that are specific to certain organisms, species, or genera from the high-quality sequencing reads. Indeed, software designed exclusively for a particular organism can also be used to check for the presence of circRNA in another organism by modifying the default parameters. A DCC pipeline developed for the identification of circRNAs from the heart (Jakobi et al., 2016), for example, is used to detect circRNAs from plants like *O. sativa* and *N. benthamiana* (Guria et al., 2019). Some of the well-known and frequently used computational pipelines for circRNA detection are CIRI (Gao et al., 2015), Circexplorer (Zhang et al., 2014), Circexplorer2 (Zhang X.-O. et al., 2016), circRNA_finder (Westholm et al., 2014), KNIFE (Szabo et al., 2015), Mapsplice (Wang et al., 2010), DCC (Cheng et al., 2016), CIRI_AS (Gao et al., 2016), Segemehl (Hoffmann et al., 2014), circseq_cup (Ye et al., 2017), and pCircRNA_finder (Chen L. et al., 2016), which check the presence of at least one backsplice event in NGS data. Of course, they all have their own advantages and limitations. As a result, genome-wide identification of circRNAs from any organism may actually under-represent the total number of circRNAs detected, and this is also accompanied by missing out on the abysmally expressed circRNAs (Szabo and Salzman, 2016). The pcircRNA_finder is, so far, the only pipeline designed for circRNA determination in plants by combining multiple software to detect backsplicing reads (Chu et al., 2018a,b). This calls for simultaneous analysis using different software to confirm the extent of similarity and number of novel circRNAs. Various animal and plant circRNA databases are being created where new additions are recorded as and when they are reported. Some of the non-exhaustive plant circRNA databases include PlantcircBase (Chu et al., 2017), which has a repository of more than 115,000 circRNAs from 16 different plants. AtcircDB, meanwhile, is meant exclusively for *Arabidopsis* circRNA (Ye et al., 2019). The PlantCircNet (Zhang P. et al., 2017) is meant for visualizing the plant circRNA-miRNA–mRNA interaction networks. Similarly, CircFunBase is the animal circRNA database and is a repository of functional circRNAs (Meng et al., 2019). CircR2Disease (Fan et al., 2018) is designed for the circRNAs involved in various diseases, and circBase (GlaŽar et al., 2014) is designed for circRNAs reported from humans, *Drosophila*, mice, and *C. elegans*. Some of the above tools can be used for the comparative expression of linear RNAs and circRNAs by calculating the read counts for each type of RNA and comparing them with total read counts. Nevertheless, experimental validation is a must in order to rule out the false positives generated from NGS analysis.

The inherent circular nature provides resistance to degradation from exoribonucleases, such as RNase R, augmenting the enrichment of circRNAs before subjecting them to NGS (Suzuki et al., 2006; Vincent and Deutscher, 2006). Treatment with RNase R is more appropriate as it will likely decrease the detection of a backsplice junction generated from template switching, trans-linear splicing, or a genome duplication event (Barrett and Salzman, 2016). Later, divergent RT-PCR/qRT-PCR and subsequent Sanger sequencing and/or northern hybridization can be performed to validate the accurate identification of the NGS-derived circRNAs (Hansen et al., 2011; Wang Z. et al., 2017; Cortés-López et al., 2018; Guria et al., 2019).

Small nuclear RNAs (snRNAs) or the highly structured double-stranded RNA, having 3′-overhangs that are shorter than 7 nt, are resistant to digestion by RNase R (Suzuki et al., 2006; Vincent and Deutscher, 2006; Pandey et al., 2019). In order to enhance the enrichment of pure circRNAs from a pool of total RNA, Poly-A tailing followed by poly-A depletion can be carried out on the leftover complex-structured linear RNA after RNase R treatment. This will categorically reduce the remaining linear RNA that is otherwise present after RNase R treatment. The efficiency of digestion can be examined by PCR for the absence/presence of linear RNA (Pandey et al., 2019).

A gel trap assay, where RNase R usage can be omitted, is yet another method using low-melting agarose that is heated and mixed with total RNA prior to loading in agarose gel. This causes the circular molecules to be trapped inside the well, which can be extracted, purified, and deep sequenced for the presence of circRNA (Jeck and Sharpless, 2014; Barrett and Salzman, 2016). Similarly, total RNA can also be run on vertical non-denaturing two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in two different directions perpendicular to each other. Electrophoretic migration of circular molecules is slow and shows an arch-like pattern, as compared to linear molecules, due to its trapping in the cross-linked gel (Jeck and Sharpless, 2014). The entire arch can be gel eluted and deep sequenced for global circRNA identification. In yet another method, use of RNase H, which can cleave a RNA–DNA hybrid, can also be employed for circRNA identification by assigning a short DNA probe that is complementary to a region of circRNA. A single nick at the bound region will make it linearized for circRNA, or two different

bands if it is linear RNA when run on an agarose gel (Jeck and Sharpless, 2014; Barrett and Salzman, 2016).

Backspliced events from total RNA can also be detected by designing a circRNA ampli-seq panel consisting of known circRNAs and their corresponding linear counterparts followed by NGS (Zaghlool et al., 2018). Thus, it has the advantage of simultaneous quantification of circRNA and its corresponding linear RNA in a specific condition. In yet another strategy, fluorescently labeled padlock probes containing the complementary region of the backspliced junction of a circRNA were designed, followed by rolling circle amplification (RCA). The amplified product was detected by epifluorescence or could be sequenced for particular circRNA identification. However, in both the cases, the sequence of the circRNA has to be known to either design the ampli-seq panel or padlock probes.

Recently, we employed a new technique where we exploited the properties of multiple displacement amplification (MDA) to harness even low-expressed circRNAs from *O. sativa* ssp. *Indica* and *N. benthamiana* for the first time (Guria et al., 2019). The MDA products were digested, cloned, and sequenced to check for the presence of junction sites by comparing the plant circRNA database. Additionally, the MDA products were deep sequenced at much lesser reads and analyzed for genome-wide circRNA identification, which resulted in 1,875 and 9,242 circRNAs from *O. sativa* ssp. *Indica* and *N. benthamiana*, respectively. This method proves to be much cheaper than the traditional RNA-seq method and can be applied on any unannotated genome organism.

ALTERNATIVE CIRCULARIZATION

Based on the computational prediction, it has been determined that a specific gene locus could yield more than one circRNA of different lengths via alternative backsplicing. This mechanism is primarily attributed to association with the competition of putative RNA pairs across introns, which favor the circularization of exons (Zhang et al., 2014; Chen and Yang, 2015; Zhang X.-O. et al., 2016). For instance, an IR sequence present in >2 introns, as shown in **Figure 4**, can result in multiple numbers of varying sizes of circRNAs, either exonic or exonic–intronic circRNA (EiciRNA). But what determines the dominance of one intronic pair over the other possibly forming pairs in a generation of circRNAs is yet to be determined. On the other hand, the presence of the same IR within a single intron results in canonical linear splicing, which then yields the sequential joining of two flanking exons (Chen and Yang, 2015) (**Figure 4**). Subsequently, EiciRNAs (**Figure 4**) can undergo another round of backsplicing reaction to generate only exonic circRNA and the skipping of intervening introns, which might undergo degradation by spliceosomal factors (Chen and Yang, 2015).

Two different types of alternative backsplicing can be possible: (a) alternative 5′ backsplicing and (b) alternative 3′ backsplicing (Zhang X.-O. et al., 2016). The presence and usage of more non-canonical backsplice junctions anywhere in the genome renders an increase in alternative circularization efficiency, which yields different types of circRNAs. The splice variants of circRNA with the same junction sites can be detected by a circRNA-RCA approach, which further provides an opportunity to identify the full-length circRNAs (Das et al., 2019). However, prior knowledge of circRNA sequence information is a must to design primers. Indeed, most of the plant circRNAs are isoforms of varying lengths from the same locus (Guria et al., 2019). Similarly, human *Camsap1* is shown to produce 7 exonic circRNA isoforms in H9 cells (Zhang et al., 2014). Large numbers of circRNA isoforms are generated from a gene having a longer transcript (Khan et al., 2016; Guria et al., 2019). For example, the human cardiac-specific *Titin* gene with 365 exons has been shown to result in 80 different circRNAs. The ryanodine receptor (*RyRs*) gene with 107 exons has been reported with around 59 RyR-specific circRNAs (Khan et al., 2016). Likewise, the S-adenyltransfer gene can produce two circRNAs that have different backsplicing sites and where both share an overlapping parental region in *P. edulis* (Wang Y. et al., 2019). However, the expression of circRNA isoforms will vary depending on the changes in physiological conditions. Thus, alternative circularization is a major criterion for circRNA diversity within a genome (Zhang X.-O. et al., 2016), and the generation of the genome is again correlated with biotic and abiotic factors.

CIRCRNA-DERIVED PSEUDOGENES

Pseudogenes from linear RNA, which are known for retrotransposition, are abundantly present in mammalian genomes, whereas they are significantly less so in plants (Prade et al., 2018). In an analogy with the former observation, a parallel concept was correlated with circRNA-derived pseudogenes (Dong et al., 2016). CircRNA can undergo reverse transcription containing a backsplice junction that can integrate randomly at multiple positions of the genome. This could be either with the complete or partial pseudogene sequence but must contain the backsplicing site, thereby altering the genome architecture. For example, 33 high-confidence pseudogenes were found to be generated from circRFWD2 containing the exon 6 and exon 2 as backsplicing sites in mice. However, the same are not found in rats or other primates as determined using a circpseudo computational pipeline, suggesting the possible occurrence of divergent evolution (Dong et al., 2016). On the other hand, a circSATB1-derived pseudogene could be found in both mice and rats, explaining the possible occurrence of evolutionarily conserved retrotransposition. Conversely, circPRKDC- and circCAMSAP1-derived pseudogenes could be found in gorillas and chimpanzees but not in rhesus monkeys. This elucidates how retrotransposition might have occurred very recently during the course of evolution. Sometimes, a pseudogene may be found in one mouse strain but not in another, which explains the strain-specific retrotransposition within the species (e.g., circDIAP3-derived pseudogene). Pseudogenes derived from circRNA generally possess few Adenosines (A) at their 3′end as compared to linear RNA-derived pseudogenes. However, the mechanism of integration remains unknown (Dong et al., 2016). Nevertheless, it may be possible that new circRNA(s) might emerge from the pseudogene-integrated locus in the genome.

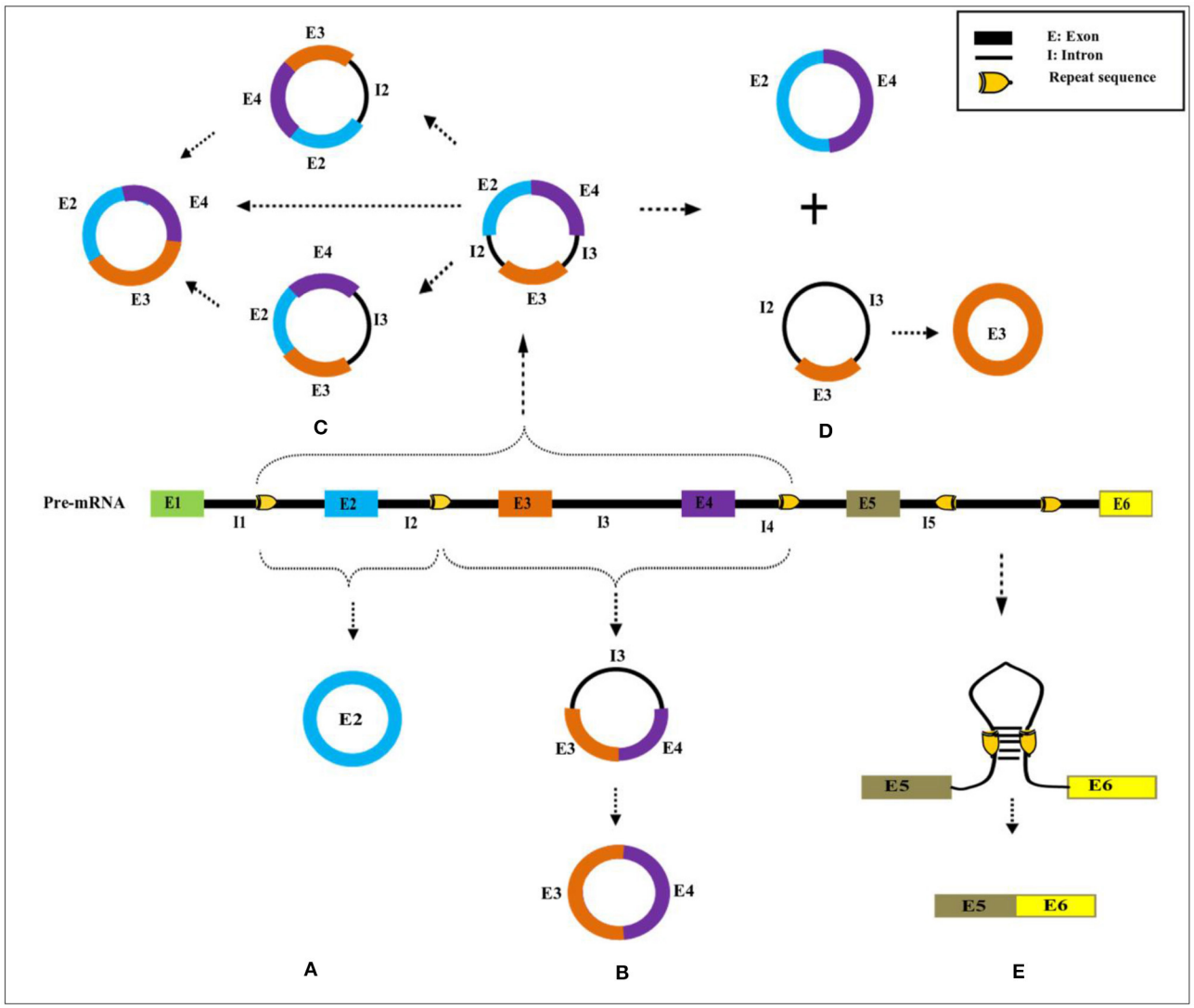

FIGURE 4 | Possible alternate backsplicing and canonical splicing. Presence of *cis* sequences across introns in pre-mRNA results in formation of circRNAs of varying sizes that are comprised of different exons with or without introns **(A–D)**. Presence of indirect repeat sequence in the same intron causes sequential joining of flanking exons to form linear RNA **(E)**. The pictorial representation does not reflect in actual scale.

Thus, a generation of circRNA followed by the retrotransposition of pseudogenes generated from circRNA is a dynamic process. The formation of new circRNA(s) from the newly integrated site, which may again be retro transposed, thereby may impart in the development of evolution. However, evidences for the same are lacking probably because a balance is being maintained by small RNAs such as hc-siRNA/PIWI RNA to counteract the effect of retrotransposition (He X-J. et al., 2011).

FUNCTIONS

MiRNA Sponging

Much evidence has emphasized the putative role of circRNAs in gene regulation. One of the proven functions of circRNA is the inhibition of the miRNA function by binding the target miRNAs directly or indirectly by a process commonly referred to as miRNA sponging. CiRS-7/circCDR1as is a classic example having 74 miR-7 binding sites in humans and at least 63 conserved binding sites across 32 vertebrates (Memczak et al., 2013; Li et al., 2018). Imperfect complementary between ciRS-7-miR-7 strongly suppresses the miR-7 availability to its mRNA targets (Hansen et al., 2013) involved in various cancers (Kefas et al., 2008; Reddy et al., 2008) (**Table 2**) and neuro-degenerative disorders such as Parkinson disease (Junn et al., 2009) and Alzheimer's disease (Lukiw, 2013). On the other hand, ciRS-7 has perfect complementarity with miR-671 between the 10–11 nt along with extended complementarity beyond the 12 nt; as a result of this, ciRS-7 is cleaved by Argonaute-2 (AGO-2) (Hansen et al., 2011). Similarly, circSRY sponges miR-138 by binding to at least 16 sites, and it thus possibly plays a role in the progression of cancer and

TABLE 2 | Functional characterization of circRNAs and their significance in disease curbing.

Disorder type	Molecular feedback axis and their expression levels	CircRNA information	Remark(s)	Computational pipeline used	References
Breast cancer (BC)	Hsa_circ_0004771 (high)/miR-653 (low)/ZEB2 (high)	13 binding sites for different miRNAs	*In vitro* and *In vivo* studies. ZEB2 protein executes EMT. MiR-653 suppresses *Zeb2* expression by binding at its 3′-UTR and acts as a tumor suppressor that is sponged by hsa_circ_0004771	Circinteractome, TargetScan	Xie et al., 2019
Lung adenocarcinoma (LUAD)	CircMTO1 (low)/miR-17 (high)/QKI-5 (low)	CircMTO1 derived from exon 2 and 3 of *Mto1* gene (318 bp)	*In vitro* and *In vivo* studies. Tumor suppressor CircMTO1 sponges miR-17, allowing for enhanced QKI-5 expression, which, in turn, retards proliferation by inactivating the Notch signaling pathway	RNAhybrid, miRanda, TargetScan	Zhang et al., 2019
Non-small cell lung carcinoma (NSCLC)	CircP4HB (high)/miR-133a-5p (low)/Vimentin (high)	Derived from exon of *P4hb* gene located on chromosome 17.	*In vitro* and *In vivo* studies. CircP4HB promotes EMT by sponging tumor suppressor miR-133a-5p.	Arraystar software	Wang T. et al., 2019
Bladder carcinoma (BCa)	CircDOCK1 (high)/hsa_miR_132-3p (low)/Sox5 (high)	CircDOCK1 has potential binding sites for five different miRNAs	*In vitro* and *In vivo* studies. Targeting circDOCK1 reduced cell viability and retarded migration of tumor	PicTar, TargetScan	Liu P. et al., 2019
	CircHIPK3 (low)/miR-558 (high)/HPSE (Heparanase) (high)	Derived from exon 2 of *Hipk3* gene (1099 bp)	*In vitro* and *In vivo* studies CircHIPK3 attenuates cell migration, invasion and angiogenesis in BCa by sponging miR-558 which down-regulates HSPE expression and its downsteam targets MMP-9 and VEGF	miRanda, PITA, RNAhybrid	Li et al., 2017
	CircTCF25 (high)/miR-103a-3p and miR-107 (low)/CDK6 (high)	Derived from exon(s) of *Tcf25* gene located on chromosome 16	*In vitro* and *In vivo* studies. miR-103a-3p and miR-107 negatively regulates CDK6, which, along with Cyclin D, controls G1 to S transition by inactivating RB1 via phosphorylation	miRanda, TargetScan, DIANA-miRPath, DAVID	Zhong et al., 2016
	Hsa_circ_0068871 (high)/miR-181a-5p (low)/FGFR3 (high)	Derived from exon 4–8 of *Fgfr3* gene	*In vitro* and *In vivo* studies Hsa_circ_0068871 sponges miR-181a-5p, which is a tumor suppressor and regulates FGFR3 expression. FGFR3 activates STAT3, which triggers tumor development	circBase, CircNet, CircInteractome, TargetScan, RNA 22v2	Mao et al., 2019
	CircITCH (low)/miR-17, miR-224 (high)/p21, PTEN (low)	Derived from several exons of *Itch* gene	*In vitro* and *In vivo* studies. CircITCH is a tumor suppressor molecule that can sponge miR-17 and miR-224, thereby downregulating p21 and PTEN involved in BCa cell proliferation	Starbase V2.0, Circinteractome	Yang C. et al., 2018
Hepatocellular carcinomas (HCC)	Cir_0005986 (low)/miR-129-5p (high)/Notch1 (low)	Derived from gene *Prdm2* (PR/SET Domain 2) localized on chromosome 1	*In vitro* studies Cir_0005986 affects proliferation via regulating G0/G1 to S phase transition	DIAN mirPath v.3, Arraystar software, miRTarBase	Fu et al., 2017
Laryngeal squamous cell carcinoma (LSCC)	CircRASSF2 (high)/miR-302b-3p (low)/IGF-1R (high)	Derived from *Rassf2* gene present on chromosome 20	*In vitro* and *In vivo* studies CircRASSF2 promotes tumorigenesis by upregulating IGF-1R, which is a target of miR-302b-3p. Overexpressed circRASSF2 secreted in serum by exosomes	TargetScan, PicTar, miRanda	Tian et al., 2019
Pancreatic ductal adenocarcinoma (PDAC)	CircRNA_100782 (high)/miR-124 (low)/IL-6, STAT3 (high)	Derived from sequence on chromosome 11	*In vitro* and *In vivo* studies CircRNA_100782 sponges miR-124, a negative regulator of proliferation in PDAC. miR-124 targets IL-6 and STAT3, which are crucial for cell growth and survival	TargetScan	Chen et al., 2017
Gastric cancer (GC)	CircRNA_100269 (low)/miR-630 (high)/LPHN2 (low)	Derived from exon of *Lphn2* gene present on chromosome 1.	*In vitro* studies miR-630 negatively regulates the circRNA_100269 and, thereby, downregulates the expression of its linear variant *Lphn2*	TargetScan, miRanda	Zhang Y. et al., 2017
Esophageal squamous cell carcinoma (ESCC)	CircITCH (low)/miR-7 (high)/ITCH (low)	Derived from exons 6-13 of *Itch* gene located on chromosome 20.	*In vitro* studies. CircITCH is an antitumor molecule, enhances *Itch* expression that further regulates the Wnt/β-Catenin pathway	TargetScan/TargetScanS, miRanda	Li F. et al., 2015

(Continued)

TABLE 2 | Continued

Disorder type	Molecular feedback axis and their expression levels	CircRNA information	Remark(s)	Computational pipeline used	References
Cardiac fibrosis	CircHIPK3 (high)/miR-29b-3p (low)/a-SMA, COL1A1, COL3A1 (high)	Derived from exon 2 of *Hipk3* gene. Localized in cytoplasm	*In vitro* studies. miR-29b-3p has an antifibrotic effect and is a target for circHIPK3	regRNA2	Ni et al., 2019
Alzheimer's disease (AD)	CircHDAC9 (low)/miR-138 (high)/Sirt1 (low)	Localized in cytoplasm	*In vitro* studies CircHDAC9 has a binding site for miR-138 for which Sirt1 is a target. Sirt1 suppresses Amyloid-β production and regulates neuro-inflammation and mitochondrial dysfunction	TargetScan 7.0, miRanda 3.3a, RegRNA 2.0	Lu et al., 2019
	CiRS-7 (low)/miR-7 (high)/UBE2A (low)	CiRS-7 is found to have more than 70 miR-7 binding sites	UBE2A is an autophagic protein involved in amyloid peptide clearance in AD, and miR-7 is known to suppress its expression. CiRS-7 sponges miR-7 and declines its effect on UBE2A expression		Lukiw, 2013; Li et al., 2018

Parkinson's disease (Hansen et al., 2013; Qu et al., 2017). A single circRNA can bind to a single or many miRNAs at one or more sites (Memczak et al., 2013; Guria et al., 2019) by either perfect or near-perfect binding. For example, circITCH can bind to miR-138, miR-17, and miR-124, whereas circFOXO3 can bind to miR-22, miR-136*, miR-138, miR-149*, miR-433, miR-762, miR-3614-5p, and miR-3622b-5p (Qu et al., 2017). However, only a small number of circRNAs were found to have a sponging effect (Chu et al., 2018a,b; Guria et al., 2019), which suggested the possibility of having simultaneous other putative functions. It is intriguing to note that all the circRNAs that display sponging activity are localized in the cytoplasm, and most of them are exonic circRNAs (Kulcheski et al., 2016).

Similarly, a total of 115,171 circRNAs have been reported in 16 different plants (Chu et al., 2017), and 102 circRNAs have been found to contain miRNA binding sites in *S. lycopersicum* (Zuo et al., 2016). Pan et al. (2018) have used DEG software to trace out 20 circRNAs connected to the miRNA-mediated expression of 91 genes under heat shock conditions in *A. thaliana*. Nevertheless, a myriad of work demonstrating the circRNA sponging function in human diseases has been well documented as compared to plants.

Out of a significant number of circRNAs discovered so far in various organisms, only a handful of them have been validated, among which few are found to serve as miRNA sponges. In order to have effective sponging activity, the stoichiometry of the circRNA has to match with the abundance of the miRNAs. This could be attained through the presence of either multiple binding sites for a particular miRNA (CiR-7) or by having high copy numbers of the sponging circRNA, preferably with large-sized circRNA (Zheng et al., 2016). Small-sized circRNAs might have an inclination for exosomal-mediated ejection from the cell, which could be a reason for their inability to sponge (Li Y. et al., 2015; Zheng et al., 2016; Preußer et al., 2018). A single gene can generate multiple circRNAs, either with a high copy number, of different sizes (e.g., PTK2 gene can produce 47 distinct circRNAs) (Zheng et al., 2016), or in a combination of both possibilities. As already observed, a single circRNA can be expressed in various tissues (e.g., circHIPK3—Li et al., 2017; Ni et al., 2019) and can possess binding sites for multiple distinct miRNAs (e.g., circFOXO3—Han et al., 2017; Stefanetti et al., 2018). Considering all the cases mentioned above, a single circRNA can bind to multiple distinct miRNAs that regulate different pathways and *vice versa*, which is proof of a network that functions interdependently to maintain cellular homeostasis.

CircRNAs as Potential Biomarkers and Therapeutic Targets

Discovering biomarkers at the early stages of a disease is a very promising path in diagnosis and prevention. While the race for detection and functional determination still continues for circRNAs, reports from many researchers are highlighting their potential as biomarkers and potential within therapeutics. Due to its high abundance, longevity, and tissue-specific expression, circRNAs could be a potential molecule used in forensic science. Reconstructing a crime scene is quite a challenging task due to limitations such as low quantity and quality of samples at the site. So far, RNA profiling is under investigation for potential to serve as a biomarker in the identification and differentiation of body fluids. Until the discovery of circRNAs, RNA-stable transcript regions and miRNAs have been studied as stable biomarkers, the latter being stable due to its small size and Argonaute binding. An effort was made to include circRNA in RNA profiling in order to enhance biomarker identification and sensitivity (Zhang Y. et al., 2018). It included circRNA of a peripheral blood-specific ALAS2 gene and a menstrual blood-specific MMP7 gene in RNA profiling to enhance identification and sensitivity. Further work is indeed needed to capitalize on its potential as a forensic biomarker. In addition to this, it has been shown that exosomes provide additional protection to circRNAs that are enclosed within it (Li Y. et al., 2015). Subsequently, an increasing amount of circRNA (up to ∼2 fold) was found in exosomes when compared to the cells (Lu and Xu, 2016). Overexpressed small-sized circRNAs have a higher tendency to be expelled from

cells as exosomes into the circulatory system, which can serve as a non-invasive diagnostics method for biomarkers. CircRNAs are found to be capable of crossing the blood–brain barrier (BBB), entering into the blood and cerebrospinal fluid (CSF) and can thus provide us with the status of Central Nervous System (CNS) disorders (Lu and Xu, 2016). On the other hand, the differential expression of plant circRNA profiles under a variety of stress conditions, such as drought in *T. aestivum* (Wang Y. et al., 2017), chilling in *S. lycopersicum* (Zuo et al., 2016), and nutrients stress, such as phosphate, iron, and zinc in *O. sativa* and *H. vulgare* (Darbani et al., 2016; Liu et al., 2017), might serve as reliable markers in plants that were previously underestimated.

Cell to Cell Communication

About 1,215 circRNAs have been identified from isolated exosomes from human serum (Li Y. et al., 2015). Recently, the circulating exosomes containing overexpressed circRASSF2 were identified from laryngeal squamous cell carcinoma (LSCC) patients (Tian et al., 2019). These circulating exosomal circRNAs are usually found to be <1,000 nt long with a median of 350 nt in length (Li Y. et al., 2015). The small-sized circRNAs may become enclosed in exosomes and circulate in the blood to serve as potential biomarkers or as a cell signaling molecules. Moreover, the sorting of circRNAs in exosomes further depends on the low levels of its miRNA target(s) in the cells (Li Y. et al., 2015). Knowing their prolonged stability, the expulsion of extracellular vesicles (EVs) containing circRNAs is one of the evident ways for the clearance of circRNA cargo (Lasda and Parker, 2016). We speculate that the transport of these EV circRNAs might be involved in metastasis and proliferation of cancer. In contrast to animals, long-distance trafficking of PSTVd via the phloem (Palukaitis, 1987; Zhu et al., 2001) highlights the possibility of the circRNAs being communicating molecules via plant vasculature. Nevertheless, extensive research is indeed further required in this direction.

Transcription Enhancer/Repressor

Recent discoveries have provided the functional aspect of circRNAs by exploring their potential as transcriptional regulators in both a *cis* and *trans* manner. Initially, the introns containing circRNAs, such as circEIF3J, circPAIP2, circANKRD52, circMCM5, and circSIRT7, are found to be interacting with the elongating RNA Polymerase II complex through positive feedback to regulate their own gene expression (Zhang et al., 2013). Similarly, nucleus-inhabiting EiciRNAs bind to U1 spliceosome components and promote expression of their own parental gene in addition to post-transcriptional regulation (Wilusz, 2017). Some circRNAs, such as circMBL, circFMN, and circDMD, can bind directly to their cognate mRNAs and thereby suppress their expression (mRNA trap) (Li et al., 2017). In plants, exon 6 *SEP3*-derived exonic circRNA tends to form an R-loop on the parental locus, thereby retarding its transcription elongation in *trans* to enhance the biogenesis of the exon-skipped circular variant (Conn et al., 2017). These studies demonstrate that the circRNAs can play an imperative role in diverse transcriptional regulation mechanisms.

Cell Cycle Regulation

The diverse mechanism behind the action of the tumor suppressor gene-derived circFOXO3 in cell cycle regulation has been studied. Having binding sites for miR-22, miR-96, miR-136, miR-138, miR-149, miR-433, miR-762, miR-3614-5p, and miR-3622b-5p (Han et al., 2017; Stefanetti et al., 2018), circFOXO3 sponges these miRNAs from binding the linear variant of *FOXO3* and relieves its suppression. Besides having miRNA binding sites, circFOXO3 has binding sites for proteins involved in cell cycle regulation, such as p21, p27, p53, CDK-2, and MDM2. Two subsequent studies by Du et al. (2016, 2017) have emphasized the role of circRNA–protein interaction in cell cycle regulation using pull-down assays. They demonstrated the formation of a ternary complex by binding of p21 and CDK-2 to circFOXO3 at adjacent sites, which inhibits activation of the CDK-2/Cyclin-E complex otherwise necessary for G1/S transition, thereby resulting in cell cycle arrest in the G1 phase (Du et al., 2016). Along with this, circFOXO3 had binding sites for p53 and MDM2 and regulated the cell cycle (Du et al., 2017). To further strengthen its role in cancer development, low levels of circFOXO3 in breast cancer cell lines and patient samples were identified. Conversely, overexpression of circFOXO3 in cancer cells induced apoptosis and inhibited tumor growth (Du et al., 2017).

The role of circRNAs in the cell cycle regulation of cardiomyocytes has also been studied. *In situ* replenishment of cardiomyocytes after cardiac injury could be a potential recovery approach from damage incurred by a myocardial infarction (MI). Studies have demonstrated a reduced level of circNfix in proliferating neonatal cardiomyocytes when compared to adult cardiomyocytes (Huang et al., 2019). The ternary complex comprising of circNfix brings Nedd41 and YbX1 into close proximity and thereby mediates the ubiquitination and consequent degradation of YbX1. Downregulation of YbX1 leads to reduced levels of its downstream target genes, such as Cyclin A2 and Cyclin B1, and this ultimately inhibits cardiomyocyte proliferation. However, considerable research is needed to understand the mechanism in detail.

Ribosomal RNA Maturation

CircRNA–protein interactions have further delineated the potential of circRNA to halt the global translational machinery in a cell besides commanding its own translation. circANRIL is one such example involved in the modulation of ribosomal RNA maturation, and it thereby dictates ribosomal biogenesis in the vascular smooth muscle cells and macrophages (Holdt et al., 2016). Using a lambda N peptide-mediated pull-down assay of circANRIL-B-Box, it was found that about 54% of the nuclear proteins were either involved in ribosomal biogenesis and its assembly or the regulation of rRNA splicing. A competitive attachment of circANRIL with a C-terminal lysine-rich domain of PES1 was also shown to prevent pre-rRNA binding. The latter resulted in dysfunction of the PeBoW complex, ultimately hindering the exonuclease-mediated rRNA maturation. Enhanced expression of circANRIL results in impaired ribosomal biogenesis due to premature rRNA accumulation as this increases p53 activation. This results in higher apoptosis and a lower rate of proliferation in humans,

thereby manifesting the atheroprotective role of circANRIL (Holdt et al., 2016).

Translation

CircRNAs are confidently grouped under long non-coding RNAs; a result of this is that the translational potential of circRNA has never been given much attention. The functional catalog of circRNAs was initially comprised of numerous evidence indicating miRNA sponging and protein sequestrating across animals and plants (Barrett and Salzman, 2016). However, recent evidence has highlighted the protein-coding potential of endogenous circRNAs due to their abundant association with the polysome (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2017). CircZNF609 from myoblasts presents in heavy polysomes and contains two in-frame start codons separated by 150 nt. It also carries a 5′ conserved internal ribosome entry site (IRES) and thus produces two similar intense proteins by cap-independent translation. Knockdown of circZNF609 using specific siRNA decreases proliferation of human and mouse myoblast cell lines, signifying its role in myogenesis (Legnini et al., 2017). Subsequently, it is investigated through an RNA-wide analysis on the m6A pattern that the IRES of circZNF609 is highly methylated (Zhao et al., 2014), which probably makes it responsible for the cap-independent translation. CircRNA translation requires the involvement of METTL3/METTL14, eukaryotic initiation factor eIF4G2, and m6A reader YTHDF3 compounded by heat stress. This leads to translocation of YTHDF2 from the cytosol to nucleus to block FTO, ultimately increasing m6A modification at its consensus motif (RRm6ACH, A–G/A and H–A/C/U) in IRES to initiate cap-independent translation (Yang et al., 2017). Therefore, replacement of circZNF609 IRES with a different UTR of same length inhibits its own translation (Legnini et al., 2017). However, a single m6A site is enough to induce circRNA translation with same efficiency as that by two m6A sites present in IRES (Yang et al., 2017). A summarized report from Yang et al. (2017) stated that 623 circRNAs were m6A methylated in the human Hs68 cell line, of which 25 circRNAs had a translation initiation site with ≥150 nt. On the other hand, 250 circRNAs were found to be associated with polysomes that correspond to ∼0.6 circRNA/million reads with translatable coding potential (Yang et al., 2017). About 72 human circRNAs have been proven to express proteins as listed in circRNADb (Chen X. et al., 2016). Similarly, another endogenous circRNA from *Drosophila*, circMBL3, encodes ∼37 KDa proteins and shows two specific bands (with and without the fifth exon). Besides, 34–158 circRNAs derived from *Drosophila,* rats, and mice were found to be associated with polysomes, emphasizing that those circRNAs might be translated into proteins (Pamudurti et al., 2017).

Protein translation from endogenous circRNAs informs the possible role of circRNAs in cancer progression. In an attempt, Zheng et al. (2019) found a ∼10 KDa circPPP1R12A-73aa (coding 73 amino acids) protein expressed from circPPP1R12A (hsa_circ_0000423) that had a 216 nt short open reading frame formed by the backsplicing of exon 24 and 25. This circPPP1R12A-73aa protein has a unique conserved peptide GRLRHVNCLSPGVQD at the C-terminal. The circPPP1R12A-73aa protein, unlike the circPPP1R12A, regulates colon cancer progression, invasion, and metastasis, as was proven from 20 different patient samples and in nude mice. *In vitro* expression of the circPPP1R12A-73aa protein in various colon cancer cell lines, such as HT-29, HT-116, SW480, SW620, LoVo, SW48, DLD-1, CaCo2, and HCT-15, was also studied. The expression of the circPPP1R12A-73aa protein was found to increase in colon cancer cell lines as compared to control cell line NCM460 (Zheng et al., 2019). Similarly, a 17 KDa novel protein, SHPRH-146aa, was expressed from 440 nt circSHPRH (having an overlapping start and stop codon) after backsplicing of exons 26–29. It also possessed a unique peptide sequence, AAILQKWK, and is present more in normal brain tissue (Zhang et al., 2018a). PINT87aa, a 10 KDa protein expressed from circPINTexon2/circLINC-PINT (hsa_circ_082389), is formed by the circularization of exon 2 (Zhang et al., 2018b). Likewise, FBXW7-185aa, which is expressed from circFBXW7 (novel_circ_022705) after the backsplicing of exon 3 and 4, is a 21 KDa protein (Yang Y. et al., 2018). Both proteins are expressed more in normal brain tissue than in glioblastoma. PINT87aa may bind to the 150–300 aa domain of the PAF1 protein complex, which in turn recruits RNA polymerase II and regulates the transcriptional elongation of downstream genes (Zhang et al., 2018b). FBXW7-185aa promotes cell cycle arrest at the G1 stage and reduces the proliferation of glioma cells (Yang et al., 2017). Since circRNA may share the same coding sequence (CDS) as their corresponding linear mRNA, it is difficult to identify the origin of the translatable product. Moreover, library construction is difficult for ribosome footprinting (RFP) circRNAs due to the limited availability of tools for the identification of the circRNA-generated peptides. So far, no proteins or peptides have been detected from plant circRNAs. However, an elaborative study is required to search for m6A sites at 5′UTR in plants, and this could pave a path for the possibility of cap-independent translation.

ROLE OF CIRCRNA IN PLANTS SO FAR

The search for the presence of circRNAs is ongoing in plants but at a slower pace than the ongoing search in animals. Though >100,000 circRNAs have been identified from different plants as listed in the plant circRNA database (Chu et al., 2017), only a fraction of those have been validated. The population of exonic circRNAs differs between plants and even species of the same plant, ranging from ∼6.5 to 86% (Lu et al., 2015; Ye et al., 2015; Wang Z. et al., 2017; Zhao W. et al., 2017; Guria et al., 2019), due to usage of various pipelines, incomplete genome annotations, and other unknown possibilities. Different circRNAs are found to be expressed at different biotic and abiotic stress conditions, as has been proven in plants like *O. sativa* (Ye et al., 2015), *S. lycopersicum* (Zuo et al., 2016), *A. thaliana* (Pan et al., 2018), *T. aestivum* (Wang Y. et al., 2017), *P. betulifolia* (Wang et al., 2018), *A. deliciosa* (Wang Z. et al., 2017), and *S. tuberosum* (Zhou R. et al., 2017), where these circRNAs could act as potential plant biomarkers.

In the context of functional significance, plant circRNAs are found to have network interaction with miRNAs as sponging or cleavage properties (Chu et al., 2018b; Guria et al., 2019). However, this observation requires further validation either by the overexpression or knockdown of those circRNAs in plants. Although only a handful of plant circRNAs are having a putative miRNA interaction ability, its scope for other unidentified function cannot be sidelined, as was claimed in animal circRNAs. For example, exon 6-skipped circRNA of *SEP3* in *A. thaliana* forms a DNA–RNA hybrid loop that negatively regulates the transcription of its host gene (Conn et al., 2017). This reveals a novel function of circRNA that incites curiosity for the possibility of a similar mechanism in other plants as well. Similarly, circRNA derived from *PSY1* (involved in carotenoid biosynthesis) is found to be differentially expressed during various stages of fruit ripening (Tan et al., 2017). However, overexpression of PSY1-circ1 leads to decreased beta-carotene and lycopene content, resulting in yellowing of the fruits. Similarly, PDS-circ1 has also been shown to regulate the ripening pathway as a decrease in PDS mRNA expression often results in photobleaching of leaves, petals, and sepals (Tan et al., 2017). No plant circRNAs have so far been found to code for any proteins, although the presence of ORF in circRNA downstream of the IRES sequence complemented by m6A could act as a potential translatory endogenous circRNA. The biogenesis of plant circRNAs does not always follow the same pattern that is found in animals, such as having fewer repetitive or complementary flanking sequences (Zhao T. et al., 2017) and the presence of more non-GT/AG backsplice junctions (Guria et al., 2019). As a result, alternative circularization is frequent (Ye et al., 2017, Ye et al., 2015, Lu et al., 2015, Tan et al., 2017, Guria et al., 2019) because of which different types of circRNAs that originate from different loci are abundant in plants (**Table 1**). It is interesting to know that ∼6 and ∼1% of circRNA found in *A. thaliana* comes from chloroplast and mitochondrial genes, respectively, which is indicative of its presence and regulation in sub-cellular organelles (Sun et al., 2016). Trans-backsplicing is also significant in plants, with a reported 13% of circRNAs in *A. thaliana* and 34% in *O. sativa* (Ye et al., 2015; Chu et al., 2018b), apart from a single circRNA in *N. benthamiana* that was identified by the MDA-cloning method (Guria et al., 2019). However, the effect of these circRNAs on gene regulation and the possible physiological changes thereof need to be thoroughly evaluated. Most of the exonic circRNAs formed in *O. sativa*, *A. thaliana*, and *G. max* are comprised of 1–4 exons (Lu et al., 2015; Ye et al., 2015; Zhao W. et al., 2017), which are possibly formed post-transcriptionally after the intervening introns are removed; this is unlike single exonic circRNA, which is derived by the co-transcriptional pathway (Chu et al., 2018b).

There is an urgent need for the development of computational pipelines designed exclusively for plants as false positives often crop up during validation using animal- or human-specific software. Circseq_cup (Ye et al., 2017) has therefore been released to accurately explore the complete sequence of circRNA in plants, and it has ∼3,000 assembled full-length circRNAs from *O. sativa*. PcircRNA_finder is the only plant-specific circRNA prediction software, although it uses multiple programs and yields only exonic circRNA (Chu et al., 2018a). Therefore, it is possible that the usage of various types of software enlists different types of circRNAs from the same plants, and this is probably due to discrete criteria for setting up the software. A vast plethora of circRNA-related research and the development of unique software are therefore required in plants as well as animals, which has immense potential in terms of unraveling various mechanisms that work together in plants.

CIRCRNA DEGRADATION

The fluctuating levels of circRNAs inside the cells upon stress stimulation (Ye et al., 2015; Liu P. et al., 2019) or during developmental differentiation have recently been studied (Mahmoudi and Cairns, 2019). Despite existing knowledge on circRNA degradation, more detailed testing is required to strengthen the lesser-known concepts. The regulation of circRNA degradation is known to be controlled by five pathways, some of which have been proven. What remains is hypothetical, which necessitates further validation.

The first pathway to mention is in accord to our previously published data (Guria et al., 2019), where we have computationally predicted a high percentage (∼85%) of perfect complementary miRNA binding sites with plant circRNAs that may subsequently be subjected toward degradation. This is in line with previous findings that mention near-perfect complementarity between miRNA and its target in plants as compared to animals (Schwab et al., 2005; Ding et al., 2012). Although the interaction of circRNA and miRNA is broadly known through sponging models, the instance of miRNA-mediated regulation of circRNA degradation has so far barely been highlighted. It has been demonstrated in HEK293 cells that the sequence specificity between miR-671 and the non-linear natural antisense transcripts of *CDR1* directs the cleavage of the latter and regulates its mRNA levels (Hansen et al., 2011). It might be possible that many circRNAs are prone to degradation by Ago2-slicer-mediated action with its target miRNA as shown previously (Hansen et al., 2011); although further research is required to validate the above concept across eukaryotes.

Secondly, circRNA degradation could be carried out through endonuclease activity. The activation of cytoplasmic endonuclease RNaseL has recently been shown to trigger PKR activation via cleavage of bound inhibitory dsRNA in systemic lupus erythematosus patients (Liu C.-X et al., 2019). These patients further showed a reduced level of circRNAs, which were found to form a 16–26 bp dsRNA imperfect duplex. The latter, when complexed to PKR, inhibits its activity. Therefore, activation of RNaseL through the 2′ and 5′-oligoadenylate synthetase system upon viral stimulation targets even the dsRNA duplexes of circRNAs, along with the viral and cellular RNA, to activate the PKR via the antiviral signaling pathway (Liu C.-X et al., 2019).

Thirdly, the exosomes, due to their 3′-5′ exoribonuclease activity, are known to participate in mRNA quality control by being actively involving in mRNA processing and degradation. Besides this, it was recently found that, out of its nine subunits,

the Rrp44 subunit possessed a PilT N-terminus (PIN)-domain with endoribonuclease activity. This may contribute to circRNA degradation, which is otherwise resistant to exonuclease activity due to its lack of linear ends (Schaeffer et al., 2009). Similarly, further detection of endo-ribonucleolytic activity of existing molecules and their interaction with circRNAs might open up the potential for a new mechanism for possible circRNA degradation.

The fourth pathway could be based on the known fact that m6A modified mRNAs that are recognized and guided by YTHDF2 into nuclear P-bodies for their degradation (Wang X. et al., 2014). Recently, it has been shown that 22% of 1,348 circRNAs interacting with YTHDF2 proteins are m6A modified in HeLa cells using RNA immunoprecipitation (RIP)-seq (Zhou C. et al., 2017). In addition, ciRS-7 has previously been found to compartmentalize in P-bodies when co-transfected with miR-7 in HEK293 and HeLa cells (Hansen et al., 2013). Therefore, it is highly possible that the interaction of YTHDF2 with m6A-modified circRNA may lead to its turnover. However, detailed investigation regarding its mechanism of action is indeed required to ascertain the proposed concept.

In addition to the above mentioned pathway, the possible fifth pathway could be EV mediated, in which EVs are membrane-bound structures capable of encapsulating cellular components, including circRNAs as mentioned previously (Yang and Li, 2018). Given their prolonged stability, the expulsion of EVs containing circRNAs is one of the ways the cell can get rid of the accumulating circRNA population (Lasda and Parker, 2016). It may become further degraded upon encountering endonucleases present in the extracellular matrix.

CONCLUSION AND PERSPECTIVES

Significant reports in the field of circRNA have illuminated the RNA world recently. Novel circRNAs are reported to have diverse functions; this includes acting as biomarkers and having therapeutic potential in uses for cancer and other diseases, which could further pave the way for effective diagnosis, treatment, and prevention. As compared to the animal system, research on plant circRNAs is minimal and is in need of more attention, especially designing of exclusive bioinformatic tools. Nevertheless, some of the proposed findings on plant circRNAs, including the mechanism of their biogenesis, which deviates from animal circRNAs, are worth mentioning; putative circRNA has, for example, a regulatory role in metabolic pathways. In the coming years, more research has to be put forward into the translational potential of circRNA. It is important to unravel any differences in the function of proteins coded by circRNA from the canonical spliced mRNA. It is equally interesting to study the possibility of protein isoforms generated from alternative backsplicing and their possible function in gene regulatory networks. It is also intriguing to know whether the secondary structure of circRNA will affect its miRNA sponging ability. Similarly, in the absence of a terminator codon in circRNA, how the protein synthesis is regulated is another interesting question to be resolved. Nevertheless, the hunt is on across the scientific community to find answers to many more burgeoning questions, including in the field of plant circRNAs.

AUTHOR CONTRIBUTIONS

AG and PS contributed equally in conceiving the review focus, conducting the literature review, summarizing the manuscript, reviewed literature, wrote the first draft, and finalized the manuscript. GP, SN, AG, and PS revised and made corrections to the manuscript. All authors approved the final version of manuscript.

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REVIEW
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Structure, Regulation, and Function of Linear and Circular Long Non-Coding RNAs

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Long non-coding RNAs (lncRNAs), including linear lncRNAs and circular RNAs (circRNAs), exhibit a surprising range of structures. Linear lncRNAs and circRNAs are generated by different pathways. Linear lncRNAs perform functions that depend on their specific sequences, transcription, and DNA elements of their gene loci. In some cases, linear lncRNAs contain a short open reading frame encoding a peptide. circRNAs are covalently closed RNAs with tissue-specific and cell-specific expression patterns that have recently been extensively investigated. Pioneering work focusing on their biogenesis and functional characterization indicates that circRNAs regulate cell development via multiple mechanisms and play critical roles in the immune system. Furthermore, circRNAs in exosomes function on target cells. As with linear lncRNAs, specific circRNAs can also be translated. In this review, we summarize current understanding and highlight the diverse structure, regulation, and function of linear lncRNAs and circRNAs.

Keywords: linear lncRNAs, circRNAs, structure, regulation, function

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INTRODUCTION

The central dogma of molecular biology describes the relationship between the informational macromolecules, DNA and RNA, and the transfer of the encoded information into proteins (Crick, 1970). However, more than 98% of the human genome is transcribed but only 2% of transcripts encode proteins (Wang et al., 2016; Kopp and Mendell, 2018). The transcripts that are not translated into proteins have been characterized as non-coding RNAs (ncRNAs). Based on their transcript size, they can be defined as small (≤200 nt) (small ncRNAs) and long ncRNAs (>200 nt) (lncRNAs), respectively. Small ncRNAs consist of transfer RNAs (tRNAs), small nucleic RNAs (snRNAs), small nucleolar RNAs (snoRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and QDE-2-interacting RNAs (qiRNAs) (Madhani, 2013; Svendsen and Montgomery, 2018). These small ncRNAs participate in gene regulation at both transcriptional and post-transcriptional levels (Lee et al., 2009; Anderson and Ivanov, 2016; Czech and Hannon, 2016; Wang et al., 2016; Meng X. Y. et al., 2017; Xing et al., 2017; Shen et al., 2018). In contrast to small ncRNAs, lncRNAs, which were once thought to merely represent noise from imprecise transcription initiation, have been proposed to carry out diverse functions in cells (Mowel et al., 2017). Most well-characterized linear lncRNAs are transcribed by RNA polymerase II (Pol II) and are presumably capped, polyadenylated, and contain exon-exon splice junctions like mRNAs (Chen, 2016). However, the 3′ ends of a number of linear lncRNAs are formed in unusual ways (Chen, 2016). It seems that the unique end structures of

linear lncRNAs protect their internal sequence and provide localization signals (Xing et al., 2017). Based on their genomic locations relative to adjacent protein-coding genes, lncRNAs are classified as sense, antisense, bidirectional, intronic, and intergenic lncRNAs (Wang et al., 2011; Chen, 2016; Wu et al., 2017; Kopp and Mendell, 2018).

Linear lncRNAs have diverse functions, including maintaining nuclear structure integrity (Clemson et al., 2009; Lei et al., 2013), positively or negatively regulating genes in *cis* or in *trans* by recruiting transcription factors or chromatin-modifying complexes to DNA targets in the nucleus (Long et al., 2017), acting as decoys to sequester RNA binding proteins (RBPs), or directly interacting with DNA (Chen, 2016; Cloutier et al., 2016). Linear lncRNAs in the cytoplasm serve as competing endogenous RNAs (ceRNAs) for miRNAs. In some cases, linear lncRNAs interact with RBPs to regulate signaling pathways (Fei et al., 2017; Jiang et al., 2018). Nevertheless, linear lncRNAs themselves do not perform sequence-specific functions but their loci are often the source of regulatory elements, such as enhancers and promoters. The process of linear lncRNAs transcription may impact the expression of nearby genes by recruiting specific protein factors (Ebisuya et al., 2008; Kopp and Mendell, 2018; Sanli et al., 2018). Like proteins, the functions of linear lncRNAs depend on their localization pattern in the nucleus and cytoplasm (Chen, 2016; Xing et al., 2017). Moreover, the short peptides produced from some specific linear lncRNAs are also functional (Anderson et al., 2015; Matsumoto et al., 2016; Nelson et al., 2016).

Recent studies suggest that some lncRNAs can form as a circle (circRNAs) and can function as a sponge to recruit miRNAs or transcriptional effectors to regulate target gene expression. Most circRNAs consist of one or more exons, termed extra-coding RNAs (ecRNAs), but some derive from the intron of the parent gene, such as circular intronic RNAs (ciRNAs) and intron retained circRNAs (exon-intron circRNAs, also known as EIciRNAs). circRNAs are more stable than linear ncRNAs because their circular structure cannot be degraded by most RNA decay machinery (Vicens and Westhof, 2014; Meng S. et al., 2017). The first circRNAs to be identified, *viroid*, was found in RNA viruses as early as 1976 (Sanger et al., 1976) and was then found in eukaryotes in 1979 (Hsu and Coca-Prados, 1979). Electron microscopy directly proved that circRNAs exist in eukaryotic cells (Hsu and Coca-Prados, 1979). Although circRNAs have attracted increasing attention, our understanding of their functions is still limited (Li X. et al., 2018). They appear to control brain function by titrating miRNAs (Piwecka et al., 2017), interacting with *RNA*-binding domains to influence cancer development (Fang et al., 2018), and protecting mRNAs from degradation (Zhu et al., 2019). They can also be biomarkers of cancer (Li Y. et al., 2015), ciRNAs and EIciRNAs are localized in the nucleus, where they promote the transcription of their parent genes (Li X. et al., 2018). Some specific circRNAs encode peptides (Legnini et al., 2017; Zhang et al., 2018), but internal ribosome entry site (IRES) elements might be necessary for this (Tatomer and Wilusz, 2017; Yun et al., 2017). Furthermore, recent work has shown that circRNAs may play critical roles in innate immune pathways (Cadena and Hur, 2017; Chen et al., 2017)

Although models of regulation are well established in many species, tissues, and cells types, the functions of linear lncRNAs and circRNAs remain elusive. Here, we highlight advances in our understanding of the multiple structures, functions, and regulation of linear lncRNAs and circRNAs.

STRUCTURES OF LINEAR LNCRNAS

The 5′ m^7G cap and 3′ poly(A) tail are the hallmark structures of eukaryotic mRNAs and most annotated linear lncRNAs are transcribed from their own loci and are spliced just like mRNAs (Wu et al., 2017). However, linear lncRNAs also originate from pre-mRNAs as a result of alternative splicing (Grelet et al., 2017) (**Figure 1A**). The maturation and stabilization of many other linear lncRNAs are achieved through several non-canonical mechanisms that are highly associated with eukaryotic RNAs processing. For example, RNase P, which is best known for its function in tRNAs maturation, recognizes the tRNAs-like structure around the 3′ end of linear lncRNAs and generates a mature 3′ end with a U•A-U triple-helical structure (Wu et al., 2017) (**Figure 1B**). The 3′-end product is further cleaved by RNase Z to form MALAT1-associated small cytoplasmic RNAs (mascRNAs), whose function is still unknown (Wilusz et al., 2008).

Study of non-polyadenylated RNA transcripts in human cells revealed that many excised introns were longer than 200 nt, leading to the discovery of novel non-coding transcripts that lack 5′ caps and 3′ poly(A) tails but that have snoRNAs at both ends (Yin et al., 2012) (**Figure 1C**). A class of linear lncRNAs derived from the Prader-Willi Syndrome (PWS) region was identified and named sno-lncRNA, whose ends correspond to positions of intronic snoRNAs. These Box C/D sno-lncRNAs accumulate near their sites of synthesis and associate strongly with Fox family splicing regulators to alter splicing patterns (Yin et al., 2012). Likewise, one linear lncRNA called (snoRNA)-ended long non-coding RNA that enhances pre-rRNA transcription (*SLERT*) has a unique Box H/ACA snoRNA at both ends. Because *SLERT* is generated from the *TBRG4* gene locus, which is located at a distance from the nucleolus, these ends are critical to protect the *SLERT* internal sequence from degradation and for translocation to the nucleolus (Xing et al., 2017). Remarkably, six types of sno-lncRNAs have been described (Wu et al., 2016; Wu et al., 2017) and their ends are capped by BoxC/D-BoxC/D, BoxH/ACA-BoxH/ACA, BoxC/D-BoxH/ACA, BoxH/ACA-BoxC/D, BoxC/D-poly(A) (5′snoRNA capped and 3′polyadenylated), and BoxH/ACA-poly(A) (**Figures 1C, D**).

REGULATIONS OF LINEAR LNCRNAS

Linear lncRNAs Regulate Gene Expression in *cis* or in *trans*

The functions of most linear lncRNAs depend on their sequence (**Figure 2A**). Various linear lncRNA functions are

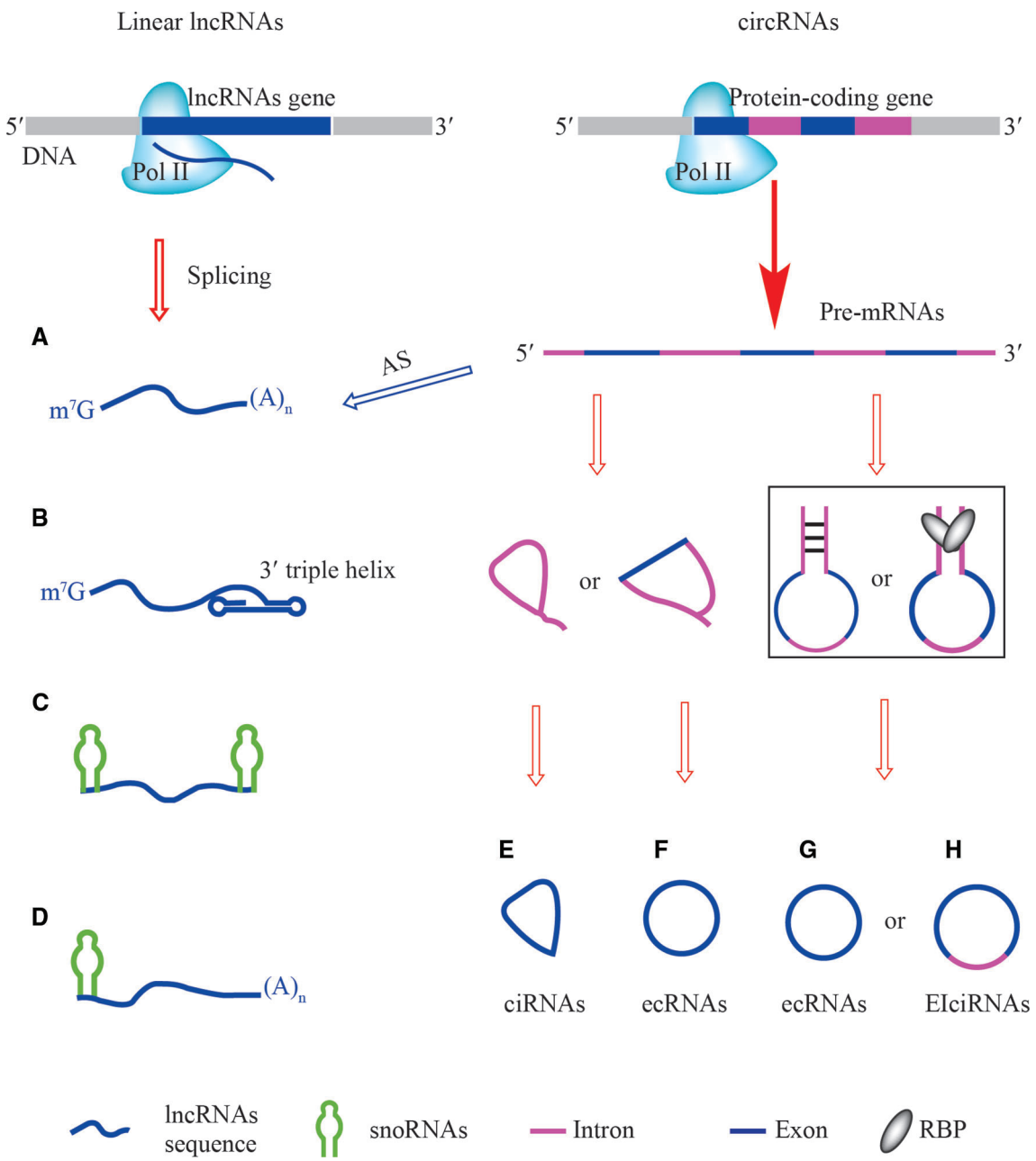

FIGURE 1 | The structures of linear lncRNAs and circRNAs. **(A)** Linear lncRNAs with 5′ m^7G and 3′ poly(A) ends are derived from specific loci (left) or alternative splicing of pre-mRNAs (right). **(B)** Linear lncRNAs with 3′ triple helix ends are alternatively processed by ribonuclease P (RNase P). **(C)** Small nucleolar RNAs (snoRNAs)-ended lncRNAs (sno-lncRNAs), their ends are capped by different structures of snoRNAs, BoxC/D-BoxC/D, BoxH/ACA-BoxH/ACA, BoxC/D-BoxH/ACA, and BoxH/ACA-BoxC/D. **(D)** The 5′ snoRNAs-ended and 3′-polyadenylated linear lncRNAs, their 5′ ends are capped by BoxC/D-poly(A) and BoxH/ACA-poly(A). **(E)** ciRNAs are derived from intron lariats of pre-mRNAs. **(F)** ecRNAs containing exon of pre-mRNAs are generated by internal back-splicing of lariats. **(G, H)** *cis*-acting and *trans*-acting factors are involved in the production of ecRNAs and ElciRNAs from pre-mRNAs.

listed in **Table 1**. The most famous and well-established example of a *cis*-acting linear lncRNA is the X-inactive specific transcript, *Xist* (Penny et al., 1996; Cerase et al., 2015). In placental mammals, one of the two X chromosomes is transcriptionally silenced in the early embryo to provide dosage compensation. During X chromosome inactivation (Galupa and Heard, 2015), *Xist* is only transcribed from the inactivated chromosome. Despite the *Xist* lncRNA having been studied for several decades, its molecular functions are still highly debated (Cerase et al., 2015). In contrast, *cis*-acting linear lncRNAs are involved in dosage compensation in male *Drosophila melanogaster* by doubling the transcription of many

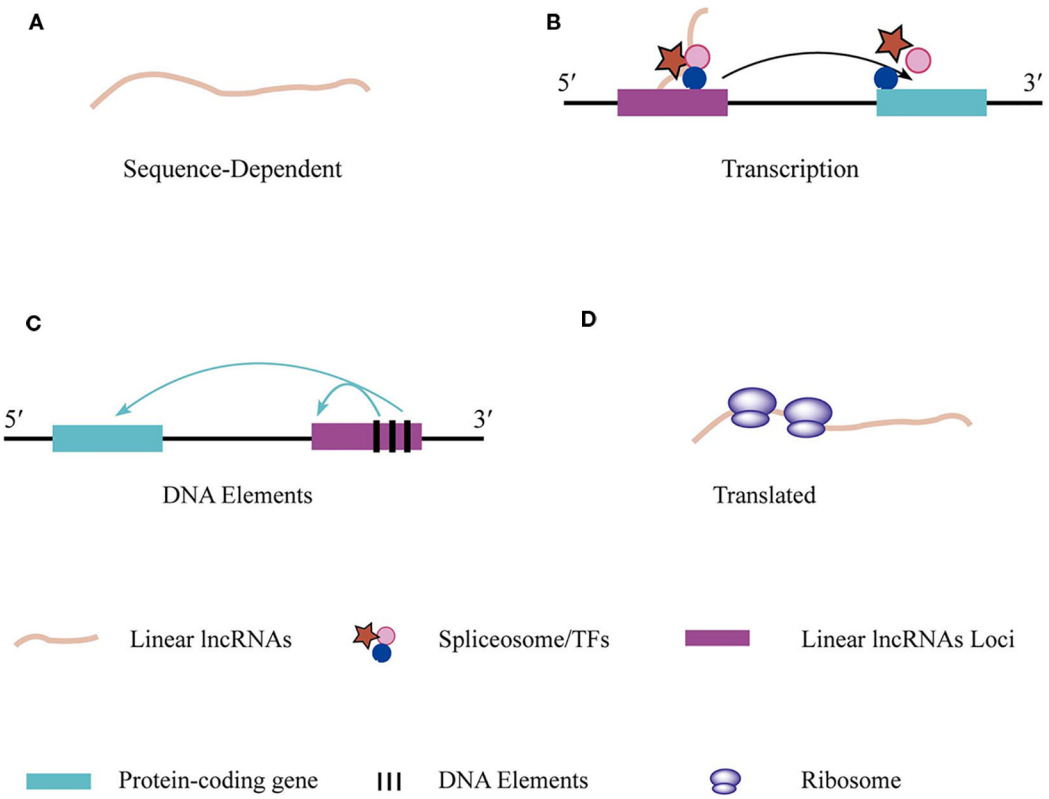

FIGURE 2 | The regulations and functions of linear lncRNAs. **(A)** Linear lncRNAs perform function based on their unique nucleotide sequence which specifically bind to DNA, RNA, and RBPs or absorb miRNAs. **(B)** The transcriptional action of linear lncRNAs regulates adjacent gene expression. **(C)** DNA elements that embed in linear lncRNAs loci are able to regulate adjacent gene transcription **(D)** Some specific linear lncRNAs that contain open reading frame can be translated.

TABLE 1 | The identified linear lncRNAs functions.

Name	Origin	Mechanism	Function	References
Xist	Placental mammals	Sequence-dependent	Gene activation	(Penny et al., 1996)
roX1/roX2	*Drosophila melanogaster*	Sequence-dependent	Gene activation	(Gelbart and Kuroda, 2009)
Linc-RAM	Mouse	Sequence-dependent	Gene activation	(Yu et al., 2017)
HOTAIR	Human	Sequence-dependent	Gene inactivation	(Portoso et al., 2017)
^{DRR}eRNA	Mouse	Sequence-dependent	Gene activation	(Li et al., 2016)
lnc-DC	Human	Sequence-dependent	Interacts with protein	(Pin et al., 2014)
lncRNA-ACOD1	Human	Sequence-dependent	Interacts with protein	(Wang et al., 2017)
lnc-Lsm3b	Human	Sequence-dependent	Decoy	(Jiang et al., 2018)
Lnczc3h7a	Human	Sequence-dependent	Scaffold	(Lin et al., 2019)
H19	Human	Sequence-dependent	miRNA sponge	(Kallen et al., 2013)
Ftx	Placental mammals	Transcription	Gene activation	(Furlan et al., 2018)
Meg3	Mouse	Transcription	Gene inactivation	(Sanli et al., 2018)
PVT1	Human	DNA elements	Gene activation	(Cho et al., 2018)
Bendr	Mammalian	DNA elements	Gene activation	(Engreitz et al., 2016)
LINC00961	Human	Can be translated	Produces a peptide	(Matsumoto et al., 2016)
HOXB-AS3	Primates	Can be translated	Produces a peptide	(Huang et al., 2017)

X-linked genes (Gelbart and Kuroda, 2009). Activating DCCs (dosage compensation complexes) consist of two linear lncRNAs (roX1 and roX2 RNAs) and five male-specific-lethal (MSL) proteins (MSL1, MSL2, MSL3, the acetyltransferase, MOF, and the RNA helicase, MLE). DCCs only form in male flies because of the male-specific expression of the core MSL2 subunit and roX RNA (Maenner et al., 2013). Despite differing in size and having little sequence similarity, roX1 and roX2 are

both thought to be scaffolds for the proper assembly of the MSL proteins. A series of conserved sequence motifs (GUUNUNCG) in the 3′ end of the roX RNA participate in the formation of a stable stem-loop structure (SLroX). The SLroX structure is important for roX RNA function (Maenner et al., 2013). The DCCs recognize the X chromosome through a limited number of "chromosomal entry" or "high-affinity" sites and the incorporation of roX RNA increases their interactions (Fang et al., 2008). Once DCCs are tethered to the active chromatin, lysine 16 of histone H4 (H4K16) is acetylated by MOF, leading to gene activation (Maenner et al., 2013).

In contrast to *cis*-acting linear lncRNAs, some linear lncRNAs also positively or negatively regulate gene transcription at distant sites. One linear lncRNA named *Linc-RAM* (Linc-RNA Activator of Myogenesis) is specifically expressed in mouse skeletal muscle cells. *linc-RAM* is transcriptionally upregulated by MyoD and directly binds to MyoD, which promotes the assembly of the MyoD-Baf60c-Brg1 activation complex on specific regulatory elements of target genes (Yu et al., 2017). Some linear lncRNAs negatively regulate gene transcription in *trans*. The HOX antisense intergenic RNA, *HOTAIR* (2.2 kb), is a spliced and polyadenylated mammalian transcript derived from the *HOXC* locus, one of four HOX gene clusters (*HOXA*, *HOXB*, *HOXC*, and *HOXD*). Using chromatin isolation by RNA purification (ChIRP), *HOTAIR* was shown to interact with the *HOXD* cluster. Although newer methods are needed to further dissect the mechanism of *HOTAIR* function, it is thought to act as a scaffold that coordinates the recruitment of a chromatin-modifying complex to the distant *HOXD* locus, thereby establishing a repressed chromatin state (Portoso et al., 2017; Kopp and Mendell, 2018).

New Insights Into eRNA Regulation

ncRNAs transcribed from active enhancers are known as eRNAs. eRNAs are an important component of transcriptional activation through their promotion of chromatin accessibility, Pol II recruitment, and enhancer-promoter contacts (Li et al., 2016). eRNAs perform their functions in *cis* or in *trans*. Upon enhancer activation, specific transcription factors binding to DNA motifs recruit transcription activators, such as histone acetyltransferases, CREB binding protein (CBP), and p300 (Goodman and Smolik, 2000; Creyghton et al., 2010). CBP and p300 are transcription co-activators that control the expression patterns of genes involved in cell growth, transformation, and development (Jin et al., 2014). Similar to polycomb repressive complex 2 (PRC2), CBP is also a chromatin-modifying enzyme whose activity can be regulated by direct binding to ncRNA (Cerase et al., 2015; Bose et al., 2017). CBP binds directly to a large number of eRNAs in cells. Steady-state histone acetyltransferase (HAT) assays revealed that the RNA binding region of CBP is the HAT domain. Briefly, eRNAs are transcribed from enhancers and act in *cis* to bind to the HAT domain of CBP to activate acetylation activity, which is required for the regulation of target genes (Bose et al., 2017).

Interestingly, the enhancer regions of mouse *MyoD*, which is located on chromosome 7, gives rise to at least two eRNAs. The core enhancer eRNA (^{CE}RNA) influences the adjacent *MyoD* gene, whereas an eRNA named distal regulatory region (DDR) eRNA (^{DRR}eRNA), which is transcribed from the enhancer of MyoD, acts in *trans* (Tsai et al., 2018). Chromatin isolation by RNA purification sequencing (ChIRP-seq) and single-molecule RNA fluorescence *in situ* hybridization (FISH) experiments indicated that ^{DRR}eRNA co-localizes with nascent Myogenin transcripts, which are located on mouse chromosome 1. ^{DRR}eRNA associates with the cohesin complex, which is required for cohesin chromatin recruitment and maintenance on chromosome 1, promoting the activation of the Myogenin gene (Tsai et al., 2018).

Transcription of Linear lncRNAs Regulates Adjacent Gene Expression

Mammalian genomes are pervasively transcribed to produce enormous amounts of linear lncRNA. In addition to sequence-specific regulation, cross-talk exists between linear lncRNA expression and the expression of nearby genes (**Figure 2B**, **Table 1**). As described above, *Xist* promotes the process of X inactivation in female mammals. However, *Xist* expression needs to be tightly controlled by the X-inactivation center (Xic), which contains many linear lncRNA genes, such as *Linx*, *Jpx*, and *Ftx*. Linear lncRNA transcribed from *Ftx* or *Ftx*-embedded miRs had no impact on *Xist* transcription (Furlan et al., 2018). Interestingly, *Ftx* transcription was needed for *Xist* transcriptional activation at the onset of differentiation (Furlan et al., 2018). *Blustr* is a linear lncRNA located 5-kb upstream of the gene, *Sfmbt2*. Prematurely terminated transcription of *Blustr* or mutation of the first 5′ splice site of *Blustr* can abolish the expression of *Sfmbt2*, indicating that the *cis*-activating effect is associated with its transcription (Engreitz et al., 2016). The linear lncRNA, upper hand (*Uph*), provides an example of the translation of a non-coding RNA having a critical impact on the transcriptional activation of a nearby gene, *Hand2*. HAND2 is a transcription factor that controls the reprogramming of fibroblasts into cardiomyocytes. Termination of *Uph* transcription independently of its transcript resulted in the loss of *Hand2* expression in the mouse heart, which leads to embryonic lethality (Anderson et al., 2016).

Interestingly, the expression of linear lncRNAs may negatively regulate nearby genes. Delta-like-1 (*Dlk1*) encodes a ligand that inhibits Notch1 signaling and plays a role in placental development, nutrient metabolism, and adipocytosis (Sanli et al., 2018). The *Dlk1-Dio3* imprinted domain, which contains three protein-coding genes, *Dlk1* (also called *Pref1*), *Rtl1*, and *Dio3*, also expresses multiple ncRNAs, such as linear lncRNA, *Meg3* (maternally expressed gene 3, also called *Gtl2*), the C/D-box snoRNA cluster, *Rian*, the miRNA cluster, *Mirg*, and the Rtl1-antisense, *Rtl1as* (Sanli et al., 2018). In a hybrid embryonic stem cell system, *Dlk1* became imprinted and was involved in transcriptional upregulation on the paternal chromosome, while the maternal *Dlk1* gene remained poised for activation during neural differentiation. It has been postulated that the genes are repressed by one of the locus' ncRNAs. Both *Meg3* expression and the H3-Lys-27 methyltransferase, EZH2, prevent *Dlk1* activation in *cis* on the maternal chromosome. However, the *Meg3* linear lncRNA was partially retained in *cis* and overlaps

with the maternal *Dlk1*. A future challenge is, therefore, to answer whether the *Meg3* linear lncRNA is involved in chromatin repression (Sanli et al., 2018).

DNA Elements of Linear lncRNAs Regulate Adjacent Gene Expression

Cis-regulatory activity may involve DNA elements of a linear lncRNA locus, such as the promoter, but be independent of the linear lncRNA transcript or the transcription of linear lncRNA in general (**Figure 2C**, **Table 1**). Plasmacytoma variant translocation 1 (*PVT1*) was the first linear lncRNA identified in human Burkitt's lymphoma (Graham and Adams, 1986). *PVT1* and the myelocytomatosis (*MYC*) oncogenes are located some distance apart on 8q24 and the *PVT1*-encoded linear lncRNA and miRNA have oncogenic functions (Cho et al., 2018). Indeed, *PVT1* performs an oncogenic function by stabilizing the MYC protein (Tseng et al., 2014). However, silencing *PVT1* using CRISPR interference (CRISPRi) technology unexpectedly enhanced cell proliferation of glioblastoma cells and induced pluripotent stem cells. Furthermore, recurrent structural rearrangements of the *PVT1* locus disrupts *PVT1* transcription in cancer genomes, indicating that the *PVT1* locus has unknown regulatory mechanisms. These findings suggest that the *PVT1* locus harbors four intragenic enhancers of *MYC*, which promote *MYC* transcription. Furthermore, the competition for enhancers between the *PVT1* and *MYC* promoters is proposed to control cell development in glioblastoma cells and induced pluripotent stem cells independent of the *PVT1* linear lncRNA (Cho et al., 2018). As another example, the promoter of *Bendr* (*Bend4*-regulating effects not dependent on the RNA) has a regulatory function for the adjacent *Bend4* gene. Inserting a polyA signal into the first intron of *Bendr* had no effect on *Bend4* expression, indicating that the regulatory function was independent of *Bendr* RNA transcription. However, deletion of the ~750 bp *Bendr* promoter-proximal region reduced expression of the adjacent *Bend4* gene by 57%. Therefore, *cis* activation of *Bend4* requires the promoter of a nearby linear lncRNA (Engreitz et al., 2016; Kopp and Mendell, 2018).

FUNCTIONS OF LINEAR LNCRNAS

Linear lncRNAs Interact Directly With Proteins, Serve as Decoys, and Act as Scaffolds

Proteins in the cytoplasm function through various mechanisms and, in some cases, their function is linked to a functional linear lncRNA (Pin et al., 2014). Early in 2014, *lnc-DC* was found to be exclusively expressed in conventional human dendritic cells (DCs) and to control human DC differentiation by directly binding to STAT3 (signal transducer and activator of transcription 3) in the cytoplasm (Pin et al., 2014). In another case, the metabolic enzyme, glutamic-oxaloacetic transaminase 2 (GOT2), was confirmed to be an *lncRNA-ACOD1* binding protein. *lncRNA-ACOD1* is induced by multiple viruses but is independent of type I interferon (IFN-I). Upon induction, *lncRNA-ACOD1* activates GOT2 and substantially changes cellular metabolism for the benefit of viruses. Down-regulation of *lncRNA-ACOD1* dramatically protects mouse and human cells from virus infection. It is considered to be a tactic by which viruses complete their life cycle and propagate (Wang et al., 2017).

Linear lncRNAs also serve as decoys to replace other kinds of RNA and then bind to host proteins. In mouse macrophages, retinoic acid-inducible gene I (*Rig1*) has the ability to bind to pathogenic RNA through its C-terminal domain (CTD) in the presence of ATP and to promote innate immunity (Jiang et al., 2018). RIGI recognizes double-stranded viral RNA (dsRNA) that has invaded cells and indirectly promotes the transcription of interferons. Strikingly, in addition to resisting virus RNA, interferons also induce the production of a linear lncRNA called *lnc-Lsm3b*. *lnc-Lsm3b* serves as decoy to replace viral RNA and it has been proposed that inhibition of RIG-I activation was probably because RIG-I oligomerization was prevented (Jiang et al., 2018). A similar model for myogenesis was reported by David Glass's group (Gong et al., 2015). One linear lncRNA, *LncMyoD*, located next to the *MyoD* gene, was directly activated by *MyoD* during myoblast differentiation, but did not bind to *MyoD*. RNA pull-down experiments identified IGF2-mRNA-binding protein 2 (IMP2) as the binding partner of *LncMyoD*. The increased level of *LncMyoD* serves as decoy to outcompete other mRNAs, such as *N-Ras* and *c-Myc*, blocking proliferation and creating a permissive state for differentiation (Gong et al., 2015).

In addition to these mechanisms of interacting with proteins, linear lncRNAs also regulate protein function by serving as a cytoplasmic scaffold. Recent attention has focused on an E3 ligase, tripartite motif 25 (TRIM25), a protein down-stream of RIGI. A linear lncRNA named *Lnczc3h7a* was detected in an RNA pull-down assay of RIGI. Overexpression of *Lnczc3h7a* increased the RIGI-TRIM25 interaction and K63-linked ubiquitination of RIGI in VSV-infected mouse fibroblast cells, but the interaction was absent in uninfected RAW264.7 cells. An RNA pull-down assay showed that *Lnczc3h7a* binds to the helicase of RIGI and the C-terminal SPRY domain within TRIM25. Individual-nucleotide-resolution cross-linking and immunoprecipitation experiments indicated that the *Lnczc3h7a* binding site of TRIM25 is around nucleotide 311, while the *Lnczc3h7a* binding sites of RIGI are around nucleotide 308 and 332. Altogether, *Lnczc3h7a* serves as a scaffold to facilitate the RIGI-TRIM25 interaction and to regulate their functions in response to virus infection (Lin et al., 2019).

Linear lncRNAs Serve as Sponges for miRNAs

With the development of the competing endogenous RNAs (ceRNAs) hypothesis, a large number of linear lncRNAs that serve as miRNAs sponges were identified to play roles in regulating translation (Mousavi et al., 2014). The linear lncRNA, *MIR100HG*, and two *MIR100HG*-derived miRNAs, miR-100 and miR-125b, play important roles in the cetuximab resistance of cetuximab-sensitive CRC cells and head and neck squamous cell carcinoma cells lines (Lu et al., 2017). The

transcription factor, GATA6, inhibits the production of the linear lncRNAs, *MIR100HG*, but one of the two miRNAs, miR-125b, provides feedback inhibition of GATA6 and relieves the repression. Thus, increased levels of *MIR100HG* produces more miR-100 and miR-125b, which coordinate to repress five Wnt/β-catenin negative regulators, resulting in increased Wnt/β-catenin signaling (Lu et al., 2017). The human linear lncRNAs, *H19*, is a ~2.3 kb, capped, spliced, and polyadenylated RNAs, predominantly located in the cytoplasm that is implicated in genetic disorders and cancer (Jia et al., 2018). However, the mechanisms by which *H19* regulates gene function remain elusive. *H19* can serve as a molecular sponge for miRNAs to modulate gene expression in the mouse myogenic C2C12 cell line. Bioinformatic analysis revealed that *H19* has one canonical and three non-canonical binding sites for the miRNAs, *let-7*, while *let-7* overexpression results in a differentiated myoblast phenotype. However, strongly induced *H19* acts as a sponge for *let-7* and hinders muscle differentiation (Kallen et al., 2013).

Some Linear lncRNAs Encode Short Peptides

Deep-sequencing technologies have led to the identification of a large number of linear lncRNAs that lack obvious long protein-coding open reading frames (ORFs). However, some linear lncRNAs with putative small ORFs of less than 100 amino acids actually code for proteins that play important roles in cells (Anderson et al., 2015; Matsumoto et al., 2016; Nelson et al., 2016) (**Figure 2D**, **Table 1**). Furthermore, in some specific cases, linear lncRNAs possess dual functions that are dependent on both the linear lncRNA itself and proteins encoded by the linear lncRNA (Anderson et al., 2015; Yu et al., 2017).

Small regulatory polypeptide of amino acid response (SPAR) is a short, 90 amino acid peptide encoded in humans and mice by the linear lncRNA, *LINC00961*. SPAR plays an important role in muscle regeneration. SPAR contains a conserved transmembrane domain at its N terminus with its C terminus extending into the cytosol. Immunofluorescence staining showed that SPAR is localized to late endosomes/lysosomes. Further work revealed that this small peptide interacts with the lysosomal v-ATPase to negatively regulate mTORC1 activation by amino acids (Roberto et al., 2011). Using CRISPR/Cas9 engineering to knockout the SPAR peptide showed that down regulation of SPAR enables efficient activation of mTORC1 and promotes muscle regeneration (Matsumoto et al., 2016).

The Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) controls the release and reuptake of Ca^{2+} from the sarcoplasmic reticulum (SR). Myoregulin (MLN) and dwarf open reading frame (DWORF), which are 46 and 34 amino acids long, respectively, are two functional mammalian polypeptides encoded by linear lncRNAs that modulate the calcium pump, SERCA, and control muscle performance (Anderson et al., 2015; Nelson et al., 2016). MLN, phospholamban (PLN), and sarcolipin (SLN) share a similar structure and function and inhibit SERCA. In contrast to MLN, DWORF enhances SERCA activity by displacing the SERCA inhibitors MLN, PLN, and SLN (Nelson et al., 2016). Deletion of MLN in mice skeletal muscle enhances Ca^{2+} handling and improves exercise performance. Overexpression of DWORF in mouse cardiomyocytes increases peak Ca^{2+} transient amplitude and SR Ca^{2+} load during each cycle of contraction-relaxation, reducing the decay time constant of cytosolic Ca^{2+} (Nelson et al., 2016). Thus, MLN may have the opposite function to DWORF. Interestingly, in addition to encoding the peptide MLN, the linear lncRNA, linc-RAM, promotes myogenic differentiation by interacting with MyoD (Yu et al., 2017).

Small peptides encoded by putative linear lncRNAs can suppress cancer cell growth. Cancer cells, including colorectal cancer (CRC) cells, exhibit distinct metabolic reprogramming patterns, which support rapid proliferation (Israelsen and Vander Heiden, 2015). The glycolytic enzyme, pyruvate kinase M (PKM), plays an important role in this process (Mayumi et al., 2012). There are two PKM isoforms, PKM1 and PKM2, resulting from alternative splicing of the *PKM* pre-mRNA. PKM2 is almost universally re-expressed in cancer cells and supports the proliferation of cancer cells, whereas the adult isoform, PKM1, is universally expressed in normal cells. The *HOXB* cluster antisense RNA 3 (*HOXB-AS3*) gene is a linear lncRNA gene that has the ability to produce a conserved 53-amino acid peptide. This peptide modulates the alternative splicing of the *PKM* pre-mRNA to yield more PKM1, which suppresses CRC cell growth (Huang et al., 2017).

STRUCTURES AND PROPERTIES OF CIRCRNAS

Most annotated circRNAs originate from nucleolar pre-mRNAs, but some circRNAs are derived from mitochondria (Liu et al., 2019). Several mechanisms are associated with the biogenesis of circRNAs. ciRNAs are derived from the failure of intronic lariat debranching during canonical splicing (Zhang et al., 2013). Bioinformatic and experimental evidence indicate that the formation of ciRNAs depends on a consensus RNA motif that contains a 7-nt GU-rich element near the 5′ ss and an 11-nt C-rich element near the branch point (Zhang et al., 2013) (**Figure 1E**). Unlike linear mRNAs, which are mainly located in the cytoplasm, human ciRNAs are preferentially localized in the nucleus (Li X. et al., 2018). Additionally, lariats containing an exon can form during exon skipping and internal back-splicing allows the formation of extra-coding RNAs (Steven et al., 2015) (**Figure 1F**). The biogenesis of circRNAs in flies and humans is highly dependent on intronic sequences. RBPs also promote exon circularization by binding to sequences in the flanking introns (Zhang et al., 2013). In some cases, the biogenesis of circRNAs is influenced by a combination of *cis*-acting elements and *trans*-acting splicing factors (Kramer et al., 2015). Although the majority of circRNAs normally contain multiple exons (**Figure 1G**), alternative splicing allows intron retention within EIciRNAs, which have been found to remain in the nucleus (Zhang et al., 2014) (**Figure 1H**).

Some protein factors can disrupt *cis*-acting elements associated with circRNA biogenesis. *Alu* elements are repetitive elements that make up more than 10% of the human genome (De

Koning et al., 2011) and which are involved in the biogenesis of endogenous circRNAs. However, the function of *Alu* elements is inhibited by nuclear RNA helicase, DHX9. DHX9 interacts directly with the interferon-inducible isoform of ADAR (p150) and disrupts circRNA biogenesis. The loss of DHX9 doubles the production of circRNAs (Aktaş et al., 2017).

Most circRNAs are exported to the cytoplasm from the nucleus, except for intron-containing circRNAs. Two proteins, spliceosome RNA helicase, DDX39B (also called DEAD box protein UAP56 or UAP56), and ATP-dependent RNA helicase, DDX39A (also called nuclear RNA helicase URH49 or URH49), are associated with transporting circRNAs. In humans, UAP56 exports circRNAs larger than 1,200 nucleotides whereas URH49 exports circRNAs smaller than 400 nucleotides (Huang et al., 2018).

So far, we know very little about the degradation mechanisms of circRNAs, but a few examples have been studied. For example, circRNA *CDR1as* (also known as ciRS-7) is degraded by the Argonaute 2 (AGO2) protein complex (Kopp and Mendell, 2018). The binding of miR-671 to *CDR1as* triggers AGO2-dependent cleavage of *CDR1as*. Evidence also shows that N^6-methyladenosine (m^6A) within circRNAs promotes their degradation (Park et al., 2019). Furthermore, the circRNA that binds to dsRNA-activated protein kinase (PKR) is degraded by RNase L (Liu et al., 2019).

REGULATIONS OF CIRCRNAS

circRNAs in Exosomes

Exosomes are small membrane vesicles of endocytic origin secreted by most cells types. Their cargos of proteins, mRNAs, and miRNAs modulate recipient cell behaviors. Recently, circRNAs have also been shown to be abundant in exosomes, where they may function as miRNA sponges (Li Y. et al., 2015) (**Figure 3A**). Indeed, circRNAs in exosomes secreted from adipocytes and cancer cells play important roles in regulating their target cells. In hepatocellular carcinoma (HCC) patients with higher body fat ratios, exosome circ-deubiquitination (*circ-DB*) from adipocytes is upregulated. Ubiquitin-specific protease 7 (USP7) is a deubiquitinating enzyme and a high level of USP7 is frequently found in HCC tissues. *circ-DB* absorbs miR-34a, which targets *USP7* mRNA and promotes the expression of USP7 (Zhang et al., 2019a). Interestingly, exosomal circRNAs derived from gastric cancer (GC) cells also function as sponges for miRNA. PR domain containing 16 (PRDM16), a zinc finger transcription factor, plays important roles in the browning of white adipose tissue (WAT) in GC patients. miR-133 has been proposed as an upstream regulator of PRDM16. The circRNA, *ciRS-133*, in exosomes derived from GC cells can sponge miR-133 and activate PRDM16 (Zhang et al., 2019b).

circRNAs Regulate Maternal Gene Transcription

Most circRNAs are derived from the middle exons of protein-coding genes, and they can affect the splicing of their linear counterparts (**Figure 3B**). The second exon of the splicing factor, muscleblind (MBL/MBNL1), is circularized in flies and humans. Interestingly, the flanking introns of the second exon contain conserved muscleblind binding sites, which are bound by MBL. The MBL levels strongly affect the circulation and production of *circMbl*. Thus, the biogenesis of *circMbl* is strongly regulated by MBL and can function in gene regulation by competing with linear splicing (Reut et al., 2014).

Arabidopsis circSEP3 is derived from exon 6 of SEPALLATA3 (SEP3). *circSEP3* binds strongly to its cognate DNA locus, forming an RNA:DNA hybrid, or R-loop. The formation of the R-loop results in transcriptional pausing and leads to the formation of alternatively spliced SEP3 mRNA with exon skipping, which in turn drives floral homeotic phenotypes (Conn et al., 2017). Another case is friend leukemia virus integration 1 (FLI1), a transcription factor that promotes tumor growth. Interestingly, a circRNA FLI1 exonic circular RNA (*FECR1*) derived from exons 4-2-3 of the *FLI1* pre-mRNA can bind to the FLI1 promoter and recruits demethylase TET1 to the promoter region of its own host gene (Chen et al., 2018).

Most circRNAs are located in the cytoplasm, but the intron lariats processed to ciRNAs or EIciRNAs are restricted to the nucleus in human cells. The nuclear retained circRNAs regulate transcription and splicing of their parent genes. The ciRNA, *ci-ankrd52*, is derived from the second intron of ankyrin repeat domain 52 (*ANKRD52*) and mainly accumulates in the nucleus, associating with the elongation Pol II machinery to positively regulate translation of its encoding gene (Zhang et al., 2013). The EIciRNA, *ElciEIF3J*, is predominantly localized in the nucleus, where it interacts with U1 snRNP via specific RNA-RNA interaction, promoting the transcription of their parental genes (Li Z. et al., 2015).

Pseudogenes Derived From circRNAs

Pseudogenes are usually derived from the integration of reverse-transcribed linear mRNAs and it is estimated that about 10% of known gene loci in humans and mice are processed pseudogenes. Although circRNAs are speculated to be stable in cells, some circRNAs can be retro-transcribed and ultimately inserted back into the host genome as processed pseudogenes (**Figure 3C**). In contrast to linear mRNAs, circRNA-derived pseudogenes have an exon-exon junction in the reverse order (non-colinear), which allows for rearrangements of exons into the host genome. For example, 33 pseudogenes are predicted to be derived from *circRFWD2* because of the existence of the non-colinear exon 6-exon 2 junction sequence (Dong et al., 2016).

circRNAs Are Involved in Innate Immune Responses

As discussed above, RIGI is necessary for activating innate immunity in response to viral infections. In addition to binding to viral dsRNA or specific self-produced linear lncRNAs, such as *lnc-Lsm3b* or *Lnczc3h7a*, RIGI also discriminates between exogenous and endogenous circRNAs. Delivery of purified *in vitro* generated circRNA to mammalian cells showed that RIGI is necessary for sensing foreign circRNAs, leading to the activation of innate immunity (Chen et al., 2017)

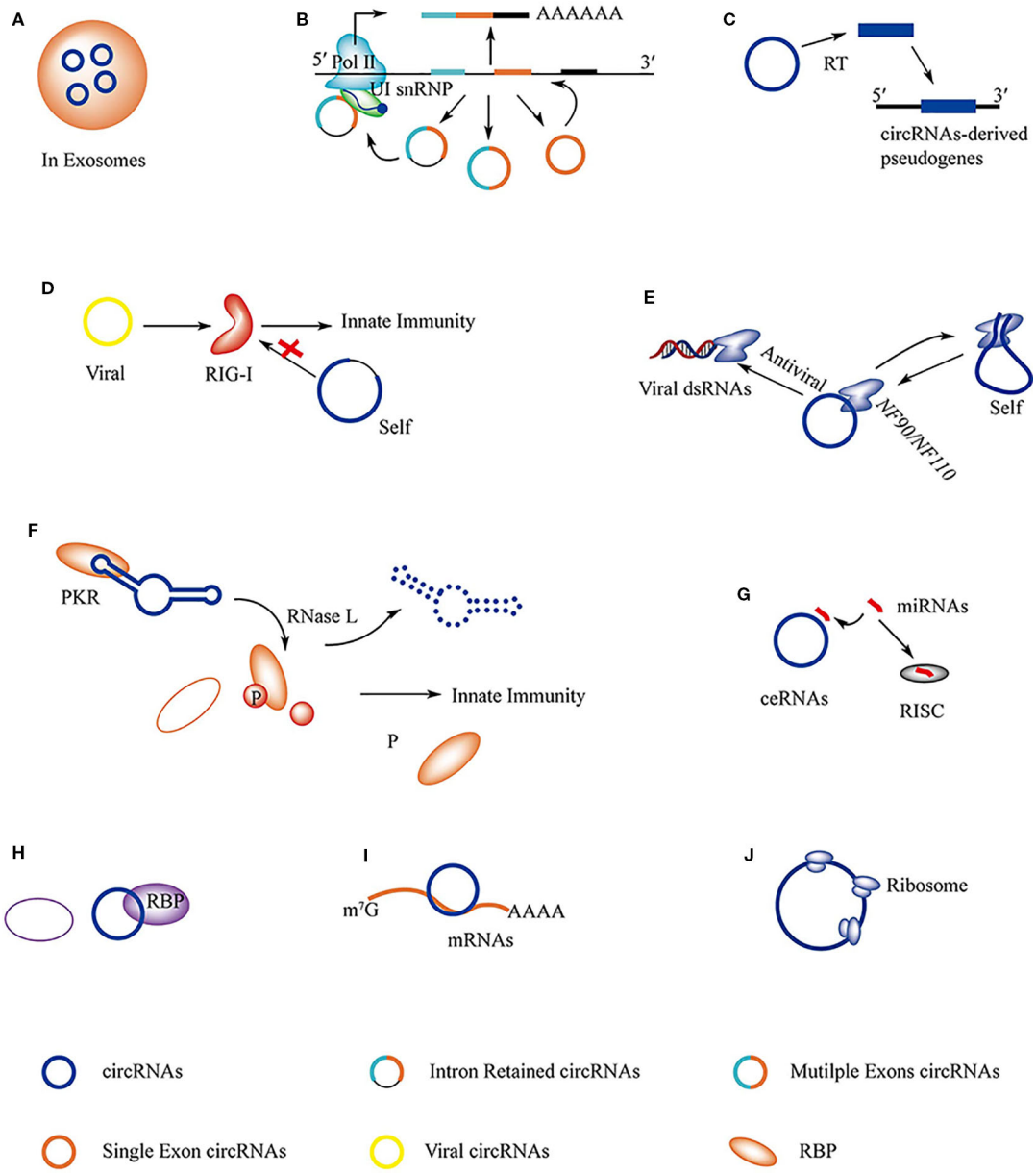

FIGURE 3 | The regulations and functions of circRNAs. **(A)** circRNAs in exosomes can be transported from host cell to target cell where circRNAs serve as miRNAs sponges to regulate gene expression. **(B)** ciRNAs, ElciRNAs, and ecRNAs are able to regulate the expression of their maternal gene in nucleous. **(C)** Pseudogenes are derived from circRNAs. **(D)** RIG-I is able to distinguish viral circRNAs from self-originated ciRNAs and activate immune response. **(E)** Immune factor *NF90/NF110* has dual function that is associated with the biogenesis of circRNAs and innate immune response. **(F)** The structure of circRNAs play important roles in stabilizing PKR activity, the degradation of circRNAs by RNase L releases the activity of PKR and results in immune response. **(G)** circRNAs serve as miRNAs sponge in cell and regulate gene expression. **(H)** circRNAs are able to interact with RBPs directly and regulate its function; circRNAs serve as decoy to competitive binding to RBPs with other RNAs, like mRNAs and viral dsRNAs; circRNAs are found to act as scaffold to interact with RBPs. **(I)** circRNAs interact with mRNAs and protect it from degradation. **(J)** Specific circRNAs can be translated into protein.

(**Figure 3D**). Interestingly, RIGI does not recognize endogenous circRNAs. Using a human intron to express a foreign circRNA sequence abrogated immune activation confirming that RIGI discriminates between self- and foreign circRNAs by intron identity (Chen et al., 2017). However, the mechanism by which RIGI recognizes exogenous circRNAs remains unknown.

Protein factors involved in the biogenesis of circRNAs can act as immune factors (Li et al., 2017). Immune factors involved in circRNA formation were identified by combining immune factors with circRNA biogenesis and performing genome-wide siRNA screening. The double-stranded RNA-binding domain containing immune factors, *NF90/NF110*,

were identified as a dual-function protein (Li et al., 2017). *NF90/NF110* promotes circRNA processing by stabilizing the flanking intronic RNA pairs in the nucleus and subsequently interacts with the mature circRNA to form the *NF90/NF110*-circRNA complex (circRNPs) in the cytoplasm. Upon viral infection, *NF90/NF110* is released from the circRNPs, resulting in an antiviral immune response (Cadena and Hur, 2017; Li et al., 2017) (**Figure 3E**).

Pioneering work by Liu et al. led to the discovery that many circRNAs play important roles in the autoimmune disease, systemic lupus erythematosus (SLE) (Liu et al., 2019). They found a global reduction in circRNAs and aberrant PKR activation in peripheral blood mononuclear cells (PBMCs) in patients with SLE. They also showed that many circRNAs in normal PBMCs tend to form 16–26 bp intra-molecularly imperfect RNA duplexes (intra-dsRNA). These unique structures can bind to PKR, a receptor that can sense viral nucleic acid and direct antiviral activity (Schlee and Hartmann, 2016; Liu et al., 2019). In the early cellular innate immune response, these circRNAs are degraded by RNase L. This releases active PKR, which is in turn linked to SLE progression (**Figure 3F**). In this case, the unique intra-dsRNA structure and the degradation of circRNA are two important factors that inhibit and activate PKR activity, respectively.

FUNCTIONS OF CIRCRNAS

circRNAs Serve as Sponges for miRNAs

In recent years, the diverse regulatory mechanisms of circRNAs have become clear, as listed in **Table 2**. To regulate mature miRNA activity some circRNAs serve as miRNA sponges. In human and mouse brains, the natural antisense transcript of cerebellar degeneration-related protein 1 (*CDR1as*) is a circRNA found in the cytoplasm of neurons. *CDR1as* contains over 70 conserved seed matches for miR-7 and one binding site for miR-671 (Jens, 2013). According to preliminary studies, *CDR1as* can absorb miR-7 and the binding sites are only partially complementary to miR-7, which ensures that *CDR1as* is not sliced by Ago2. However, *CDR1as* is almost fully complementary to miR-671, thus the miR-7 cargo can be released by miR-671-mediated slicing of *CDR1as* (Kopp and Mendell, 2018). The deregulation of miR-7 results in the upregulation of immediate early genes (IEGs), which are strongly linked to increased neuron activity (Piwecka et al., 2017) (**Figure 3G**). However, *CDR1as* also functions in non-brain tissues, such as in islet and HCC cells, where it targets miR-7 expression (Xu et al., 2015; Yu et al., 2016), and in bladder cancer tissues where it sponges miR-135a (Li P. et al., 2018). With development of the ceRNA hypothesis, additional circRNAs have been discovered to act as miRNA sponges in cells (Zheng et al., 2016; Shan et al., 2019; Wang et al., 2019).

circRNAs Interact Directly With Proteins, Serve as Decoys, or Act as Scaffolds

Similar to linear lncRNAs, circRNAs also have the ability to interact with proteins and regulate protein function (**Figure 3H**). The circRNA, *circ-Ccnb1*, can inhibit breast cancer progression and was enhanced by mutant p53 (Yuan et al., 2006; Fang et al., 2018). The tumor suppressor p53 is a transcription factor that contains 393 amino acids but mutant p53 enhances cancer progression and malignancy. The malignancy consequences that result from the many possible p53 mutations are complicated making the study of downstream p53 signaling difficult. Two key proteins, H2AX and Bclaf1, which function in DNA repair and cell mitosis, play roles in cancer development (Sone et al., 2014; Zhou et al., 2014). Mutant p53 does not bind to H2AX enabling a new approach to repress malignant tumor progression caused by mutant p53. A model of *circ-Ccnb1*-protein interactions was proposed, in which *circ-Ccnb1* interacts with H2AX and wild-type p53 to sustain cell proliferation and survival. However, in the mutant p53 cell (in which *circ-Ccnb1* was down-regulated), ectopically delivered *circ-Ccnb1* interacts with H2AX and Bclaf1, which induces the death of the p53 mutant cancer cells (Fang et al., 2018).

A recent study indicated that circRNAs can serve as decoys that compete with mRNAs in binding to proteins (Abdelmohsen et al., 2017). HuR is a RBP that associates with a wide range of RNAs to regulate protein expression patterns (Lebedeva et al., 2011). HuR positively regulates the translation of Poly(A)-

TABLE 2 | The identified circRNAs functions.

Name	Origin	Structure	Function	References
circHIPK3	Human	Single exon	miRNA sponge	(Zheng et al., 2016)
circ-Ccnb1	Human	Multiple exons	Interacts with protein	(Fang et al., 2018)
circACC1	Human	Multiple exons	Scaffold	(Li et al., 2019)
circMbl	*Drosophila melanogaster*	Single exon	Regulates maternal gene	(Ashwal-Fluss et al., 2014)
CircSEP3	*Arabidopsis*	Single exon	Regulates maternal gene	(Conn et al., 2017)
FECR1	Human	Multiple exons	Regulates maternal gene	(Chen et al., 2018)
ci-ankrd52	Human	Intronic	Regulates maternal gene	(Zhang et al., 2013)
ElciEIF3J	Human	Exon-intron	Regulates maternal gene	(Li Z. et al., 2015)
circRFWD2	Mouse	Multiple exons	Pseudogene host	(Dong et al., 2016)
circPan3	Mouse	Multiple exons	Protects mRNAs	(Zhu et al., 2019)
circ-SHPRH	Human	Multiple exons	Produces a peptide	(Zhang et al., 2018)
Circ-ZNF609	Human, Mouse	Multiple exons	Produces a peptide	(Legnini et al., 2017)

binding protein nuclear 1 (*PABPN1*) mRNA. Interestingly, the circRNA, *CircPABPN1*, which is derived from the *PABPN1* pre-mRNAs, can regulate the translation of its linear counterparts. High levels of *CircPABPN1* can suppress HuR binding to *PABPN1* mRNA, leading to decreased translation. *CircPABPN1* also acts as a decoy to competitively bind to HuR and negatively regulate *PABPN1* mRNA translation (Abdelmohsen et al., 2017) (**Figure 3H**). The CCHC-type zinc finger nucleic acid binding protein, CNBP, binds to the *HuR* promoter and promotes its transcription, which plays an important role in GC. However, *circ-HuR* derived from *HuR* interacts with CNBP and inhibits its binding to the *HuR* promoter, resulting in repression of tumor progression. In this case, *circ-HuR* might also be a decoy to regulate the function of CNBP (Yang et al., 2019).

circRNAs have also been observed to act as scaffolds, promoting protein assembly (Du et al., 2016; Li et al., 2019). AMP-activated protein kinase (AMPK) is a tri-complex consisting of an α catalytic subunit and β and γ regulatory subunits that is a critical sensor of cellular energy status (Hardie et al., 2012). During serum deprivation, *circACC1* derived from *ACC1*-pre-mRNA regulates the assembly of AMPK, promoting β-oxidation and glycolysis. Upon metabolic stress, *circACC1* is up-regulated in cells and interacts with the regulatory β and γ subunits to form a ternary complex, which promotes lipid metabolism (Li et al., 2019). In this case, the circRNA may serve as a scaffold to facilitate the assembly of AMPK and increase its enzyme activity as part of an efficient strategy to cope with environmental pressure (**Figure 3H**).

circRNAs Protect mRNAs From Degradation

In adult mice, the intestinal epithelium is renewed from multipotent intestinal stem cells (ISCs) and is the most rapidly self-renewing tissue with a turnover of 5 days (Zhu et al., 2019). ISCs located at the base of intestinal crypts are capable of giving rise to all epithelial lineages and exhibit long term self-renewal (Toshiro et al., 2009). Innate lymphoid cells (ILCs) located on mucosal surfaces potentiate the immune system, sustain mucosal integrity and tissue homeostasis (David and Hergen, 2015). IL-13 secreted by ILC2s engages with IL-13Rα1, which is an IL-13 receptor subunit on crypt ISCs, and activates Wnt-β-catenin signaling (Zhu et al., 2019). Interestingly, a circRNA named *circPan3* derived from the *Pan3* gene transcript is highly expressed in mouse and human ISCs. *circPan3* protects *Il13ral* mRNA from KSRP (an mRNA decay protein) mediated degradation and promotes the production of IL-13Rα1 in crypt ISCs, resulting in the reception of more IL-13 (Zhu et al., 2019) (**Figure 3I**).

circRNAs With Protein-Coding Ability

Although circRNAs lack the 5′ end 7-methylguanosine (m^7G) cap structure and the 3′ poly(A) tail that are necessary for mRNA translation, they have the potential to produce proteins. In 1995, an artificial circRNA was demonstrated to be translatable in eukaryotic cells (Chen and Sarnow, 1995). Recent studies have shown that specific endogenous circRNAs also code for proteins. To yield proteins, circRNAs need IRES elements, which directly bind initiation factors or the ribosome itself to drive translation of the ORF (Tatomer and Wilusz, 2017) (**Figure 3J**).

The human SNF2 histone linker PHD RING helicase (*SHPRH*) gene, an E3 ligase that targets the proliferating cell nuclear antigen (PCNA) for degradation (Akira et al., 2006; Ildiko et al., 2006), is located in the 6q24 chromosomal region. The loss of heterozygosity in this region is associated with a wide variety of cancers. Interestingly, this region also produces a novel circRNA named *circ-SHPRH*, which encodes a 17 kDa protein, SHPRH-146aa. Interestingly, SHPRH-146aa shares the same amino acid sequence as the C-terminal 1520–1651 residues of full-length SHPRH. The common amino acid sequence of SHPRH-146aa reduces the likelihood of SHPRH degradation by another E3 ligase, DTL. Overexpression of SHPRH-146aa reduces malignancy and tumorigenicity both *in vitro* and *in vivo* (Zhang et al., 2018). Thus, SHPRH-146aa might protect full-length SHPRH from DTL-induced ubiquitination (Zhang et al., 2018).

circ-ZNF609 is another example of a protein-coding circRNA identified in murine and human myoblasts that specifically controls myoblast proliferation. *circ-ZNF609* originates from the second exon of its host gene and contains a 753-nt ORF from the start codon to an in-frame STOP codon. Sucrose gradient fractionation experiments proved that *circ-ZNF609* binds to polysomes. Using an expression vector and the CRISPR/Cas9 system, a 3xFLAG-coding sequence was inserted upstream of the STOP codon *in vitro* and *in vivo*. Western blot experiments then showed that *circ-ZNF609* has the ability to produce a protein (Legnini et al., 2017).

CONCLUSIONS AND PERSPECTIVES

RNA-seq has revealed thousands of functional lncRNA molecules in diverse species. The mechanisms of lncRNAs regulation are much more diverse than previously thought. The discovery of functional lncRNAs might shed new light on embryonic development, psychological disorders, and physical diseases. In the past few decades, novel end structures have been shown to play important roles in the functions of linear lncRNAs. With the development of integrated approaches, there is great potential to discover new types of linear lncRNA. lncRNAs seem to perform their functions based on their unique nucleotide sequence, which enables specific binding to DNA, RNA, and RBPs or absorption of miRNAs. Nevertheless, we should not ignore the transcriptional activity and DNA elements of some linear lncRNA loci to influence neighborhood gene expression or the potential of linear lncRNAs to encode peptides.

Nowadays, circRNAs are considered to be effective at regulating cell progression via multiple mechanisms. The study of *CDR1as* revealed that circRNAs regulate mammalian brain function by absorbing miRNAs. This mode of action is also found in other cells, especially cancer cells. Otherwise, the regulatory activities of circRNAs seem to be related to functional RBPs. The

interactions between circRNAs and RBPs play critical roles in gene regulation and signal transduction. Remarkably, given the vast number of circRNAs, we still need to learn more about the roles of circRNAs in cells, in healthy and diseased tissues. However, it is difficult to determine circRNA structures because of the large sequence overlap between circRNAs and their linear cognate RNAs (Liu et al., 2019). The development of new methodologies will facilitate progress in this field (Li X. et al., 2018).

Further painstaking work is needed to reveal the detailed molecular mechanisms by which linear lncRNAs and circRNAs regulate biological process. However, the biggest challenge is the transfer of research findings to clinical application and trials. To date, clinical trials of miRNA therapeutics have been conducted based on an extensive body of literature and a simple regulatory model (Janssen et al., 2013), but this is difficult for linear lncRNAs and circRNAs because of the complexity of their structures and regulatory mechanisms. Nevertheless, linear lncRNAs and circRNAs are expected to be subjected to clinical trials in the near future (Chen et al., 2016; Brandenburger et al., 2018; Vo et al., 2019).

AUTHOR CONTRIBUTIONS

TQ, JL, and K-QZ conceived this manuscript. TQ wrote the draft manuscript. All authors read and approved the manuscript.

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circGNB1 Facilitates Triple-Negative Breast Cancer Progression by Regulating miR-141-5p-IGF1R Axis

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As an intriguing class of RNA, circular RNAs (circRNAs) are vital mediators of various diseases including cancers. However, the biological role and underlying mechanism of the majority of circRNAs are still ambiguous in the progression of triple-negative breast cancer (TNBC). In this study, we characterized and further investigated hsa_circ_0009362 (circGNB1) by reanalyzing the circRNA microarray profiling in our previous study. Validating by qRT-PCR, circGNB1 was overexpressed in TNBC cell lines and high expression of circGNB1 was associated with worse clinical features and survival outcomes. The expression of circGNB1 was positively correlated with tumor size and clinical stage, and high expression of circGNB1 was an independent risk factor for TNBC patients. Cell proliferation, colony formation, wound-healing and mouse xenograft assays were carried out to investigate the functions of circGNB1. Both *in vitro* and *in vivo* assays revealed that knockdown of circGNB1 significantly suppressed cell proliferation, migration and tumor growth. Subsequently, we performed luciferase reporter assays and RNA immunoprecipitation assays to elucidate the underlying molecular mechanism of circGNB1. The results showed that circGNB1 sponges miR-141-5p and facilitates TNBC progression by upregulating IGF1R. Altogether, our study demonstrated the pivotal role of circGNB1-miR-141-5p-IGF1R axis in TNBC growth and metastasis though the mechanism of competing endogenous RNAs. Therefore, circGNB1 may have the potential to be a therapeutic target and novel prognostic biomarker for TNBC.

Keywords: circGNB1, circular RNAs, IGF1R, competitive endogenous RNAs, triple negative breast cancer

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INTRODUCTION

According to the global estimated cancer statistic, breast cancer is the most common malignancy and second major cause of cancer-related deaths among women worldwide (Bray et al., 2018). Regarded as a high heterogeneous disease, breast cancer can be divided into four major different molecular subtypes (Harbeck and Gnant, 2017). Among these four subtypes, triple-negative breast cancer (TNBC) is characterized by the loss of expression of human epidermal growth factor

Abbreviations: 3′-UTR, 3′-untranslated region; ceRNA, competing endogenous RNAs; circRNA, circular RNA; IGF1R, insulin like growth factor 1 receptor; miRNAs, microRNAs; RIP, RNA immunoprecipitation; SD, standard deviation; siRNA, small interfering RNA; TNBC, triple-negative breast cancer.

receptor 2 (HER2) and hormonal receptors, which accounts for approximately 15% of all breast cancers (Carey et al., 2010). Lacking of effective therapeutic target, TNBC has the highest metastatic rate and worst prognosis which occurs more frequently in young women (Foulkes et al., 2010; Xiao et al., 2018a). Therefore, it is urgent to elucidate the underlying mechanisms which contribute to the progression of TNBC and identify new treatment strategies for patients with TNBC.

In recent years, circular RNAs (circRNAs) were widely studied in the fields of the life sciences for its various biological functions in cells. By regulating the expression of key genes, circRNAs play important roles in the development and progression process of different kind of cancer (Kristensen et al., 2018). As a type of endogenous non-coding RNAs (ncRNAs), circRNAs are widely present and expressed in mammalian cells with a linear structure (Jeck et al., 2013). circRNAs are highly conversed and formed by the back splicing of exons or introns without a 5′-cup or 3′-poly A tail, which are more stable and abundant than linear mRNAs (Zhang et al., 2014). Once were regarded as the byproducts of splicing errors, circRNAs are mediators of cell biological activities by versatile mechanisms, including sponging microRNA (miRNA), binding proteins and encoding novel small proteins (Li et al., 2018). RNAs (mRNAs, long non-coding RNAs, circRNAs and etc.) can serve as competing endogenous RNAs (ceRNAs) and communicate with each other by binding miRNAs, according to the ceRNA hypothesis (Salmena et al., 2011; Tay et al., 2014). Accumulating evidence indicates that circRNAs regulate the cell biological process by acting as miRNA sponges. For example, the most well-known circRNA CDR1as promotes proliferation and invasion of different tumors by blocking miR-7 (Hansen et al., 2013b; Weng et al., 2017; Pan et al., 2018; Zou et al., 2019a). circFAT1 suppresses gastric cancer progression by sponging miR-548g and upregulates RUNX1 tumor suppressor (Fang et al., 2019). In previous study, circKIF4A, circRAD18 and circPLK1 were also identified and proved to be oncogenic regulators in the progression of breast cancer by interacting with miRNAs (Kong et al., 2019; Tang et al., 2019; Zou et al., 2019b). Despite the progress and advancements in the study of circRNAs, the potential functions and the underlying molecular mechanism of the most circRNAs are still remained unclear.

In the present study, we identified a frequently upregulated novel circRNA (hsa_circ_0009362, circGNB1) in TNBC by analyzing our previous circRNA microarray profiling. A series of experiments and bioinformatic analysis were conducted to study the biogenesis, functions and mechanisms of circGNB1 in TNBC. Generally, our study demonstrated the pivotal role of circGNB1-miR-141-5p-IGF1R axis in TNBC growth and metastasis though the mechanism of competing endogenous RNAs. Thus, circGNB1 may have the potential to be a therapeutic target and novel prognostic biomarker for TNBC.

MATERIALS AND METHODS

Clinical Data and Patient Samples

Fresh breast cancer samples were collected from patients at Sun Yat-sen University Cancer Center (SYSUCC, China). All the resected breast cancer tissues were immediately infiltrated into RNAlater reagent (Ambion, Texas). This study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center Health Authority and conducted in accordance with the Declaration of Helsinki. Written informed consent was collected from all patients before participation in this study.

Cell Culture

All cell lines (MCF-10A, MDA-MB-231, BT549, HCC1806, HCC38, MCF-7, T47D, BT474, SKBR-3, and MDA-MB-361) used in this study were obtained from the ATCC. Being cultured and passaged for less than 6 months, all of the above cell lines were proofed free of mycoplasma infection verifying occasionally by DNA fingerprinting.

qRT-PCR Analysis

Total RNA of cells and samples were extracted by TRIzol reagent (Invitrogen). The cytoplasmic and nuclear RNA in the cells were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). qRT-PCR analysis was performed using SYBR Premix Ex Taq (Takara). The sequences of primers used in qRT-PCR analysis is listed in **Supplementary Table S2**.

Cell Oligonucleotide Transfection

Transfection of MDA-MB-231 and BT-549 cells was performed with Lipofectamine 3000 (Invitrogen). The miRNA mimics and inhibitors were provided by GeneCopoeia (Rockville). The small interfering RNAs (siRNAs) were synthesized by RiboBio (Guangzhou,China).

CCK-8 Assay

The transfected cells were seeded into the well of a 96-well plate at a density of 2×10^3 and cultured for 2 days. Ten microliters of CCK-8 reagent (Dojindo Corp, Japan) was added to each well. After incubation for 2 h at 37°C, the absorbance at a wavelength of 450 nM was measured using a microtiter plate reader.

Colony Formation Assay

A total of 1×10^3 cells were resuspended and replanted into a 6-well plate. After incubation for 10 days in an appropriate condition, the cell colonies were fixed with methanol and stained with 0.3% crystal violet for half an hour. Images were obtained right away after staining. Image J software was utilized to count and recorded the number of colonies in each well.

Wound Healing Assay

Generally, cells were cultured in 6-well plates. At least three artificial linear wounds were made by scratching with a 200 μL pipette tip and the position was marked afterward. At the time period of 0 and 24 h, each wound was imaged with an inverted microscope.

Dual Luciferase Reporter Assay

At a density of 5×10^3 cells per well, MDA-MB-231 and BT549 cells were added to 96-well plates. The putative

miRNA binding site of circGNB1 and 3′-UTR of IGF1R was mutated. Constructed plasmids (wild-type or mutant) and miR-141-5p mimics were cotransfected into MDA-MB-231 and BT549 cells for 2 days. Then, luciferase activity was measured by the dual luciferase reporter assay system (Promega) according to the instructions of manufacturer. Independent experiments were conducted in in triplicate.

RNA Immunoprecipitation (RIP)

The RIP assay for Ago2 was conducted with a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, United States), an anti-Ago2 antibody (Millipore, United States) and a normal IgG (Millipore, United States). The level of RNA was quantified after the RNA complexes were purified. The relative abundance of circGNB1, IGF1R and miR-141-5p was determined by qRT-PCR analysis after purification.

Western Blot Analysis

Total protein of cells was extracted by RIPA lysis buffer, separated by SDS-PAGE and subsequently transferred to PVDF membranes (Millipore). Membranes were blocked with 5% skim milk at room temperature for 1 h and subsequently incubated with the primary antibody anti-IGF1R (1:1000, Abcam, United States) and β-actin antibody (1:1000, Affinity, United States) at 4°C overnight. A secondary antibody (CST) was used and detected by chemiluminescence.

Mouse Xenograft Model

All animal procedures and care were performed according to the guidelines of the institutes and the approval of the Institute Research Ethics Committee of SYSUCC. MDA-MB-231 and BT549 cells (1×10^7) were subcutaneously injected into the dorsal flanks of female BALB/c nude mice ($n = 5$ for each group) and the mice were treated with an intratumoral injection (50 μL si-NC or si-circGNB1) every 4 days. After 5 weeks, mice were euthanized, and tumors were weighed and recorded. For the lung metastasis experiments, 1×10^5 cells (transfected with si-NC or si-circGNB1) were intravenously injected into the tail vein of mice ($n = 5$ for each group). After 8 weeks, all the mice were euthanized and the lungs were excised. The numbers of lung metastases were counted visually and subsequently confirmed via microscopy of hematoxylin and eosin (HE)-stained sections.

Statistical Analysis

All the data were analyzed with SPSS 24.0 software (SPSS Inc., Chicago, IL, United States). Quantitative data are presented as the form of mean ± standard deviation (SD). We used two-tailed Student's *t*-test to compare the difference of two groups. Kaplan-Meier analysis and the log-rank test were implemented to generate the overall survival curves and compare differences between the two cohorts, respectively. $P < 0.05$ was considered statistically significant.

RESULTS

circGNB1 Is Upregulated in TNBC and Correlated With Poor Clinical Outcomes

We reanalyzed the circRNA microarray profiling in our previous study (Chen et al., 2018), we founded that hsa_circ_0009362 was frequently upregulated in TNBC tissues compared to the adjacent normal mammary tissues (**Supplementary Table S1**). By browsing the circBase database and University of California, Santa Cruz (UCSC) Genome Browser, we found that hsa_circ_0009362 is generated from exons 2 and 3 of GNB1 with no intron (chr1:1756835-1770677) which is located on chromosome 1p36.33. Therefore, we named it circGNB1 and designed the divergent primers. By using qRT-PCR analysis, we validated that the expression level of circGNB1 was upregulated in breast cancer cell lines compared to normal mammary cell lines MCF-10A (**Figure 1A**). To evaluate the prognostic value of circGNB1, a total of 222 patients with TNBC was recruited and divided into two cohorts according to the expression circGNB1 assessed by qRT-PCR analysis. Kaplan-Meier survival analysis showed that high expression level of circGNB1 was associated with a poor overall survival (OS) and disease-free survival (DFS) outcomes (**Figures 1B,C**). To investigate the correlation between the circGNB1 expression level and clinicopathological characteristics in TNBC, we did further statistical analysis. The expression of circGNB1 was positively correlated with tumor size and clinical stage, and high expression of circGNB1 was an independent risk factor for TNBC patients (**Tables 1**, **2**). RNase R digestion experiment and Actinomycin D assay was conducted to verify the circular characteristics of circGNB1 in MDA-MB-231 and BT549, respectively (**Figures 1D,E**).

Downregulation of circGNB1 Suppresses the Proliferation and Metastasis of TNBC Cells *in vitro*

To investigate whether circGNB1 was involved in the progression of TNBC, we next performed loss-of-function assays. We designed siRNA targeting the back-splicing region of circGNB1 and validated its efficacy by qRT-PCR analysis (**Figure 2A**). qRT-PCR analysis showed that the siRNA could only target the back-splice junction of circRNA and have no effect on linear GNB1 mRNA expression (**Supplementary Figure S1A**). Next, we conducted CCK-8 assays and colony formation assays to assess the influence of circGNB1 on cell proliferation and the ability of colony-forming. We found that knock down of circGNB1 could significantly inhibit the growth and colony-forming ability in MDA-MB-231 and BT-549 cell lines (**Figures 2B,C**). Wound healing assay revealed that silencing of circGNB1 markedly reduced the migration ability of these two TNBC cell lines (**Figure 2E** and **Supplementary Figure S1B**). To further assess the biological roles of circGNB1 in vivo, mouse xenograft models were established. Consistent with the findings in cell experiments, downregulation of circGNB1 could reduce the tumor volume (**Figures 2F,G**) and decrease the total number of lung metastases (**Figures 2H,I**). These results demonstrated the pro-cancerous function of circGNB1 in TNBC.

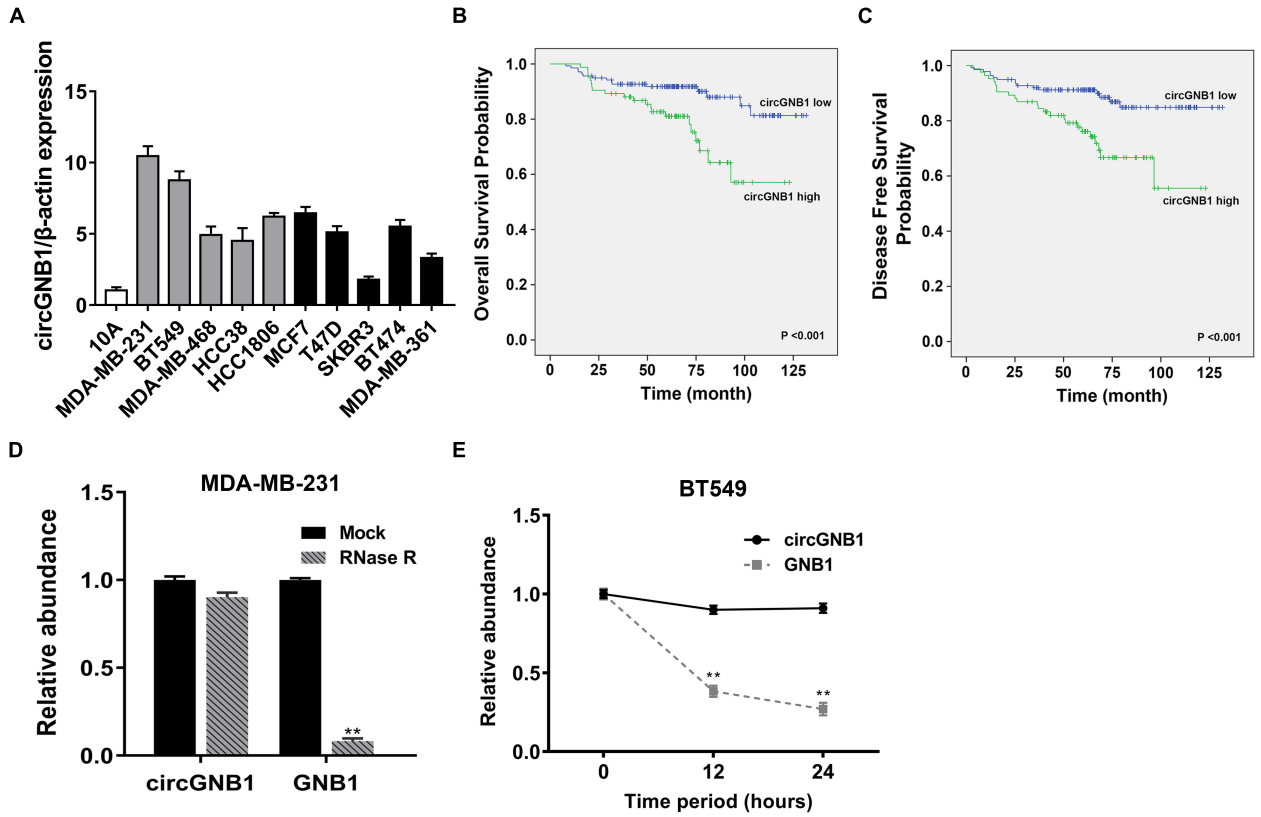

FIGURE 1 | circGNB1 is upregulated in TNBC and correlated with poor clinical outcomes. **(A)** The expression level of circGNB1 in normal mammary cell line MCF-10A and breast cancer cell lines. Gray bar and black bar represent for TNBC and non-TNBC cell lines, respectively. **(B,C)** Kaplan–Meier analysis of the **(B)** overall survival and **(C)** disease-free survival of 222 TNBC patients with circGNB1 high (green) or low (blue) expression levels. **(D)** Relative abundance of circGNB1 and GNB1 mRNA after treatment with RNase R in MDA-MB-231 cells. **(E)** Relative abundance of circGNB1 and GNB1 mRNA after being treated with Actinomycin D in BT-549 cells.

circGNB1 Functions as a Sponge of miR-141-5p

Given that circRNA has been proven to be a miRNA sponge in multiple cancers, we next predicted the potential binding miRNA of circGNB1 to elucidate the underlying molecular mechanism. According to miRNA response elements (MREs) analysis, miR-141-5p was predicted to have the potential to interact with circGNB1 (**Figure 3A**). By analyzing the miRNA microarray data derived from TCGA, we found that high expression of miR-141-5p was associated with better OS in TNBC patients[1] (**Figure 3B**). According to the reported research, downregulation of miR-141-5p was associated with progression and trastuzumab resistance in breast cancer (Finlay-Schultz et al., 2015; Li et al., 2017; Han et al., 2019). Detected by qPCR analysis, miR-141-5p was downregulated in TNBC cell lines compared to that in mammary epithelial cell lines (**Supplementary Figure S2A**). Additionally, detected by qRT-PCR analysis, circGNB1 was predominantly existed in the cytoplasm where miRNAs were mostly located in **Figure 3C**. Therefore, we conducted dual luciferase reporter assays to determine the interaction between miR-141-5p and circGNB1. We cotransfected a full-length of circGNB1-wild-type (WT) or a circGNB1-mutant (mutation of putative miRNA binding site) luciferase reporter plasmid with miR-141-5p mimics or control mimics into MDA-MB-231 and BT549 cells. The results revealed that miR-141-5p mimics could reduce the relative luciferase activity of the WT reporter but not the mutant reporter (**Figures 3D,E**). Moreover, clone-formation assays indicated that the blockage of miR-141-5p significantly enhanced the colony-forming ability of BT549 cells, and this effect could be reversed by circGNB1 silencing (**Figure 3F** and **Supplementary Figure S2B**).

circGNB1 Promotes TNBC Cell Growth and Proliferation via circGNB1-miR-141-5p-IGF1R Axis

To identify the downstream targets of miR-141-5p, TargetScan algorithm was used to predicted potential oncogenes. Among these candidate genes, IGF1R was predicted which has been confirmed as a robust oncogene in breast cancer, including TNBC (Klinakis et al., 2009; Castano et al., 2013; Obr et al., 2018; **Figure 4A**). By analyzing the public online database, high expression level of IGF1R (probe: 225330_at and 243358_at) was associated with poor OS in patients

[1]http://kmplot.com/analysis/

TABLE 1 | Correlation of circGNB1 expression with clinicopathologic characteristics of triple-negative breast cancer patients.

Variables	Cases	circGNB1		*P*-value
		Low	High	
Age (*y*)				
>50	132	80 (60.6%)	52 (39.4%)	
≤50	90	58 (64.4%)	32 (35.6%)	0.563
Menopause				
Yes	132	81 (61.4%)	51 (38.6%)	
No	90	57 (63.3%)	33 (36.7%)	0.766
Tumor size				
≤2.0 cm	59	50 (84.7%)	9 (15.3%)	
>2.0 cm	163	88 (54.0%)	75 (46.0%)	0.001*
Lymph node status				
Negative	107	73 (68.2%)	34 (31.8%)	
Positive	115	65 (56.5%)	50 (43.5%)	0.072
TNM stage				
I-II	172	115 (66.9%)	57 (33.1%)	
III-IV	50	23 (46.0%)	27 (45.0%)	0.007*

**P < 0.05, statistically significant.*

with breast cancer, which was consisted with the previously reported study (**Figure 4B**). Detected by qPCR analysis, IGF1R was upregulated in breast cancer cell lines compared mammary epithelial cell lines (**Figure 4C**). Subsequently, dual luciferase reporter assays were carried out to confirm the binding between miR-141-5p and the 3′-UTR of IGF1R transcription. The results showed that the luciferase density was decreased after cotransfection with miR-141-5p mimics and the WT-3′-UTR IGF1R plasmid, compared to the mutant 3′-UTR IGF1R reporter in both MDA-MB-231 and BT549 cells (**Figures 4D,E**). In addition, RIP assays were performed to further demonstrate the direct interaction between miR-141-5p and IGF1R mRNA. circGNB1, IGF1R, and miR-141-5p were predominantly enriched on Ago2 which is the component of RNA-induced silencing complex (RISC) (**Figures 4F,G**). The enrichment of circGNB1 was decreased, while IGF1R expression was increased after knockdown of circGNB1 assessed by RIP assay (**Figures 4H,I**). Inhibition or overexpression of miR-141-5p could increase or decrease the expression of IGF1R determined by western blot analysis (**Figure 4J**). Furthermore, the result of qRT-PCR and western blot analysis revealed that si-circGNB1 could not only reduce the mRNA level of IGF1R but also change its protein expression level (**Figures 4K,L**).

DISCUSSION

As an intriguing class of RNA, circRNAs have attracted tremendous attention of the researchers and become one of the hottest topics in the field of biomedicine. Compared to linear RNAs, circRNAs are characterized for their covalently closed structure with no head or tail (Li et al., 2018). Advances made in novel bioinformatics algorithms and high-throughput sequencing technology make it easier for scientists to detect and identify circRNAs (Salzman et al., 2013). Several circRNA databases were established to identify and characterized thousands of circRNAs, including CircBase (Glazar et al., 2014), CIRCpedia (Zhang et al., 2016) and MiOncoCirc database (Vo et al., 2019). In recent years, an increasing number of circRNAs have been identified and well-studied in the cancer research. As the most well-known circRNA, CDR1as (also termed as ciRS-7) was firstly uncovered as a mediator of biological processes containing over 70 conventional binding sites for miR-7 (Hansen et al., 2013a). CDR1as/ciRS-7 was found to be an oncogenic molecule which promotes proliferation, metastasis and may regulate the tumor microenvironment of multiple cancers by sponging miR-7 (Hansen et al., 2013b; Weng et al., 2017; Pan et al., 2018; Zou et al., 2019a). Similarly, circRNA can also act as a tumor suppressor in the development and progression of cancer. For example, circFBXW7 inhibits tumor growth and metastasis in glioma and breast cancer by encoding a 21kDa novel protein FBXW7-185aa and blocking miR-197-3p (Yang et al., 2018; Ye et al., 2019). circMTO1 suppresses malignancy progression by serving as the sponge of oncogenic miR-9 to upregulate p21 expression in human hepatocellular carcinoma (Han et al., 2017). However, the functions and roles of the majority of circRNAs are still remained unknown.

In the current study, we validated that circGNB1 was overexpressed in TNBC cell lines and high expression of circGNB1 was associated with worse clinical features and

TABLE 2 | Univariate and multivariate Cox regression analysis of circGNB1 and survival in patients with triple-negative breast cancer.

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (>50 vs. ≤50 years)	0.632	0.304–1.312	0.219	NA		
Menopause (Yes vs. No)	0.670	0.329–1.362	0.258	NA		
Histological grade (G3 vs. G1-2)	1.434	0.740–2.782	0.286	NA		
Tumor size (>2.0 cm vs. ≤2.0 cm)	3.141	1.110–8.889	0.013*	2.072	0.708–6.064	0.184
Lymph node status (Positive vs. Negative)	2.807	1.350–5.836	0.006*	1.514	0.645–3.553	0.340
TNM stage (III–IV vs. I–II)	3.365	1.740–6.510	0.001*	2.703	1.360–5.373	0.005*
circGNB1 expression (High vs. Low)	2.759	1.409–5.404	0.003*	2.148	1.070–4.310	0.031*

*NA: not analyze; *P < 0.05, statistically significant.*

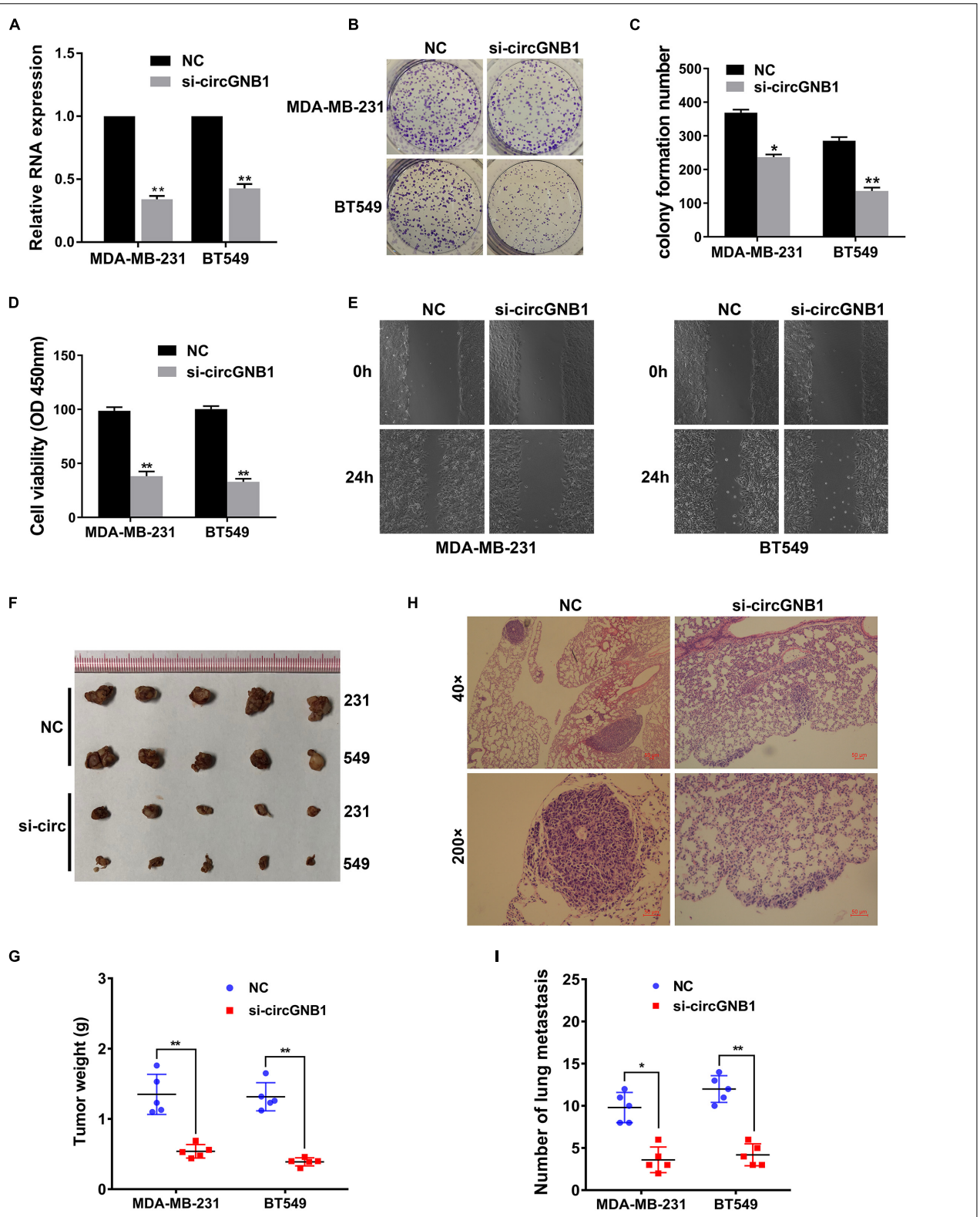

FIGURE 2 | Downregulation of circGNB1 suppresses the proliferation and metastasis of TNBC cells *in vitro*. **(A)** Efficacy of siRNA targeting circGNB1 was assessed by qRT-PCR analysis. **(B,C)** circGNB1 inhibits the colony forming ability of MDA-MB-231 and BT549 cells. **(D)** Cell proliferation was detected by CCK-8 assays. **(E)** Wound-healing assays assessed the impact of circGNB1 on cell migration ability. **(F)** Mouse xenograft models were established. **(G)** Tumor weight were measured and recorded. **(H)** Hematoxylin-eosin staining was conducted and sections of lung metastases are showed. **(I)** The number of lung metastases was counted and recorded. $^{*}P < 0.05$; $^{**}P < 0.01$.

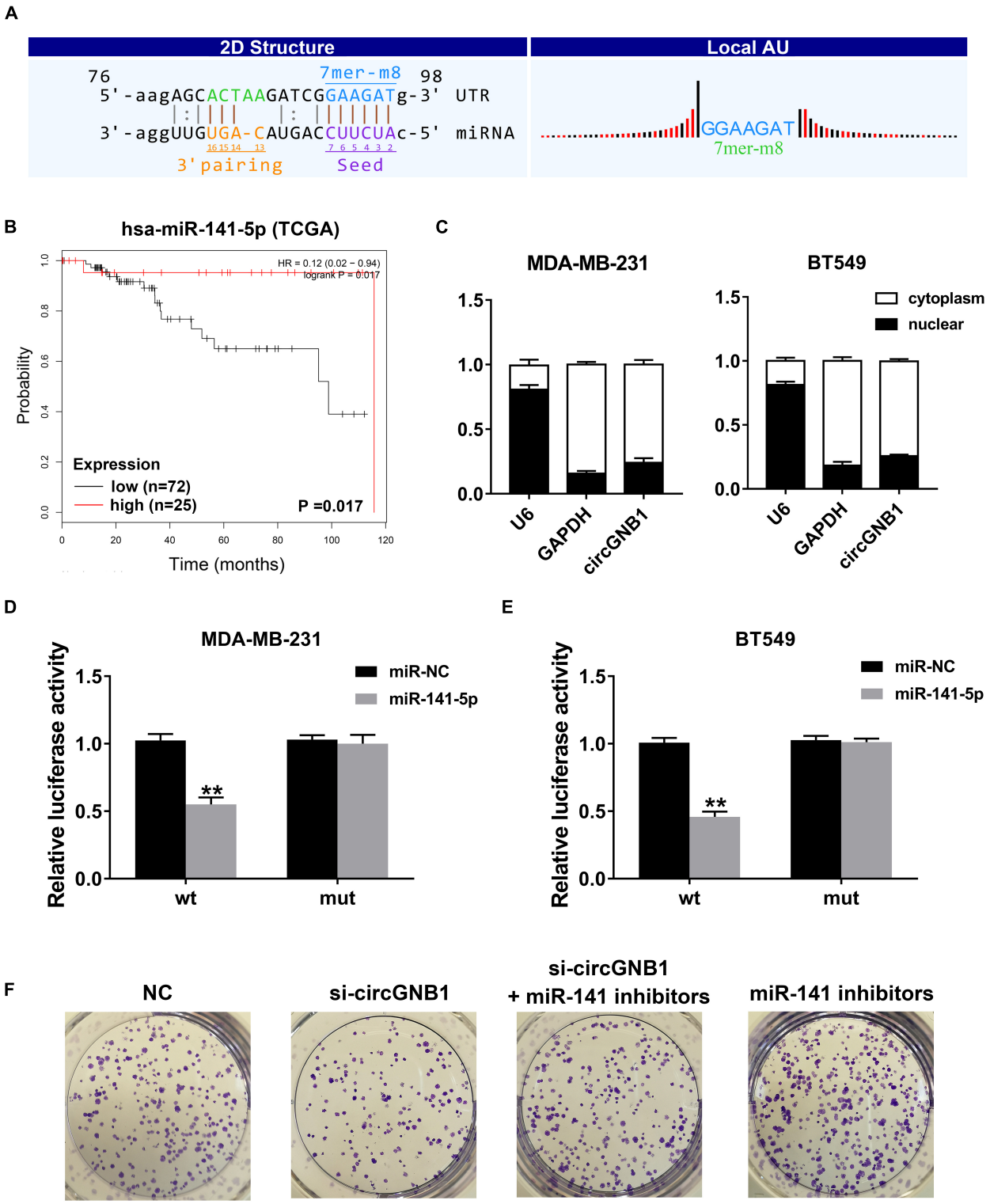

FIGURE 3 | circGNB1 functions as a sponge of miR-141-5p. **(A)** Predicted binding site of miR-141-5p within circGNB1 according to MRE. **(B)** Kaplan-Meier analysis of the association between miR-141-5p and overall survival in patients with triple negative breast cancer from TCGA public online database (http://kmplot.com/analysis). **(C)** U6 (nuclear control transcript), GAPDH (cytoplasmic control transcript) and circGNB1 in nuclear and cytoplasmic fractions analyzed by qRT-PCR. **(D–E)** Luciferase reporter assays of MDA-MB-231 and BT549 cells co-transfected with miR-141-5p mimics and circGNB1 wild type or mutant luciferase reporter plasmid. **(F)** The colony formation ability enhanced by miR-141-5p inhibitors were reversed after co-transfected with si-circGNB1 using colony formation assay. *$P < 0.05$; **$P < 0.01$.

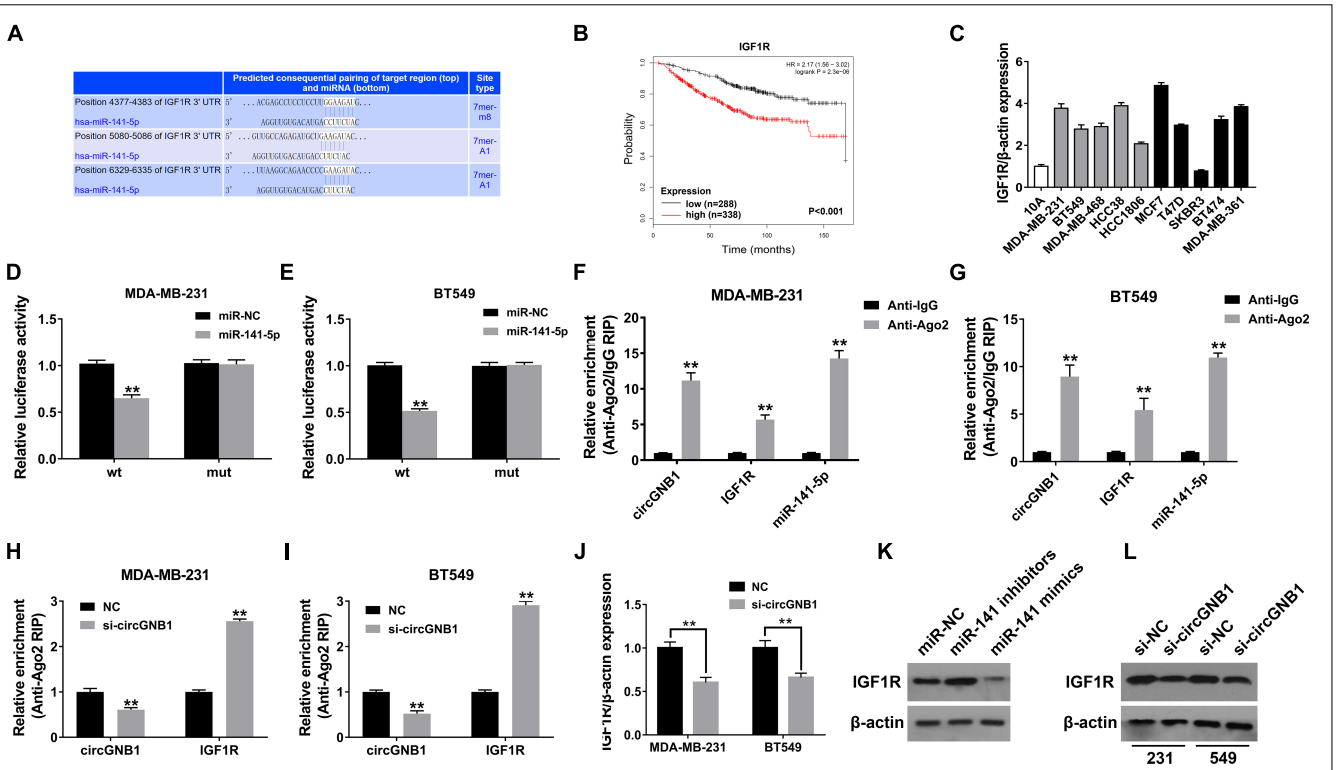

FIGURE 4 | circGNB1 promotes TNBC cell growth and proliferation via circGNB1-miR-141-5p-IGF1R axis. **(A)** TargetScan algorithm was used to predict binding sites of miR-141-5p within the 3′-UTR of IGF1R mRNA (http://www.targetscan.org). **(B)** Kaplan-Meier analysis of the association between miR-141-5p and overall survival in patients with breast cancer from public online database (http://kmplot.com/analysis). Two probes were used in this analysis (225330_at and 243358_at). **(C)** The relative expression level of IGF1R in breast cancer cell lines. Gray bar and black bar represent for TNBC and non-TNBC cell lines, respectively. **(D,E)** Luciferase reporter assay of MDA-MB-231 and BT549 cells co-transfected with miR-141-5p mimics and the 3′-UTR of IGF1R wild type or mutant luciferase reporter. **(F,G)** Enrichment of circGNB1, IGF1R and miR-141-5p on Ago2 assessed by RIP assay. **(H,I)** The enrichment of circGNB1 was decreased, while IGF1R expression was increased after knockdown of circGNB1 assessed by RIP assay. **(J)** Expression of IGF1R was decreased after transfection with si-circGNB1 detected by qPCR. **(K)** Expression of IGF1R was assessed by western bolt analysis after transfected with miR-141-5p mimics or inhibitors. **(L)** The impact of knockdown of circGNB1 on IGF1R protein expression in MDA-MB-231 and BT549 cells. $^{*}P < 0.05$; $^{**}P < 0.01$.

survival outcomes. The expression of circGNB1 was positively correlated with tumor size and clinical stage, and high expression of circGNB1 was an independent risk factor for TNBC patients. Both *in vitro* and *in vivo* functional assays revealed that knock down of circGNB1 significantly suppressed cell proliferation, migration and tumor growth. Further mechanically experiments showed that circGNB1 sponges miR-141-5p and inhibits TNBC progression by upregulating oncogene IGF1R expression. According to the bioinformatic analysis and public database, miR-141-5p was predicted as the downstream of circGNB1 and low expression of miR-141-5p was associated with an improved overall survival clinical outcome. Downregulation of miR-141 contributes to the cancer cell growth, migration, trastuzumab resistance and stem cell expansion in breast cancer (Finlay-Schultz et al., 2015; Li et al., 2017; Han et al., 2019). IGF1R is a receptor binding insulin-like growth factor with a high affinity with tyrosine kinase activity, which is highly overexpressed in most malignant tissues and enhances cell survival (Pollak, 2008). IGF1R signaling can stimulate cell proliferation and protect cell from stress in triple negative breast cancer and is regarded as a therapeutic target for treatment (Klinakis et al., 2009; Castano et al., 2013; Obr et al., 2018). We found that knockdown of circGNB1 can significantly decrease the expression of IGF1R indicating that circGNB1 could become a potential target for the inhibition of TNBC. All these results demonstrated the pivotal role of circGNB1 and its prognostic value for TNBC patients.

Lacking of effective therapeutic target, TNBC is the breast cancer subtype with the highest metastatic rate and worst prognosis. Metastasis is the main cause of mortality in patients with breast cancer, which accounts for over 90% of the death cases (Arnedos et al., 2015). Metastatic TNBC is unresectable and the treatment is limited to chemotherapy and burgeoning immunotherapy with a general therapeutic efficacy and frequent adverse effect (Santa-Maria and Gradishar, 2015; Schmid et al., 2018; Xiao et al., 2018b). Decryption of circGNB1 may provide new strategies for TNBC therapy or diagnosis. Thus, delivery of siRNA may also become an ideal strategy for TNBC treatment in the near future.

In conclusion, our study identified circGNB1 as an oncogenic molecule and demonstrated the pivotal role of circGNB1-miR-141-5p-IGF1R axis in TNBC growth and metastasis. Therefore,

circGNB1 may have the potential to be a therapeutic target and novel prognostic biomarker for TNBC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center Health Authority and conducted in accordance with the Declaration of Helsinki. Written informed consent was collected from all patients before participation in this study.

AUTHOR CONTRIBUTIONS

YK and WW designed the experiments and reviewed and revised the manuscript. PL, XL, and JZ performed the experiments. PL, AY, and FY analyzed and interpreted the data. YZ and PL was the major contributors in writing the manuscript. All authors read and approved the final version of the 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.2020.00193/full#supplementary-material

FIGURE S1 | Downregulation of circGNB1 suppresses the proliferation and metastasis of TNBC cells *in vitro*. **(A)** Expression of linear GNB1 mRNA was evaluated by qRT-PCR analysis after transfection with si-circGNB1. **(B)** Statistical graph of the wound-healing assays.

FIGURE S2 | circGNB1 functions as a sponge of miR-141-5p. **(A)** The relative expression level of miR-141-5p in breast cancer cell lines. Gray bar and black bar represent for TNBC and non-TNBC cell lines, respectively. **(B)** Statistical graph of the colony formation assay.

TABLE S1 | The full array data of the circRNA.

TABLE S2 | The sequences of qRT-PCR primers used in this study.

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ORIGINAL RESEARCH
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Circular RNA-Expression Profiling Reveals a Potential Role of Hsa_circ_0097435 in Heart Failure via Sponging Multiple MicroRNAs

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Circular RNAs represent a new type of non-coding RNA molecules that influence the occurrence and development of various human diseases by sponging microRNAs, although their roles in heart failure have not been clarified. In this study, peripheral blood samples from 5 patients with heart failure and 4 healthy volunteers were analyzed by next-generation sequencing (NGS) to screen for differentially expressed Circular RNAs. Fifty-six differentially expressed Circular RNAs were identified, of which 29 were up-regulated and 27 were down-regulated. Dysregulated expression of 6 Circular RNAs was verified by quantitative polymerase chain reaction (PCR) analysis, and hsa_circ_0097435 expression was confirmed to be significantly up-regulated in 40 patients with heart failure. Further study with extracted exosomes showed that hsa_circ_0097435 expression was significantly higher in patients with heart failure. In cardiomyocytes, hsa_circ_0097435 was up-regulated after doxorubicin treatment, promoting cardiomyocyte apoptosis. Hsa_circ_0097435 overexpression promoted cardiomyocyte apoptosis, and silencing hsa_circ_0097435 inhibited apoptosis. Moreover, RNA-pulldown experiments and AGO2-immunoprecipitation experiments revealed that hsa_circ_0097435 potentially served a role in heart failure by sponging multiple microRNAs. Collectively, these results suggest that hsa_circ_0097435 can be used as a biological blood marker and revealed a new pathway involved in regulating myocardial cell injury. Our findings may provide a rational basis for developing new treatments for heart failure.

Keywords: circular RNA, heart failure, miRNA sponge, NGS – next generation sequencing, cardiomyocyte apoptosis

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INTRODUCTION

Almost all cardiovascular diseases eventually lead to heart failure (HF). Despite advances in clinical and drug interventions that have increased the survival time of patients with HF, mortality rates remain high (Targher et al., 2017). Recent research has provided guidance for the diagnosis, treatment, and prognosis of HF. Circulating blood biomarkers are becoming increasingly important in daily clinical practice because of the high sensitivity and accuracy of the associated non-invasive

methods (Devaux et al., 2017). Natriuretic peptides, especially b-type natriuretic peptide (BNP), are blood biomarkers that are frequently measured for HF-related diagnosis and prognosis, but are not applicable to all types of heart failure (Brunner-La Rocca et al., 2015). Therefore, a new blood biomarker is needed to better diagnose, treat, and predict the prognosis of HF.

Circular RNA (circRNA) molecules have a stable, closed-ring structure and are widely present in whole blood, plasma, and extracellular vesicles (Koh et al., 2014; Li P. et al., 2015; Vausort et al., 2016). CircRNAs are regulated during cardiac development and failure (Khan et al., 2016; Werfel et al., 2016), so they offer great potential as HF biomarkers. Previous findings have revealed various circRNAs that were deregulated during HF caused by myocardial infarction in mice, which suggests a potential role of circRNAs in HF (Wu et al., 2016). However, little is known about the expression profiles and functions of circRNAs in HF. CircRNA molecules are rich in microRNA (miRNA)-binding sites and act as an miRNA sponge in cells, thereby removing the inhibitory effects of miRNAs on their target genes and increasing the target gene-expression levels. This mechanism is known as the competitive endogenous RNA (ceRNA) mechanism. A recent study revealed a novel regulatory pathway comprising circNCX1, miR-133a-3p, and CDIP1, which was found to be involved in cardiomyocyte apoptosis (Li et al., 2018). This pathway may serve as a therapeutic avenue for ischemic heart diseases. Apoptosis has important structural and functional effects on heart failure. The study of apoptosis is helpful for the treatment and prevention of heart failure. Understanding the mechanism of cardiac apoptosis and the mechanism of ceRNA is of great significance for preventing cardiac injury and treating HF. Exosomes are small membranous vesicles that can be isolated from serum and urine (Bae et al., 2018). It has been reported that plasma circRNAs can be encapsulated in exosomes (Dou et al., 2016; Tang et al., 2018). In HF, the expression of circRNAs in plasma exosomes also needs to be further studied.

In this study, next-generation sequencing (NGS) was performed to identify differentially expressed circRNAs between HF and healthy control subjects. We aimed to identify circRNA profiles in peripheral blood cells from patients with HF and to explore the roles of circRNAs in HF pathogenesis. We found that hsa_circ_0097435 expression was markedly increased in patients with HF. Further study showed that the level of hsa_circ_0097435 in exosomes from patients with HF was significantly increased. In cardiomyocytes, hsa_circ_0097435 was up-regulated after doxorubicin (DOX) treatment, promoting apoptosis in cardiomyocytes. The expression level of hsa_circ_0097435 participates in regulating apoptosis. Hsa_circ_0097435 overexpression promoted apoptosis in cardiomyocytes, and silencing hsa_circ_0097435 expression inhibited apoptosis in cardiomyocytes. Hsa_circ_0097435 overexpression resulted in significant increases in the recovery of 5 miRNAs in RNA-pulldown assays, and the AGO2 protein was significantly pulled down by hsa_circ_0097435. Immunoprecipitation of AGO2 from AC16 cells showed that AGO2 protein directly bound to 4 miRNAs. Therefore, we predicted that hsa_circ_0097435 could bind to miRNAs through the AGO2 protein and act as a sponge for multiple miRNAs.

MATERIALS AND METHODS

Collection of Samples

Forty-five patients with HF and 44 healthy volunteers who underwent physical examinations in the Affiliated Hospital of Qingdao University were included in this study, and informed consent forms were signed by all participants. Among the patient samples, we selected 5 from patients with HF and 4 from volunteers for sequencing. The inclusion criteria were clinical symptoms, b-mode ultrasound results, and laboratory examinations. Routine blood-examination results from all participants were within the normal range. Six milliliters of Trizol reagent (M5 Liquid Sample Total RNA Extraction Reagent, Mei5 Bio, Beijing, China) was added to each 2 mL peripheral blood sample immediately after collection, and the samples were preserved at –80°C for subsequent RNA extraction.

RNA Quantification and Qualification

RNA degradation and contamination, especially DNA contamination, was monitored on 1.5% agarose gels. RNA concentrations and purities were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). RNA integrity was assessed using the RNA Nano 6000 Assay Kit and the Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, United States). The optical density (OD) ratio (absorbance at 260 nm divided by that at 280 nm) of pure RNA ranged between 1.8 and 2.1. All quality standards set by the manufacturer were met.

Library Preparation for CircRNA Sequencing

For each sample, 1.5 μg RNA was used as input material for rRNA removal using the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, United States), followed by digestion of linear RNA using RNase R. Sequencing libraries were generated using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® [New England BioLabs (NEB), United States] following the manufacturer's recommendations, and index codes were added to attribute the sequences to each sample. Briefly, fragmentation was performed using divalent cations at an elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First-strand complementary DNA (cDNA) was synthesized using random hexamer primers and reverse transcriptase. Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3′ ends of the DNA fragments, NEBNext Adaptors with a hairpin loop structure were ligated to prepare for hybridization. To select for insertion fragments, preferentially 150–200 base pairs in length, the library fragments were purified using AMPure XP Beads (Beckman Coulter, Beverly, MA, United States). Then, the cDNA fragments were treated with 3 μL USER Enzyme (NEB, United States) at 37°C for 15 min before amplification by polymerase chain reaction (PCR). PCR was performed with

Phusion High-Fidelity DNA polymerase, Universal PCR primers, and the Index(X) Primer. Finally, the PCR products were purified (AMPure XP system) and the quality of each library was assessed on an Agilent Bioanalyzer 2100 and by performing quantitative PCR (qPCR).

Clustering and Sequencing

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina), according to the manufacturer's instructions. After cluster generation, the prepared libraries were sequenced using an Illumina platform, and reads were generated.

CircRNA-Profiling Analysis

CircRNAs were predicted based on the number of junction reads identified using the CircRNA Identifier (CIRI) tool and find_circ software. Differential-expression analysis of two conditions/groups was performed using the DESeq R package. DESeq provides statistical routines for determining differential expression in digital gene-expression data using a model based on the negative binomial distribution. The resulting *P*-values were adjusted using the Benjamini–Hochberg approach for controlling the false-discovery rate. Genes with an adjusted $P < 0.01$ and an absolute $\log_2$ (fold-change) > 1 found using DESeq were assigned as being differentially expressed.

Plasma Exosome Extraction

All exosome-extraction steps were performed following the instructions of the Hieff^{TM} Quick Exosome Isolation Kit (for Serum/Plasma; Yeasen Biotechnology, Co., Ltd., Shanghai, China). Blood samples (1 mL each) were transferred to individual centrifuge tubes and centrifuged for 10 min at 3000 × *g* and 4°C, after which the particles were discarded and each supernatant was transferred to a new centrifuge tube. After pretreatment, 4 volumes of 1× phosphate-buffered saline were added to each tube, and the samples were mixed evenly.

Validation by Real-Time qPCR

Six upregulated circRNAs found in blood cells from 40 HF patients and 40 healthy controls were verified by real-time qPCR. Total RNA was used for synthesizing cDNAs with the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). First-strand cDNA (2 mL) was used for PCR experiments (performed in triplicate), with TB Green Premix Ex Taq II (Takara, Tokyo, Japan). Beta-actin was detected as an internal reference for circRNAs to avoid potential aberrances in concentrations and transcription efficiencies. Relative circRNA-expression levels were measured using the $2^{-\Delta\Delta CT}$ method for each circRNA. The relative expression of miRNAs was calculated using the same real-time qPCR method as verified with circRNAs. U6 RNA was detected as an endogenous control gene for miRNAs.

Construction of Overexpression Vectors

Hsa_circ_0097435 and hsa_circ_0097435-MS2 were synthesized by total gene synthesis, and cloned individually into the pLC5-ciR vector using the *Eco*RI and *Bam*HI restriction enzyme sites. The chromatograms were normal, without any hybrid peaks or superimposed bands, and the sequence comparison was consistent, indicating that hsa_circ_0097435 and hsa_circ_0097435-MS2 were successfully inserted into pLC5-ciR, and the overexpression vectors were successfully constructed. The overexpressed plasmids and the parental vector were separately transfected into ac16 cells using the Lipofectamine^{TM} 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, United States), and overexpression of the target gene was measured by qPCR.

SiRNA Knockdown

Small-interfering RNA (siRNA) oligonucleotides specific for hsa_circ_0097435 were designed using Ambion's siRNA design tool and purchased from GenePharma Co., Ltd. (Shanghai, China). The siRNA sequence for hsa_circ_0097435 was 5′-ACUUGUGAUGCUGACUUGGTTCCAAGUCAGCAUCA CAAGUTT-3′.The specificity of the oligonucleotides was confirmed through comparing with all other sequences in GenBank using Nucleotide BLAST. Transfection of siRNAs was performed using the Lipofectamine^{TM} 3000 transfection reagent (Thermo Fisher Scientific), according to the manufacturer's instructions.

Apoptosis Assays

Apoptosis was determined by performing terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using the TUNEL Apoptosis Detection Kit (Yeasen Biotechnology, Co., Ltd., Shanghai, China), per the manufacturer's instructions. The samples were stained with anti-fluorescence attenuation sealant containing DAPI (Solarbio, Beijing, China) and detected with a Zeiss LSM510 META microscope. The percentage of apoptotic nuclei was calculated by dividing the total number of TUNEL-positive nuclei by the total number of DAPI-positive nuclei.

Prediction of miRNAs Association With Hsa_circ_0097435 and Their Target mRNAs

Go to http://circinteractome.nia.nih.gov. Select the "miRNA Target Sites" tab. CircInteractome (Panda et al., 2018) uses the TargetScan to predict the miRNAs which have sequence complementarity with hsa_circ_0097435. Furthermore, miRNAs associated with hsa_circ_0097435 were identified using circMir program (purchased from http://www.bioinf.com.cn/) that uses miRanda (Betel et al., 2008) and RNAhybrid (Rehmsmeier et al., 2004) to predict miRNAs. Finally, miRNAs predicted by at least two softwares including TargetScan, miRanda and RNAhybrid were selected. Targetscan (Lewis et al., 2003) and miRanda were then used to predict the target mRNAs of miRNAs.

Pulldown Assays

MS2 is a tag sequence that can specifically bind to the capture protein, MS2-CP. The MS2 labeling system was used to express hsa_circ_0097435-MS2 in cells. The highly

specific and stable interaction between hsa_circ_0097435-MS2 and the captured protein MS2-CP was employed to capture hsa_circ_0097435 and its interacting molecules. We constructed both an hsa_circ_0097435-overexpression plasmid (C97435) and an hsa_circ_0097435-MS2-overexpression plasmid (C97435-MS2). The expression vectors C97435-MS2 and MS2-CP were transfected in the cells to induce transient MS2-CP expression. MS2-CP and MS2-labeled circRNAs could specifically bind to form an MS2-CP-MS2–circRNA complex. The MS2-CP-MS2–circRNA complex was pulled down, and the capture products were detected to identify proteins or miRNA molecules that may interact with hsa_circ_0097435.

AGO2 Immunoprecipitation

An AGO2-specific antibody was used for AGO2 immunoprecipitation, and an IgG antibody was selected as the negative control. Mouse monoclonal anti-AGO2 (Abcam, Cambridge, England) or mouse normal IgG antibody (Abcam, Cambridge, England) were preincubated with Magna Bind goat anti-mouse IgG Magnetic Bead slurry (Thermo Fisher Scientific, Waltham, MA, United States) and used for immunoprecipitation. In brief, cells were lysed in 150 mM KCl, 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% Triton X-100, and 5 mM DTT supplemented with RNase inhibitor (Takara, Tokyo, Japan) and proteinase inhibitor cocktail (Roche Applied Science, Basel, Switzerland). The lysate was mixed with antibody-coupled Sepharose beads and left under rotation for 4 h at 4°C. Beads were subsequently washed six times in lysis buffer and the RNA was extracted using Trizol reagent(Takara, Tokyo, Japan).

GO and KEGG Analysis

The Gene Ontology (GO) database is a structured standard biological annotation system established by the GO Consortium in 2000, which is aimed at establishing a standard vocabulary of knowledge regarding genes and their products that is applicable to various species. GO function classification statistics of the mRNAs targeted by the miRNAs associated with hsa_circ_0097435 was implemented using the clusterProfiler R packages. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding the high-level functions and utilities of biological systems, such as a cell, an organism, or an ecosystem, based on molecular-level information, especially large-scale molecular datasets generated by genome sequencing

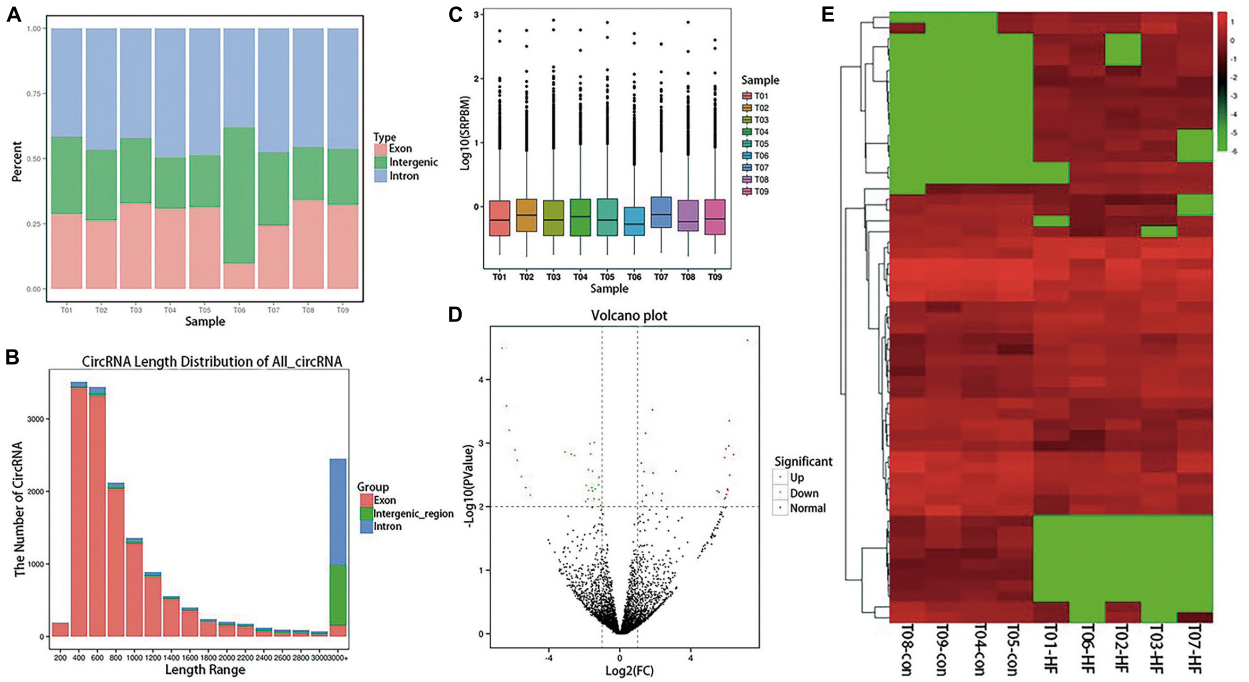

FIGURE 1 | Expression profiling of circRNAs in patients with HF. **(A)** A histogram of read distributions in different regions of the genome. Each column represents a sample, and the height of the region represents the percentage of all mapped reads mapping to that region. **(B)** CircRNA length-distribution map. The horizontal coordinate represents the length interval of circRNA. The ordinate represents the number of circRNAs in the length interval. **(C)** SRPBM boxplot of each sample. The expression of circRNA in each sample was counted and normalized by SRPBM algorithm. In the figure, the abscissa represents different samples, and the ordinate represents the logarithm value of normalized sample expression quantity **(D)** Differential-expression volcano diagram. Each point in the differential-expression volcano diagram represents a circRNA, and the abscissa represents the log value of the differential multiple of a certain circRNA in the two samples. The y-coordinate represents the negative log of the *p*-value. In the figure, the green data points represent down-regulated differentially expressed circRNAs, the red data points represent up-regulated differentially expressed circRNA, and the black data points represent non-differentially expressed circRNAs. **(E)** CircRNA cluster heatmap with differential expression. The abscissa represents the sample name and the clustering result for each sample, whereas the ordinate represents different circRNAs and the clustering results for the circRNAs. The different columns in the figure represent different samples, and the different rows represent different circRNAs. The color represents the $\log_{10}$ of the circRNA-expression level in each sample.

and other high-throughput experimental technologies[1]. The clusterProfiler R packages were used to identify which KEGG pathways the target genes were significantly enriched in.

Statistical Analysis

Data are expressed as mean ± standard error of the mean of at least three independent experiments. One-way analysis of variance was used for multiple comparisons. Student's unpaired *t*-test was used for comparisons of two groups. A $p < 0.05$ was considered to reflect a statistically significant difference ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). GraphPad Prism software, version 8 (GraphPad Software, San Diego, CA, United States) was applied for data management and analysis.

RESULTS

CircRNA Analysis

After sequencing quality control, 102.45 gb of clean data was obtained, and the percentage of Q30 bases in each sample was not less than 95.16%. RNA sequencing data was deposited in the NCBI Sequence Read Archive (BioProject: PRJNA574863). After obtaining clean reads, the sequences were aligned with the reference genome to determine their locations within the reference genome or gene, as well as the sequence characteristics unique to the sequencing samples. Alignments were performed using the Burrows–Wheeler Aligner (Gao et al., 2015). Reads that were mapped to a specified reference genome are referred to as mapped reads, and the corresponding data are referred to as mapped data. The numbers of mapped reads in different regions (exons, introns, and intergenomic regions) of the specified reference genome were counted, and a mapped reads-distribution histogram was generated for each sample with different regions of the genome (**Figure 1A**). The CIRI (**Supplementary Table S1**) and find_circ (**Supplementary Table S2**) were used to predict circRNA, and the intersection of the two software prediction results was taken. Distribution statistics were conducted for all circRNA lengths identified (**Figure 1B**). The circRNA-expression levels in each sample were statistically analyzed. Not only could differences in the expression-level distributions be seen in individual samples, but the overall expression level of different samples could be intuitively compared (**Figure 1C**).

Expression Profiling of CircRNAs in Patients With HF

Differential-expression analysis between the HF and normal control (NC) groups was performed using the DESeq R package. DESeq provides statistical routines for determining differential expression in digital gene-expression data using a model based on

[1]http://www.genome.jp/kegg/

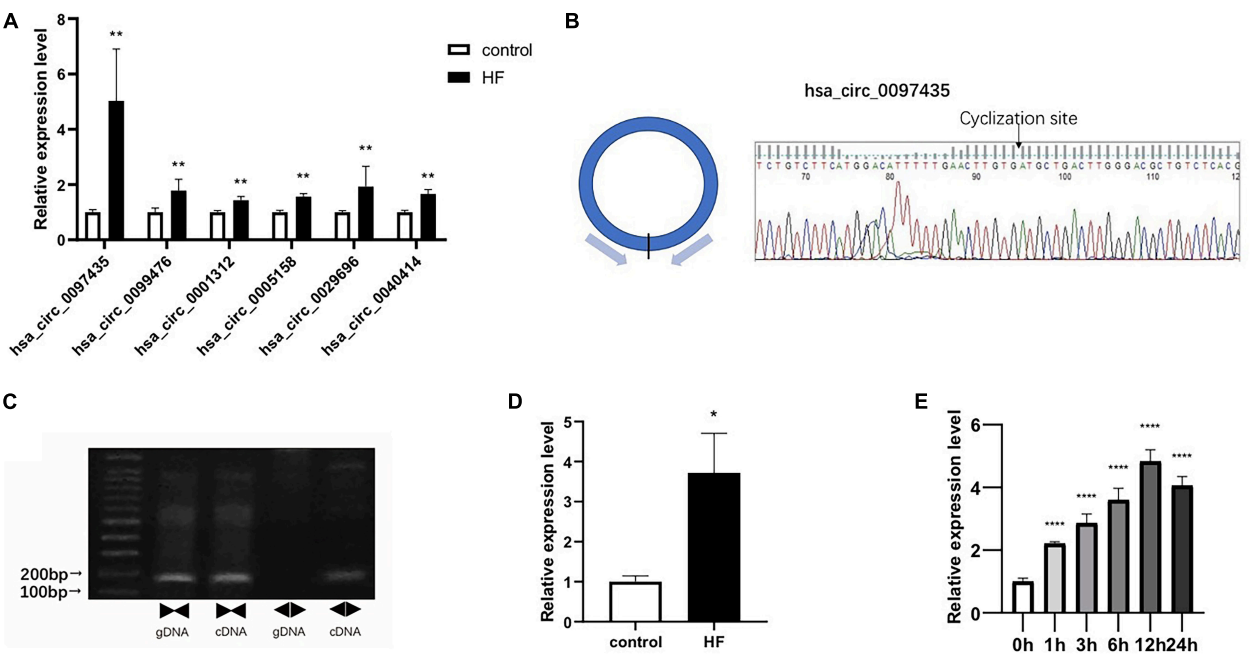

FIGURE 2 | Verification of selected circRNAs. **(A)** Six circRNAs were upregulated in the blood of patients with HF compared to the NC group. These six circRNAs included hsa_circ_0097435 ($^{**}p < 0.01$), hsa_circ_0099476 ($^{**}p < 0.01$), hsa_circ_0001312 ($^{**}p < 0.01$), hsa_circ_0005158 ($^{**}p < 0.01$), hsa_circ_0029696 ($^{**}p < 0.01$), and hsa_circ_0040414 ($^{**}p < 0.01$). **(B)** The amplification products of hsa_circ_0097435 were sequenced by Sanger sequencing to verify the cyclization site and ring-formation mode of hsa_circ_0097435. **(C)** Use of the divergent-primer strategy to verify the circular structure of hsa_circ_0097435. **(D)** The levels of hsa_circ_0097435 in exosomes of patients with HF was significantly higher than those in normal volunteer subjects. The hsa_circ_0097435 level was analyzed by qRT-PCR. $^{*}P < 0.05$ versus control. $n = 15$. **(E)** AC16 cells were treated with 2 μM DOX. Total RNA was isolated and reverse-transcribed. The hsa_circ_0097435 level was analyzed by qRT-PCR. $^{****}P < 0.0001$ versus 0 h. $n = 3$.

the negative binomial distribution. The resulting *P*-values were adjusted using the Benjamini–Hochberg approach for controlling the false-discovery rate. Genes with an adjusted $P < 0.01$ and an absolute $\log_2$ (fold-change) > 1 found by DESeq were considered to be differentially expressed. Finally, 56 circRNAs with significant differences in expression were identified (**Supplementary Table S3**). Compared with the NC group, 29 circRNAs in the HF group were significantly up-regulated and 27 were significantly down-regulated, as shown in the volcano diagram (**Figure 1D**) and cluster heatmap (**Figure 1E**).

Verification of Selected CircRNAs

Real-time qPCR was used to verify the peripheral circRNAs of 40 patients with HF and 40 healthy volunteers. For further study, we focused on upregulated circRNAs with certain circBase IDs, and selected 6 circRNAs for verification with RT-PCR. The primer sequences were detailed in **Supplementary Table S4**. The levels of hsa_circ_0097435 ($p < 0.01$), hsa_circ_0099476 ($p < 0.01$), hsa_circ_0001312 ($p < 0.01$), hsa_circ_0005158 ($p < 0.01$), hsa_circ_0029696 ($p < 0.01$), and hsa_circ_0040414 ($p < 0.01$) were significantly higher in the HF group than in the NC group, and the expression difference of hsa_circ_0097435 was most obvious (**Figure 2A**).

We detected the expression of hsa_circ_0097435 as a circRNA by amplifying it from the cDNA of AC16 cardiomyocytes, using divergent primers (**Figure 2C** and **Supplementary Figure S1**). In addition, sequencing the amplification product of hsa_circ_0097435 by Sanger sequencing was used to verify the circular connection of circRNA (**Figure 2B**). To verify the differential expression of hsa_circ_0097435 in HF, plasma exosomes of 15 patients with HF and 15 healthy volunteers were extracted to detect the hsa_circ_0097435 levels in exosomes. Significantly higher levels of hsa_circ_0097435 ($p < 0.05$) were detected in exosomes from patients with HF than in those from normal volunteers (**Figure 2D**). To understand the role of hsa_circ_0097435 in cardiomyocyte apoptosis, we further examined its production in DOX-treated cardiomyocytes. A time-dependent increase in hsa_circ_0097435 levels was observed in AC16 cells following treatment with 2 μM DOX, which peaked at 12 h after DOX treatment (**Figure 2E**). Therefore, the level of hsa_circ_0097435 increased after myocardial cell injury.

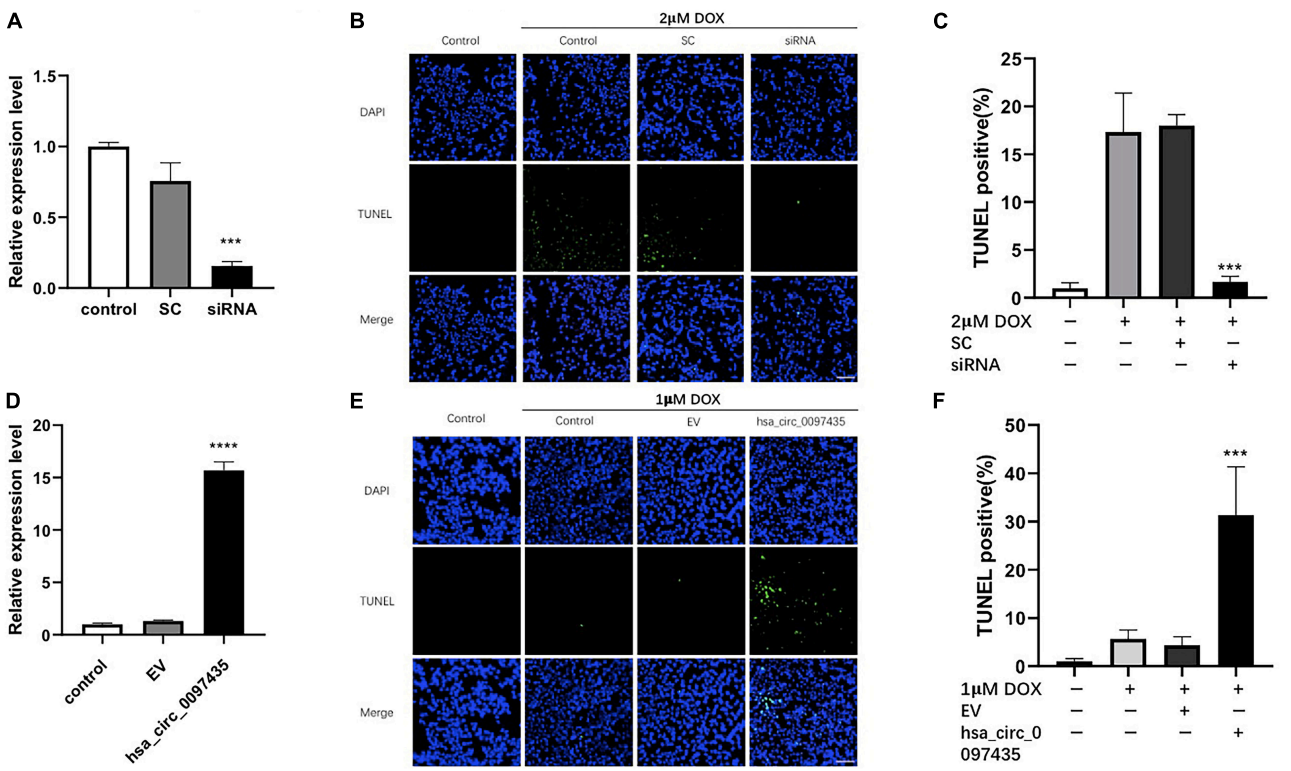

FIGURE 3 | hsa_circ_0097435 promoted apoptosis in cardiomyocytes. **(A)** AC16 cells were transfected with an siRNA vector targeting hsa_circ_0097435. The expression level of hsa_circ_0097435 was detected by qRT-PCR. SC, scramble control; siRNA, siRNA targeting the junction region in hsa_circ_0097435. ***$P < 0.001$ versus SC. $n = 3$. **(B)** AC16 cells were transfected with an hsa_circ_0097435 siRNA-expression vector and treated with 2 μM DOX for 12 h. Apoptosis was evaluated by performing TUNEL assays. Green, TUNEL-positive nuclei; blue, DAPI-stained nuclei. Scale bars, 100 μm. **(C)** The apoptosis rate calculated from three independent experiments. ***$P < 0.001$ versus SC. $n = 3$. **(D)** AC16 cells were transfected with an hsa_circ_0097435-overexpression vector. The expression level of hsa_circ_0097435 was detected by qRT-PCR. EV: empty vector. ****$P < 0.0001$ versus EV. $n = 3$. **(E)** AC16 cells were transfected with the hsa_circ_0097435-overexpression vector and treated with 1 μM DOX for 12 h. Apoptosis was detected by performing TUNEL assays. Green, TUNEL-positive nuclei; blue, DAPI-stained nuclei. Scale bars, 100 μm. **(F)** The rate of apoptosis was calculated using data from three independent experiments. ***$P < 0.001$ versus EV. $n = 3$.

Hsa_circ_0097435 Promoted Apoptosis in Cardiomyocytes

To verify whether the increased hsa_circ_0097435 level was related to myocardial apoptosis, we conducted a series of validation analyses using loss-of-function and gain-of-function experiments. Specifically targeting siRNA at the junction region of hsa_circ_0097435 was used to down-regulate hsa_circ_0097435 expression in AC16 cells (**Figure 3A**). Silencing hsa_circ_0097435 effectively inhibited DOX-induced apoptosis in AC16 cells (**Figures 3B,C**). Next, we successfully overexpressed hsa_circ_0097435 in AC16 cells (**Figure 3D**). We found that AC16 cells treated with a low DOX concentration only showed a slight induction of apoptosis, whereas AC16 cells were more sensitive to DOX treatment after hsa_circ_0097435 overexpression (**Figures 3E,F**). In summary, these results suggest that hsa_circ_0097435 promoted apoptosis in cardiomyocytes.

Hsa_circ_0097435 Acted as a Sponge for Multiple miRNAs

CircRNA plays important regulatory roles in diseases by interacting with disease-related miRNAs. Based on the gene-sequence information of hsa_circ_0097435, miRNA predictions were performed using CircInteractome, miRanda and RNAhybrid (**Supplementary Table S5**). CircInteractome uses the TargetScan to predict the miRNAs which have sequence complementarity with hsa_circ_0097435. Finally, 5 miRNAs predicted by at least two softwares including TargetScan, miRanda and RNAhybrid were selected (**Supplementary Table S6**). Binding sites of these miRNAs in the hsa_circ_0097435 region were found (**Figure 4A**). To verify that hsa_circ_0097435 acts as a miRNA sponge in cells, we conducted circRNA-pulldown experiments. We constructed both an hsa_circ_0097435-overexpression plasmid (C97435) and an hsa_circ_0097435-MS2-overexpression plasmid (C97435-MS2). After transfection, the transfection efficiency of C97435-MS2 (**Figure 4B**) and the expression efficiency of MS2-CP (**Figure 4C**) were detected. MS2 is a tag sequence that can specifically bind to the captured protein, MS2-CP. The expression vectors C97435-MS2 and MS2-CP were transfected in the cells to induce transient MS2-CP expression. MS2-CP and MS2-labeled circRNAs could specifically bind to form an MS2-CP-MS2–circRNA complex. The MS2-CP-MS2–circRNA complex was pulled down, and the target miRNAs were detected by RT-qPCR (**Figure 4D**). The experimental results demonstrated that these miRNAs could indeed bind to hsa_circ_0097435.

To verify the binding mode between hsa_circ_0097435 and miRNAs, we conducted western blot (WB) experiments on the pulled-down products, which showed that AGO2 protein was significantly pulled down by hsa_circ_0097435

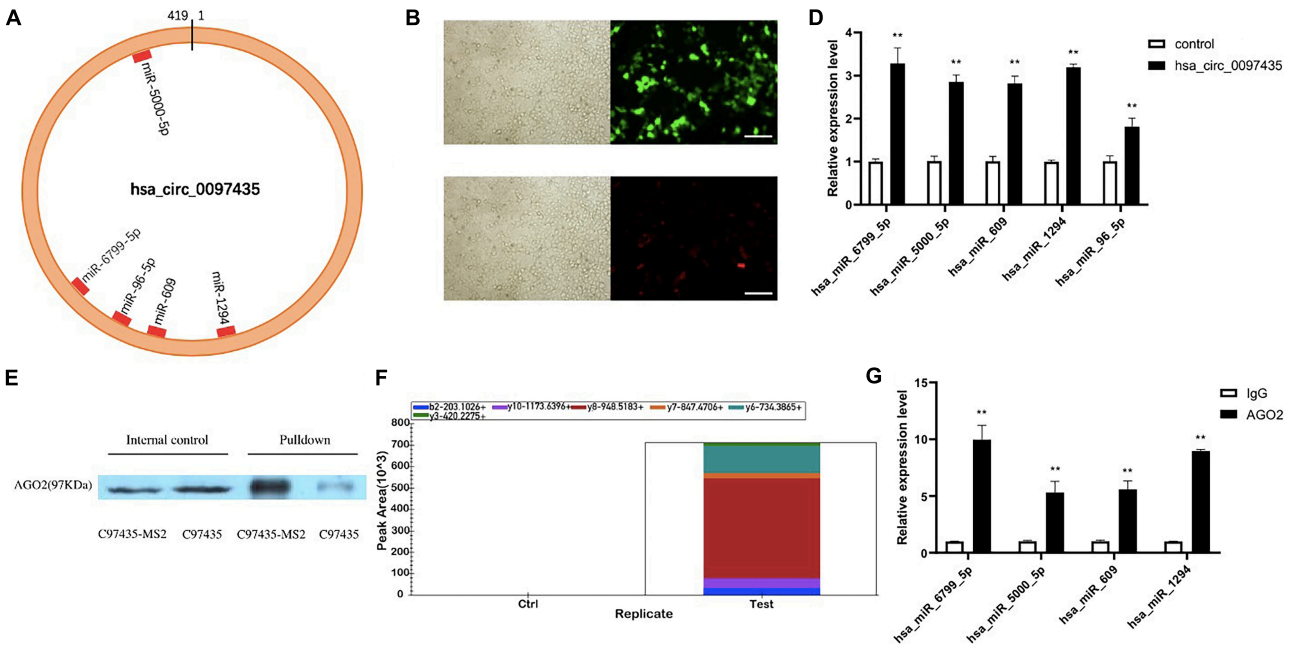

FIGURE 4 | Hsa_circ_0097435 acted as a sponge for multiple miRNAs. **(A)** The potential binding sites of miRNAs related to hsa_circ_0097435 are shown. **(B)** Detection of transient, efficient expression of C97435-MS2 (green fluorescence), Scale bars, 100 μm. **(C)** Detection of efficient expression of MS2-CP (red fluorescence), Scale bars, 100 μm. **(D)** Compared with the negative-control group, 5 miRNAs in the circRNA-enrichment group were upregulated, including hsa_miR_6799_5P (**$p < 0.01$), hsa_miR_5000_5P (**$p < 0.01$), hsa_miR_609 (**$p < 0.01$), hsa_miR_1294 (**$p < 0.01$), and hsa_miR_96_5P (**$p < 0.01$). **(E)** WB analysis of pulldown products. The AGO2 protein was significantly pulled down by hsa_circ_0097435. The amount of AGO2 protein in the C97435-MS2 pulldown group was significantly higher than in the C97435 pulldown group. **(F)** Histogram showing quantitative comparison of the polypeptide TTPQTLSNLCLK between the sample groups. AGO2 immunoprecipitation was followed by mass spectrometry, and 3–8 fragment ions of the specific peptide TTPQTLSNLCLK were selected for quantitative analysis. **(G)** Immunoprecipitation of AGO2 from AC16 cells. Compared with the IgG group, 4 miRNAs in the AGO2 group were upregulated, including hsa_miR_6799_5P (**$p < 0.01$), hsa_miR_5000_5P (**$p < 0.01$), hsa_miR_609 (**$p < 0.01$), and hsa_miR_1294 (**$p < 0.01$).

(**Figure 4E** and **Supplementary Figure S2**). Further, we conducted AGO2-immunoprecipitation experiments. The AGO2 protein was analyzed by mass spectrometry (**Figure 4F**). Immunoprecipitation of AGO2 from AC16 cells showed that AGO2 protein directly bound to 4 miRNAs (**Figure 4G**). Therefore, we predicted that hsa_circ_0097435 could bind to miRNAs through the AGO2 protein and act as a sponge for multiple miRNAs.

Prediction of miRNA Association With CircRNA and Their Target mRNAs

Based on the sequence information of known miRNAs, miRanda (**Supplementary Table S7**) and TargetScan (**Supplementary Table S8**) were used to predict target genes. Intersection target genes of two software and target genes related to cardiac function were selected (**Figure 5A** and **Supplementary Table S9**). Then, check the levels of the miRNAs upon hsa_circ_0097435 silencing (**Figure 5B**). The intersection target genes of miRanda and TargetScan were used for GO analysis and KEGG analysis. The GO annotation system generates a directed acyclic diagram containing three main branches, namely, Biological Processes, Molecular Functions, and Cellular Components. As shown in **Figure 5D**, GO functions were found out by GO enrichment analysis using hypergeometric testing, and these functions were significantly enriched compared to the entire genome background. In organisms, different gene products coordinate to perform biological functions. Pathway-annotation analysis of the mRNAs targeted by the miRNAs associated with hsa_circ_0097435 is helpful to further interpret the functions of genes. Ten pathways connected with the functions of the mRNAs targeted by the miRNAs associated with hsa_circ_0097435 were defined, based on the KEGG analysis (**Figure 5C**).

DISCUSSION

HF is becoming an increasingly prevalent epidemic. Due to the aging of the population and medical advances, increasing number of people are diagnosed with HF and their survival time has been extended, although the mortality rate remains high. As a blood biomarker for HF-related, high-frequency diagnosis and prognosis, BNP is not applicable to all types of HF patients. Another blood biomarker or a group of blood biomarkers is needed to better diagnose and treat HF and predict prognosis. Mounting evidence suggests that HF is mainly caused by genetic variations, that HF has a complex genetic basis (Creemers et al., 2011; Dorn, 2011), and that many biological molecules can serve as potential biomarkers (Maisel and Choudhary, 2012; Wang et al., 2014) or therapeutic targets (Tamargo and Lopez-Sendon, 2011). However, the molecular mechanisms that cause HF are

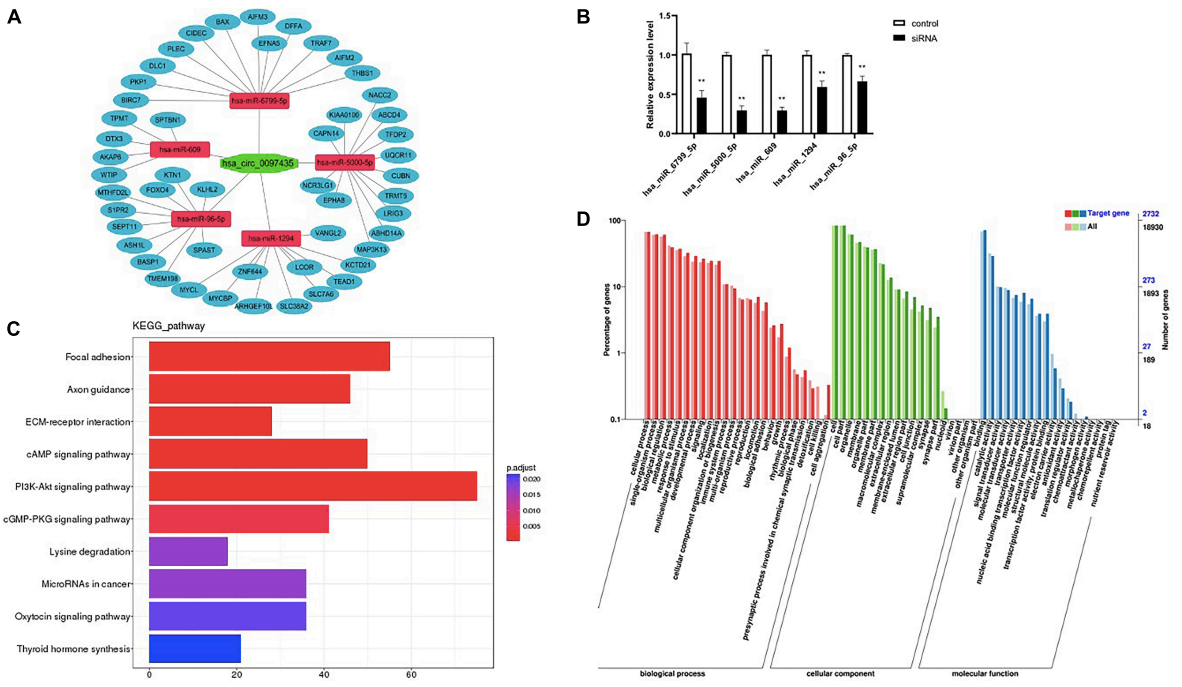

FIGURE 5 | Prediction of miRNA association with circRNA and their target mRNAs. **(A)** The possible interaction of hsa_circ_0097435, miRNAs and mRNAs. **(B)** Compared with the negative-control group, 5 miRNAs upon circ_0097435 silencing were downregulated, including hsa_miR_6799_5P (**$p < 0.01$), hsa_miR_5000_5P (**$p < 0.01$), hsa_miR_609 (**$p < 0.01$), hsa_miR_1294 (**$p < 0.01$), and hsa_miR_96_5P (**$p < 0.01$). **(C)** KEGG classification statistics of the mRNAs targeted by the miRNAs associated with hsa_circ_0097435. The abscissa represents the number of genes, the ordinate represents the pathway, and the color of the column represents the corrected *p*-value. **(D)** GO function classification statistics of the mRNAs targeted by the miRNAs associated with hsa_circ_0097435. The abscissa shows the GO classification, the left ordinate shows the percentage of the number of genes, and the right ordinate shows the number of genes.

largely unknown. CircRNAs represent a kind of non-coding RNA molecule without a 5′ end cap or 3′ end poly(A) tail, which forms a ring structure with covalent bonds. Its closed ring structure is not easily degraded by exonuclease RNase R, making it more stable than linear RNA. Therefore, circRNAs molecules are potential biomarkers for future HF diagnosis.

NGS was used to screen for differentially expressed circRNAs associated with HF. Fifty-six differentially expressed circRNAs were identified, 29 of which were up-regulated and 27 were down-regulated. To our knowledge, this is the first report describing circRNA-expression profiles in peripheral blood samples from HF patients. Our results can enrich the understanding of HF pathogenesis and provide a theoretical basis for in-depth discussions on the roles of circRNAs in HF. To further verify the up-regulated circRNAs, we increased the sample size and selected 6 circRNAs for qPCR verification. Among the six selected circRNAs, hsa_circ_0097435 ($p < 0.01$) was significantly more abundant in the HF group than in the NC group. The circular structure of hsa_circ_0097435 was verified by Sanger sequencing and a divergent-primer strategy. Further study showed that the hsa_circ_0097435 levels in exosomes from patients with HF was significantly higher ($p < 0.05$) than those from normal volunteers. Based on these experiments, we postulate that most hsa_circ_0097435 is encapsulated in exosomes, which otherwise could be decomposed by the large amount of RNase present in plasmas. This postulate is the same as previous studies found that circRNAs may be encapsulated in exosomes (Li Y. et al., 2015; Zheng et al., 2016).

To understand the role of hsa_circ_0097435 in cardiomyocyte apoptosis, we further examined its expression in DOX-treated cardiomyocytes. Hsa_circ_0097435 was the highest at 12 h after DOX treatment. To verify whether this increase was related to myocardial apoptosis, we conducted a series of validation analyses using loss-of-function and gain-of-function experiments. Hsa_circ_0097435 overexpression promoted cardiomyocyte apoptosis, and silencing hsa_circ_0097435 expression inhibited cardiomyocyte apoptosis. CircRNAs play important regulatory roles in diseases by interacting with disease-related miRNAs. To investigate the mechanism of action between hsa_circ_0097435 and miRNAs, further studies were carried out. In the RNA-pulldown assays, we identified 5 miRNAs that were significantly pulled down following hsa_circ_0097435. In addition, the AGO2 protein was significantly pulled down by hsa_circ_0097435. Immunoprecipitation of AGO2 from AC16 cells showed that the AGO2 protein could directly bind to miRNAs. Among the 4 selected miRNAs, the levels of hsa_miR_6799_5P ($p < 0.01$), hsa_miR_5000_5P ($p < 0.01$), hsa_miR_609 ($p < 0.01$), and hsa_miR_1294 ($p < 0.01$) were significantly higher in the AGO2 group than in the IgG group. Therefore, we predicted that hsa_circ_0097435 could bind to miRNAs through the AGO2 protein and act as a sponge for multiple miRNAs.

To the best of our knowledge, our results provide the first overview of circRNA-expression profiles in HF and the association between hsa_circ_0097435 and multiple miRNAs in HF. Our experiments showed that hsa_circ_0097435 played a crucial role in HF occurrence and development. Based on the hsa_circ_0097435-expression levels observed in exosomes, we speculate that most hsa_circ_0097435 was encapsulated in exosomes. Further functional studies demonstrated that after DOX treatment, myocardial apoptosis increased, and hsa_circ_0097435 could promote apoptosis. In addition, RNA-pulldown experiments and AGO2-immunoprecipitation experiments revealed the potential role of hsa_circ_0097435 in HF via sponging multiple miRNAs. In conclusion, hsa_circ_0097435 can be used as a blood biomarker, and the results further reveal a new pathway that regulates myocardial cell injury.

This study has several limitations. The regulation of hsa_circ_0097435 binding site with miRNA and indirect regulation of the expression of downstream target genes of miRNA become the focus of further research. Experimental identification and characterization of their associated molecules, such as miRNAs or proteins, are suggested in the future. Another limitation of this study is that the effect of hsa_circ_0097435 in exosomes on HF and its mechanism have not yet been determined.

DATA AVAILABILITY STATEMENT

RNA sequencing data was deposited in the NCBI Sequence Read Archive (BioProject: PRJNA574863).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of affiliated hospital of Qingdao university. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JH and LZ analyzed the sequencing results of circRNA, completed cell experiment, and wrote this manuscript. LH, HY, FX, BY, RZ, and YZ completed clinical sample collection. YA conceived and designed the research, and revised the 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.2020.00212/full#supplementary-material

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REVIEW
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Characterization and Function of Circular RNAs in Plants

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CircRNAs are covalently closed-loop single-stranded RNA molecules ubiquitously expressing in eukaryotes. As an important member of the endogenous ncRNA family, circRNAs are associated with diverse biological processes and can regulate transcription, modulate alternative splicing, and interact with miRNAs or proteins. Compared to abundant advances in animals, studies of circRNAs in plants are rapidly emerging. The databases and analysis tools for plant circRNAs are constantly being developed. Large numbers of circRNAs have been identified and characterized in plants and proved to play regulatory roles in plant growth, development, and stress responses. Here, we review the biogenesis, characteristics, bioinformatics resources, and biological functions of plant circRNAs, and summarize the distinct circularization features and differentially expression patterns comparison with animal-related results.

Keywords: circRNA, plant, circularization, characterization, regulation, stress response, bioinformatics

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INTRODUCTION

Circular RNAs (circRNAs) are a unique group of endogenous non-coding RNAs (ncRNAs) for their distinct closed-loop structures. CircRNAs are formed in the non-canonical "back-splicing" event, in which the 5′ and 3′ ends are attached by covalent bond (Jeck et al., 2013; Memczak et al., 2013; Jeck and Sharpless, 2014). Different from linear RNAs, circRNAs form closed-loop structures by covalent bond linking the 5′ and 3′ ends. Therefore, this type of ncRNAs is more stable than linear RNAs and not easily degraded by Ribonuclease R (RNase R) (Suzuki et al., 2006). Although circRNAs have been observed for decades, they are typically considered as by-products of aberrant RNA splicing (Nigro et al., 1991; Cocquerelle et al., 1993). In the recent decade, numerous circRNAs have been identified and annotated in diverse species as high-throughput sequencing technologies and bioinformatics tools advance. The published research exhibited that circRNAs are ubiquitous and abundant in all eukaryotes, such as mammals, worms, insects, fish, plants, fungi, and protists, and even in prokaryotic archaea (Danan et al., 2012; Jeck et al., 2013; Memczak et al., 2013; Wang et al., 2014; Westholm et al., 2014; Ivanov et al., 2015; Ye et al., 2015).

The majority of circRNAs are generated from protein-coding genes and consist of a single exon or multiple exons (Guo et al., 2014). Despite lack of 5′ caps and poly(A) tails, circRNAs produced in the nucleus are normally transported to the cytoplasm (Salzman et al., 2012), while a few circRNAs that are generated from excised intron lariats preferentially localize to the nucleus (Zhang et al., 2013). Although back-splicing is generally less efficient than linear splicing and their expression level are relatively low, circRNAs can accumulate in specific cell types or tissues in a temporally regulated manner (Rybak-Wolf et al., 2015; Veno et al., 2015) owing to their high stability (Jeck et al., 2013; Memczak et al., 2013). These results suggest the potential functions of circRNAs. Emerging evidence has shown that circRNAs are involved in the regulation of gene expression at

transcriptional and post-transcriptional levels. They are reported to function as miRNA sponges (Hansen et al., 2013) or RNA-binding proteins (RBP) sponges (Ashwal-Fluss et al., 2014) and are ideal biomarkers (Lukiw, 2013). It is disclosed in the latest research that proteins and peptides could derive from circRNAs comprising internal ribosomal entry sites (IRESs) (Pamudurti et al., 2017; Yang et al., 2017).

The characterization of circRNAs in plants is comparatively less than the comprehensive systematic analysis in animals. Thousands of circRNAs have been identified in nearly 30 plant species, including the model plants, crops, and Chinese herbal medicines (Ye et al., 2015; Wang et al., 2016; Zuo et al., 2016; Dong et al., 2019). Plant circRNA information is available in various databases, such as PlantCircNet (Zhang et al., 2017) and PlantcircBase (Chu et al., 2018), which collect more than 200,000 circRNAs from different research groups in total. Studies have shown that there are several differences in the circularization mechanisms of circRNAs in plants and animals. Flanking intronic complementary sequences of circularized exons have been demonstrated to be significantly important for circRNA biogenesis in animals (Zhang et al., 2014). Nonetheless, enrichment of repetitive elements or reverse complementary sequences do not appear to be found in flanking sequences of identified plant circRNAs (Lu et al., 2015; Ye et al., 2015). Thus, the circularization of plant circRNAs may be regulated by alternative mechanisms that are yet to be found.

In this review, we outline the recent advances in the biogenesis and functions of circRNAs in plants. The distinct features and circularization mechanisms of plant circRNAs are summarized by comparing them with related studies in animals. Then available bioinformatics resources, expression patterns and diverse functions of plant circRNAs are described. Particularly, the putative roles of plant circRNAs in responses to biotic and abiotic stresses are well summarized in **Table 2**.

BIOGENESIS AND DISCOVERING OF PLANT CircRNAs

Although the mechanisms of circRNA biogenesis are not very clear, two well-known models have been proposed for circRNA formation. CircRNAs are usually circularized from canonical splicing sites (Jeck et al., 2013; Memczak et al., 2013), which means back-splicing requires canonical splicing machinery (Ashwal-Fluss et al., 2014; Starke et al., 2015). Moreover, back-splicing is normally mediated by base pairing between inverted repeat elements locating in upstream and downstream introns (Zhang et al., 2014; Ivanov et al., 2015), or by dimerization of RBPs that combine with specific motifs in the flanking introns (**Figure 1A**) (Conn et al., 2015). CircRNAs are also generated from lariat precursors during exon-skipping or from intron lariats that escape debranching (**Figure 1B**) (Kelly et al., 2015).

rRNA-depleted total RNA-seq is widely used in early genome-wide profiling studies of circRNAs. Previous studies have exploited this type of publicly available datasets for circRNA identification and characterization in plants (Ye et al., 2015). Considering circular structures and relatively low expression levels of circRNAs, an additional protocol that using linear RNase R for library preparation has become the preferred method for greatly enriching circRNAs. This method has been widely used in plant circRNA studies and yielded high confidence circRNAs in maize, soybean, bamboo, tomato, and grape (Zuo et al., 2016; Chen L. F. et al., 2018; Gao et al., 2019; Luo et al., 2019; Wang Y. S. et al., 2019).

As mentioned, circRNAs are created through back-splicing where the downstream 3′ donor splice sites are joined to the upstream 5′ acceptor splice sites (**Figure 1A**). Two terminal sequences flanking the back-splice sites constitute the sequencing reads crossing back-splice junctions, which is normally ignored by mapping algorithms. These features have been exploited newly in developed bioinformatics tools to specifically detect circRNAs. These tools differ in the strategy of identifying circRNAs, so that they could be assigned to two categories (Chen et al., 2015). The first one is pseudo-reference-based approach, which relies entirely on the accurate genome annotation with all alternatively spliced mRNA isoforms, such as KNIFE (Szabo et al., 2016) and NCLscan (Chuang et al., 2016). Prior to reading mapping, all possible circRNA sequences are reconstructed based on genome annotation by shuffling exon-exon junctions (Szabo et al., 2016). The second group termed fragment-based approach, identify back-splice junctions from mapping information of multiple split alignments, including MapSplice (Wang et al., 2010), find_circ (Memczak et al., 2013), and CIRI (Gao et al., 2015). This approach has been extensively used in plant circRNA research, which infers all possible back-splicing events based on reads mapping information rather than the prior knowledge of gene annotation.

The locus-specific profiling of circRNAs that using genomic positions of back-splice sites, can be adapted for validating, quantitating, and investigating previously characterized circRNAs. For example, reverse transcription and PCR (RT-PCR) is more capable for circRNAs validation than northern blotting (Kristensen et al., 2019), droplet digital PCR (ddPCR) and reverse transcription quantitative PCR (RT-qPCR) are both available for circRNAs quantitation (Maheshwari et al., 2017; Li et al., 2018). However, genome-wide profiling and locus-specific methods could detect unique back-splice sites in circRNAs, while they cannot determine internal splicing patterns of circRNA formation.

CHARACTERIZATION OF PLANT CircRNAs

Circularization

CircRNAs can arise from a wide range of genomic positions and combinations, including exons, introns, or intergenic regions. The biogenesis of circRNAs basically depends on back-splicing of pre-mRNAs, which is conserved in eukaryotes. Significant enrichment of reverse complementary sequences comprising short repeat elements can be detected in flanking introns of circularized exons in animals, which are essential for circRNA circularization (Ashwal-Fluss et al., 2014; Liang and Wilusz, 2014). For example, circularized exons are bracketed by long introns comprising Arthrobacter luteus (Alu) elements in humans (Jeck et al., 2013). In addition, RNA secondary

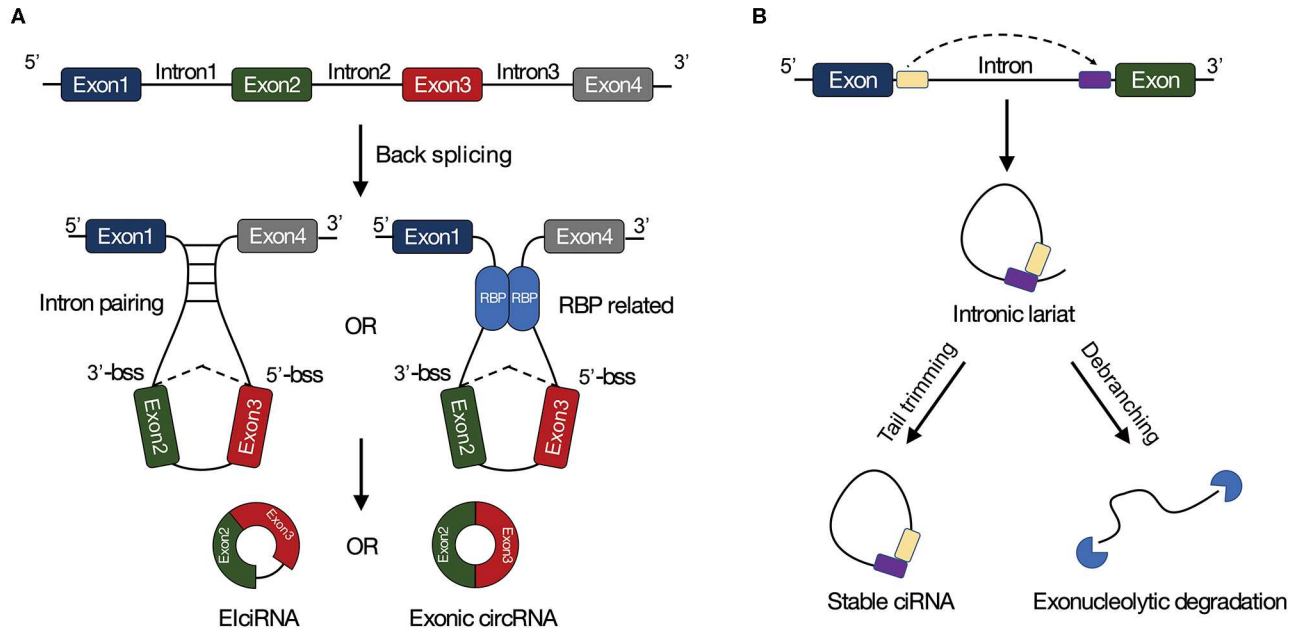

FIGURE 1 | Biogenesis of circRNAs. **(A)** Long flanking introns, inverted repeat elements, and RNA binding proteins are facilitated to back-splicing. **(B)** Circular intronic RNAs (ciRNAs) can be generated from intronic lariat precursors that escape from debranching. Bss, back-splice site; EIciRNA, exon-intron circRNA; ciRNA, circular intronic RNA.

structures and RNA-binding proteins are associated with circRNA biogenesis, and the sequence length of the flanking introns of circularized exons significantly alter circularization efficiency (Conn et al., 2015; Kramer et al., 2015). Unlike animal circRNAs, comparatively few plant circRNAs contain reverse and repetitive complementary sequences in the intronic sequences flanking exons (Lu et al., 2015; Ye et al., 2015; Zhao T. et al., 2017; Zhu et al., 2019), suggesting that intron-pairing-driven circularization may not be the primary mechanism for plant circRNA biogenesis. It has been proved that transposons and their reverse-complement counterparts enrich in maize circRNA flanking regions, showing their affection to circRNA biogenesis (Chen L. et al., 2018).

CircRNAs are generated from back-splicing which covalently link spliced down-stream splice donors with upstream splice acceptors. In the main spliceosome for splicing of most introns of eukaryotes, GT and AG terminal dinucleotides locate in the 5′ and 3′ end, respectively (Szczesniak et al., 2013). However, the mechanisms for spliceosomes selection and splicing signals of circularization are poorly characterized. In plants, circRNAs have been shown to harbor non-GT/AG splicing signals, while the majority exonic circRNAs are spliced by canonical GT/AG signal in animals (Ye et al., 2017). In Arabidopsis, grape and cotton, the majority of identified circRNAs are spliced by canonical splicing signal (Sun et al., 2016; Zhao T. et al., 2017; Gao et al., 2019), which is disagreement with the results in rice. Hundreds of circRNAs are shared with non-canonical splicing signals in rice (Ye et al., 2017), such as GC/CG, CT/GC, and GC/GT, as well as in cucumber (Zhu et al., 2019) and chloroplast of *A. thaliana* (Liu et al., 2019). Interestingly, the circRNA identification methods (find_circ and CIRI2) only consider canonical GT/AG splicing in cucumber and chloroplast of *A. thaliana*. Recent studies have revealed that back-splice sites of circRNAs are flexible and the alternative splicing of circRNAs is prevalent and much of the alternative splicing of circRNAs occurred nearby canonical splicing sites (Starke et al., 2015; Chen et al., 2016; Szabo et al., 2016). Thus, the splice signal patterns of circRNA circularization need to be verified in more plant species.

Properties and Conservation

CircRNAs are derived from all the chromosomes, as well as mitochondrial and chloroplast genomes in plants. The length of circRNAs ranges from <200 bp to longer than 100 kb, normally <1 kb. In plants, circRNAs are mainly between 200 and 600 bp, only a few of them >2 kb (Ye et al., 2017). Consistent with animals, most plant circRNAs are generated from exons of a single gene, some of them from introns, intergenic regions, untranslated regions (UTRs) or more than one gene (Ye et al., 2015). The results of gene structure annotations demonstrated that most host genes prefer to produce one circRNA than produce more than one circRNA, and few of circRNAs are generated from different loci of the same gene. CircRNA isoforms can be derived from the same locus through alternative circularization. The expression of circRNAs are much lower than their linear counterparts and correlates with the expression of their parental gene transcripts. Though, the internal regulatory mechanism of these correlations needs further research. For example, the expression of Ac_ciRNA_04842 and its derived gene *Achn372061* is positively correlated in kiwifruit (Wang et al., 2017); in rice, overexpressing Os08circ16564 significantly

inhibits the expression of its parental gene AK064900 (Lu et al., 2015). CircRNAs are highly conserved in different plant species. More than 700 orthologous genes pairs that produce circRNAs were found between Arabidopsis and rice (Ye et al., 2015). Another study of eight plant species has shown that circRNA host orthologous genes accounting for nearly 20% of genes that produce circRNAs (Zhu et al., 2019).

Bioinformatics Resources for Plant CircRNAs

High-throughput sequencing technology enables millions of circRNA sequencing reads to be accumulated in a short time period. To deal with the large number of RNA-seq datasets and have deeper understanding of circRNA biogenesis, new algorithms for efficient and accurate identification of circRNA are constantly being developed, including find_circ (Memczak et al., 2013), CIRCexplorer (Zhang et al., 2014), KNIFE (Szabo et al., 2015), and CIRI (Gao et al., 2015). However, these tools have differential performance in terms of sensitivity, accuracy, and computational costs when detecting circRNAs from RNA-Seq datasets. Comparative analyses of circRNA identification tools revealed that CIRI, KNIFE, and CIRCexplorer had better performance in terms of balancing precision and sensitivity (Zeng et al., 2017) and combining different tools could achieve more reliable predictions (Hansen et al., 2016). These comparison results provide useful guidance for improving algorithms and using current tools by researchers. PcircRNA_finder is developed specifically for plant circRNA detection, which combined five different tools to provide a more comprehensive, precise, and sensitive prediction method (**Table 1**) (Chen et al., 2016). CircPro is developed for investigating the protein-coding ability of circRNAs, which is an automated analysis pipeline that integrates five tools (**Table 1**) (Meng et al., 2017).

Increasing numbers of circRNA datasets have been generated and exploded in plants, several databases have been established to effectively organize and manage these datasets (**Table 1**). Comparing with animals, the plant circRNA databases are relatively insufficient in datasets and types. PlantcircBase (Chu et al., 2017, 2018) and PlantCircNet (Zhang et al., 2017) are comprehensive resources for plant circRNAs, containing published and new identified circRNAs information from different plant species, genome browsing, and putative mRNA-miRNA-circRNA interaction networks in corresponding species. Besides, PlantcircBase provides structure visualization of specific circRNA and validation information by PCR and sequencing. AtCircDB (Ye et al., 2019) is developed for analyzing the tissue specificity of circRNAs in Arabidopsis, while circRNA information in crops response to abiotic stress is summarized in CropCircDB (Wang K. et al., 2019). Though only two crops, maize and rice, are collected in the database. CircFunBase (Meng et al., 2019) provides circRNA information with experimentally validated and computationally predicted functions and visualized circRNA-miRNA interaction networks. ASmiR (Wang H. Y. et al., 2019) contains alternative splicing information form linear and circular RNAs in plants and their interaction information with miRNAs. However, plant species, information of phylogenetic conservation, cell-type, tissue or development stage expression, functional annotation, and interaction with other molecules are still insufficient in various databases, which will greatly promote circRNA research in plants.

FUNCTION OF PLANT CircRNAs

CircRNAs Act as miRNA Sponges

Due to previous studies in animals, the most striking function of circRNAs is to act as miRNA sponges or participate in miRNA-related pathways to regulate gene expression (**Figure 2IV**). The transcripts that contain multiple miRNA-binding sites and inhibit miRNA activity are called miRNA sponges (Ebert et al., 2007), also called competing endogenous RNAs (ceRNAs) in animals or target mimicry in plants (Franco-Zorrilla et al., 2007). These transcripts could be used to explore the function of miRNAs. Due to their loop structures, circRNAs are resistant to RNA exonucleases. These high stabilities make circRNAs have an advantage as miRNA sponges, with half-lives more than 48 h, while the half-lives of their linear counterparts are $<$20 h (Jeck et al., 2013). The circRNA CiRS-7 is a canonical miRNA sponge playing roles in inhibiting miR-7. In humans, ciRS-7 (also known as CDR1as) contains more than 70 conserved miR-7 binding sites and can strongly suppress miR-7 activity, resulting in increased levels of miR-7 targets (Hansen et al., 2013). Some studies have shown that circRNAs are putative miRNA sponges in plants, but few direct experimental evidence has been proposed. Moreover, compared with animals, plant circRNAs acting as miRNA sponges account for a smaller proportion of the total circRNAs and have lesser miRNA-binding sites (Ye et al., 2015; Zuo et al., 2016).

It is reported that only 6.6 and 5.0% of circRNAs contained putative miRNA-binding sites in rice and Arabidopsis, respectively (Ye et al., 2015). Another study in rice has been shown that 31 circRNAs harbors two or more putative miRNA-binding sites, while in total 235 circRNAs have miRNA-binding sites (Lu et al., 2015). Besides, 53 sea buckthorn circRNAs, 30 cucumber circRNAs, 25 chinese cabbage circRNAs, and 9 pepper circRNAs are predicted to act as miRNA sponges (Wang H. Y. et al., 2019; Zhang G. Y. et al., 2019; Zhu et al., 2019; Zuo et al., 2019), which required further experimental validation. In *Arabidopsis thaliana*, five circRNAs derived from flowers may function as miRNA sponges while one of them has been experimentally validated (Frydrych Capelari et al., 2019). Based on the hypothesis that circRNAs and mRNAs are targeted by the same miRNA, the putative ceRNA networks have been investigated in Arabidopsis leaves, which suggests the regulatory roles of circRNAs in leaf senescence base on differential expression patterns of mRNAs, circRNAs, and miRNAs (Meng et al., 2018). By interacting with miRNA, circRNAs may play regulatory roles in a variety of processes, including metabolic processes, developmental processes, reproductive processes, abiotic, and bionic stress response. Nevertheless, their authenticity requires further experimental validation and crosslinking immunoprecipitation and high-throughput sequencing (CLIP-seq) can help for it (Chi et al., 2009).

TABLE 1 | An overview of bioinformatics resources for plant circRNAs.

Name	Description	Web links	Latest release	Reference
PcircRNA_finder	An integrated software for circRNA prediction in plants.	http://ibi.zju.edu.cn/bioinplant/tools/manual.htm	2017	Chen et al., 2016
CircPro	An integrated tool for circRNA protein-coding potential.	http://bis.zju.edu.cn/CircPro/	2017	Meng et al., 2017
AtCircDB	A tissue-specific database for Arabidopsis circular RNAs.	http://genome.sdau.edu.cn/circRNA	2018	Ye et al., 2019
PlantCircNet	A database of plant circRNA-miRNA-gene regulatory networks.	http://bis.zju.edu.cn/plantcircnet/	2018	Zhang et al., 2017
ASmiR	A comprehensive database of miRNA targets in alternatively spliced linear and circRNAs.	http://forestry.fafu.edu.cn/bioinfor/db/ASmiR	2019	Wang H. Y. et al., 2019
CropCircDB	A database for crops in response to abiotic stress.	http://deepbiology.cn/crop/	2019	Wang K. et al., 2019
PlantcircBase	A comprehensive database of plant circRNAs in 16 organisms.	http://ibi.zju.edu.cn/plantcircbase/index.php	2019	Chu et al., 2017, 2018
CircFunBase	A database for functional circular RNAs.	http://bis.zju.edu.cn/CircFunBase/index.php	2019	Meng et al., 2019

TABLE 2 | Studies of circRNAs in plant stress responses.

	Plant Stress	Plant Species	Tissues	Number of Differentially Expressed circRNAs	Year	Reference
Biotic	Pseudomonas syringae pv. *actinidiae* infection	Kiwifruit	Root/Leaf	584	2017	Wang et al., 2017
	TYLCV infection	Tomato	Leaf	115	2018	Wang J. Y. et al., 2018
	MIMV-Infected	Maize	Leaf	160	2018	Ghorbani et al., 2018
	Verticillium wilt	Cotton	Root/Stem	280	2018	Xiang et al., 2018
Abiotic	Nutrient Depletion	*Oryza sativa* L., *Arabidopsis thaliana*	Root	27	2015	Ye et al., 2015
	Cold	Tomato	Fruit	163	2016	Zuo et al., 2016
	Dehydration	Wheat (*Triticum aestivum* L.)	Leaf	62	2017	Wang et al., 2016
	Low-nitrogen	Wheat (Triticum aestivum L.)	Root	6	2018	Ren et al., 2018
	Drought	Birch-leaf pear (*Pyrus betulifolia* Bunge)	Leaf	33	2018	Wang J. et al., 2018
	Chilling	Bell peppers (*Capsicum annuum* L. cv. Jingtian)	Fruit	36	2018	Zuo et al., 2018
	Heat	Cucumber (*Cucumis sativus* L.)	Leaf	6	2018	He et al., 2019
	Heat	*Arabidopsis thaliana*	Seedling	1583	2018	Pan et al., 2018
	Heat	Radish	Leaf	3	2019	Yang et al., 2019
	Copper	Citrus	Root/Leaf	45/17	2019	Fu et al., 2019
	Drought	Arabidopsis (*Arabidopsis thaliana*), maize (*Zea mays*)	Leaf	1843/1283	2019	Zhang P. et al., 2019
	Cold	Grape (*Vitis vinifera*)	Leaf	475	2019	Gao et al., 2019
	Salt	Cucumber (*Cucumis sativus*)	Root/Leaf	1934/44	2019	Zhu et al., 2019
	Calcium	Chinese cabbage (*Brassica rapa* L. ssp. pekinensis)	Leaf	616	2019	Wang W. H. et al., 2019
	Low-Phosphorus Stress	Soybean	Root	120	2020	Lv et al., 2020

CircRNAs in Stress Response

Previous studies have demonstrated that plant circRNAs exhibit specific cell-type, tissue, or developmental stage expression patterns and circRNAs expression is usually induced under various environmental stresses (**Figure 2V**; **Table 2**), including drought, chilling, heat, nutrient deficiency, or pathogen invasion. These suggest that circRNAs may like other ncRNAs, such as miRNAs and lncRNAs, which are crucial to plant growth and development, as well as biotic or abiotic stresses response. Evidence has shown that those differentially expressed circRNAs could regulate plant in responses to stresses by interacting with miRNAs and regulating the expression of stimulus-responsive genes. The regulatory roles and potential functions of circRNAs could be inferred and experimentally verified

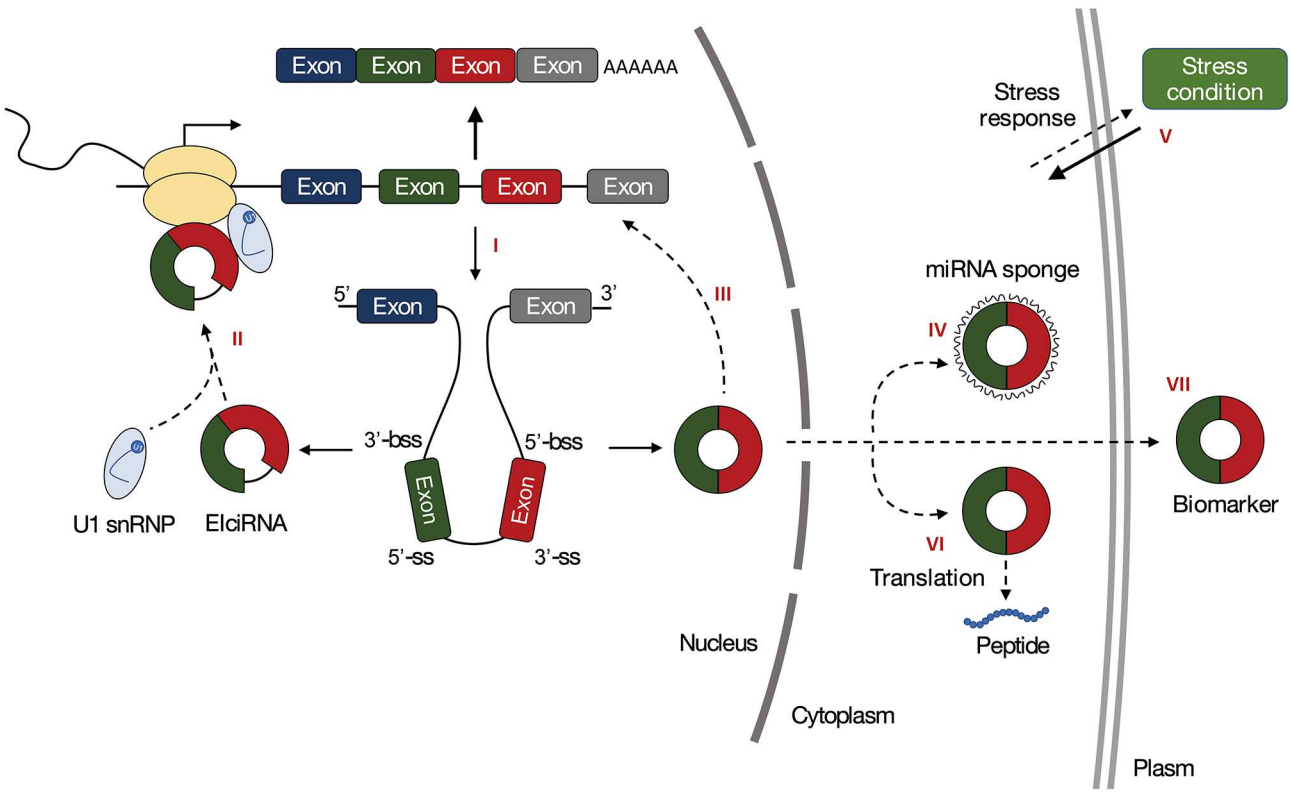

FIGURE 2 | Functions of circRNAs in plants. **(I)** The processing of circRNAs can affect the splicing of their linear counterparts. **(II)** CircRNAs can regulate transcription of their parental genes. **(III)** CircRNAs can regulate the splicing of their linear cognates. **(IV)** CircRNAs can act as miRNA sponges. **(V)** CircRNAs can regulate gene expression in response to biotic or abiotic stresses. **(VI)** CircRNAs can be translated. **(VII)** CircRNAs are promising biomarkers.

by establishing the circRNA-mediated ceRNA network under stress conditions.

CircRNAs were firstly identified under the biotic stress condition was in Arabidopsis leaves under pathogenic interaction (Sun et al., 2016). In kiwifruit, circRNAs were also identified differentially expressed under pathogen invasion (Wang et al., 2017). In total, 584 circRNAs have been shown differential expression patterns during *Pseudomonas syringae* pv. *actinidiae* (Psa) infection and their expression are related to the stage of infection (Wang et al., 2017). Besides, a list of circRNAs related to plant defense response have been identified by network analysis (Wang et al., 2017). Later studies indicate that circRNAs can function as negative regulators of tomato yellow leaf curl virus (TYLCV) interaction in tomato (Wang J. Y. et al., 2018), play regulatory roles in the Verticillium wilt response in cotton (Xiang et al., 2018) and are response to maize Iranian mosaic virus (MIMV) infection in maize (Ghorbani et al., 2018).

CircRNAs have been shown to be differentially expressed under abiotic stresses, including nutrient depletion, high light, heat, chilling, drought, or salt. However, the regulatory mechanism and specific biological significance of plant circRNAs during these conditions remain to be elucidated. In the samples from Oryza sativa roots with phosphate-starvation condition and *A. thaliana* leaves with light treatment, circRNAs were firstly identified in plants, and 27 circRNAs in rice were found to display stress-specific expression patterns under phosphate deficiency condition, of which 6 were up-regulated and 21 were down-regulated (Ye et al., 2015). These results indicated the potential roles of plant circRNAs in response to stress, furthermore, the stress-specific expression patterns are also found in other plant species with different biotic stress conditions. Analyses have shown that 36 and 163 differentially expressed circRNAs were identified in chilled bell pepper (Zuo et al., 2018) and chilled tomato fruit (Zuo et al., 2016) respectively, 475 differentially expressed circRNAs were identified in grape leaves under cold stress (Gao et al., 2019). The grape Vv-circATS1, derived from *glycerol-3-P acyltransferase*, has been found to improve cold tolerance in *Arabidopsis* by regulating the expression of stimulus-responsive genes, such as *CSD2*, *PRXCA*, *PME41*, *LOX3*, and *WRKY48*. Under dehydration-stressed conditions, differentially expressed circRNAs have been detected in wheat (Wang et al., 2016), pear (Wang J. et al., 2018), maize, and Arabidopsis (Zhang P. et al., 2019). Moreover, the similar patterns of expressional changes were observed in crops under nutrient depletion (Darbani et al., 2016; Wang W. H. et al., 2019; Lv et al., 2020), metal ion toxicity (Fu et al., 2019) or salt (Zhu et al., 2019). In addition, researchers found that the stress condition could alter lengths of circRNAs, numbers of circularized exons

and alternative circularization events in Arabidopsis (Pan et al., 2018).

CircRNAs Regulate Gene Expression

Accumulating evidence has proved that circRNAs are involved in the regulation of gene expression. The processing of circRNAs can affect the splicing of their linear counterparts (**Figure 2I**), and regulate the transcription of their parental genes (**Figure 2II**). In human, exon-intron circRNAs (EIciRNAs) enhance transcription of circRNA host genes through association with U1 snRNP. EIciRNAs have been identified in plants and implicated in gene regulation in biotic stress response (Zhao W. et al., 2017). In Arabidopsis, CircSEP3 that derived from SEPALLATA3 (SEP3) gene has been shown to regulate transcription and splicing of their linear counterparts (**Figure 2III**). CircSEP3 can bind strongly to its cognate DNA locus and form an RNA:DNA hybrid, whereas the linear RNA with the same sequence bind to the DNA much more weakly. The circRNA:DNA formation would result in transcriptional pausing and leading to the formation of alternatively spliced SEP3 mRNA with exon skipping (Conn et al., 2017). These together suggest that circRNAs can modulate gene expression at both transcription and splicing levels. In addition, the correlations between circRNAs and their parental genes have been indicated in studies of plant circRNAs, the mechanistic basis of these correlations needs further experimental validation.

CircRNAs Can Be Translated

Due to the lack of key components for the canonical cap-dependent translation, such as 5′ caps or poly(A) tails, circRNAs are considered untranslatable. However, recent research in mammals revealed that circRNAs translation could be driven by IRESs (Abe et al., 2015) and promoted by N6-Methyladenosine (m6A) RNA modification (Meyer et al., 2015; Yang et al., 2017) (**Figure 2VI**). Although a number of circRNAs have been predicted to contain putative open reading frames (ORFs) with IRESs, a few circRNAs have proven to act as protein templates, and functions of peptides derived from circRNAs remain to be explored (Legnini et al., 2017; Pamudurti et al., 2017; Zhang et al., 2018). For example, peptides FBXW-185aa that are translated from circ-FBXW7 may inhibit tumorigenesis of brain cancer (Yang et al., 2018). FBXW-185aa has been shown to interact with the deubiquitinating enzyme USP28, protecting USP28 from binding to a key regulator of tumorigenesis, FBXW7α. Therefore, FBXW-185aa prevents FBXW7α-induced degradation by antagonizing USP28-induced c-Myc stabilization. Moreover, circRNAs that contain large ORFs and m6A-modified sites in junction sequences have been detected to encode hundreds of peptides (Tang et al., 2020). For example, m6A-modified circE7 could be translated to produce E7 oncoprotein, which is directly responsible for HPV-induced carcinogenesis (Zhao et al., 2019). The latest evidence also shows that m6A modified circRNA could inhibit innate immunity (Chen et al., 2019). However, there is no circRNA in plants has been reported to be translated. The studies of m6A modification in Arabidopsis have been reported (Zhou et al., 2017), translatable circRNAs and their biological functions would be proposed in plants as research progresses.

CircRNAs Act as Biomarkers

It has been illustrated by previous research that circRNAs are universal in diverse cell types, conserved in different species and exhibit specific expression patterns, which makes them potential biomarkers (**Figure 2VII**). Moreover, circRNAs have been shown to participate in diverse physiological and pathophysiological processes, including cancers (Li et al., 2015; Qin et al., 2016; Zhu et al., 2017), cardiovascular disease (Satoh et al., 2013), neurological disease (Grapp et al., 2013) and diabetes (Zhao Z. et al., 2017), suggesting that circRNAs are becoming the emerging biomarkers for diagnosis and treatment of human diseases. CircRNAs are also regarded as aging biomarkers in *Drosophila* research (Westholm et al., 2014). Biomarkers have been investigated and used in breeding applications for a couple of years in plants (Yang et al., 2011). Due to their characteristics, circRNAs are emerging biomarkers in plants. In Arabidopsis, circRNAs have been shown to act as bona fide biomarkers of alternative splicing variants (Conn et al., 2017). The study in maize and Arabidopsis suggests that circRNAs play essential roles in plant drought response, and can be used as effective biomarkers in genetic improvement of crop drought tolerance (Zhang P. et al., 2019). Hence, the biomarker is an interesting research topic in plant circRNAs.

CONCLUSIONS AND FUTURE PERSPECTIVES

With large numbers of circRNAs being identified, more insights have been given into circRNAs, which has become a promising research hotspot. CircRNAs usually exhibit specific expression patterns and are also induced by stress conditions, suggesting that circRNAs may be a new regulatory factor at the transcriptional and post-transcriptional levels. Evidence indicates that circRNAs participate in diverse biological processes, they can act as miRNA sponges, interact with many different RBPs, be translated into peptides or act as promising biomarkers. Compared with related studies in animals, the research of plant circRNAs are relatively less, and lack of experimental and functional verification. The formation mechanism of plant circRNAs is based on bioinformatics analysis rather than convincing experimental evidence, which is still in the theoretical stage. The characterization and function of plant circRNA have been proposed and verified, but there are still quite a few prediction results, especially the interactions between circRNAs and miRNAs. In addition, the coding ability of circRNAs and their individual function roles in plant growth and developments, such as leaf senescence, flower development, fruit maturation, and response to biotic and abiotic stresses, have not been investigated so far, which may account for primary research topics of circRNAs in plants. We still need more observation and validated results about the biogenesis mechanism, specific regulatory roles, and functions of these molecules, as well as large-scale quantification, full-length sequencing and functional

verification of circRNAs. In addition, the differences between plant and animal circRNAs will also be an interesting topic.

AUTHOR CONTRIBUTIONS

PZ and MC wrote and revised the manuscript. SL prepared the figures and revised the manuscript. All authors read and approved the final manuscript.

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Circular RNAs in Blood Malignancies

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Circular (circ)RNAs influence a wide range of biological processes at least in part by interacting with proteins and microRNAs. CircRNAs expressed in the hematopoietic compartment have been increasingly recognized as modulators of physiological and pathological features of hematopoetic stem cell (HSC)-derived populations. In particular, several circRNAs were found to enhance or suppress tumor progression in blood malignancies such as leukemias and lymphomas. Moreover, numerous circRNAs have been proposed to help confer resistance to the conventional treatments used in hematopoietic cancers. Here, we review the most important circRNAs described thus far in acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), lymphomas, and multiple myeloma (MM). We discuss the usefulness of circRNAs as diagnostic and prognostic markers and their potential value as therapeutic targets.

Keywords: circular RNAs, blood malignancies, leukemias, lymphomas, non-coding RNA

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INTRODUCTION

The ENCyclopedia Of DNA Elements (ENCODE) project revealed that only 2% of the human genome encodes proteins (ENCODE Project Consortium, 2012). Subsequently, there has been a growing interest in studying RNAs without apparent coding function, collectively named non-coding RNAs (ncRNAs). Several ncRNAs, such as ribosomal (r)RNAs, transfer (t)RNAs, small nuclear (sn)RNAs, and small nucleolar (sno)RNAs are well-known for their role in "housekeeping" cell functions (Zhang et al., 2019). In addition, ncRNAs include heterogeneous transcripts with lesser-known regulatory functions, including microRNAs (miRNAs), piwi-interacting (pi)RNAs, small interfering (si)RNAs, and long non-coding (lnc)RNAs (Zhang et al., 2019).

Circular (circ)RNAs are a vast class of ncRNAs with covalently closed ends and lengths between ∼100 to thousands of nucleotides (Kristensen et al., 2019). Unlike lncRNAs, circRNAs are usually well conserved among different species. They mainly originate through a process called backsplicing, in which the 3' and 5' ends of a precursor RNA are cleaved and ligated by the splicing machinery. Given the lack of 5' and 3' ends, circRNAs are not degraded by RNA exonucleases and therefore they are quite stable. Most of the circRNAs described to-date originate from exonic sequences (Chen et al., 2015), however they may also contain introns and intergenic sequences. CircRNAs can be localized in both the cytoplasm and the nucleus, as well as outside the cell in extracellular vesicles (Li et al., 2015). They often display a tissue-specific distribution and their expression may be altered in cancer and other pathologies (Kristensen et al., 2019; Liu et al., 2019; Mei et al., 2019; Vo et al., 2019). A growing body of evidence suggests that circRNAs play a role as potential prognostic and diagnostic biomarkers in cancer, given their high stability and specific expression patterns. CircRNAs are present in human body fluids like blood, urine, and saliva, and thus could be easily detected through non-invasive biopsies (Wang et al., 2018; Su et al., 2019; Verduci et al., 2019).

A number of functions have been described for circRNAs, including synthesis of short polypeptides, recruitment of proteins to DNA, and scaffolding between specific enzymes and substrates (D'Ambra et al., 2019; Kristensen et al., 2019; Lei et al., 2020). Importantly, many circRNAs have been proposed to act as a "sponge" or decoy for microRNAs (miRNAs) and RNA-binding proteins (RBPs) (D'Ambra et al., 2019; Jamal et al., 2019; Kristensen et al., 2019; Verduci et al., 2019). CircRNAs bind and thereby functionally "neutralize" or inactivate miRNAs, thus restoring the translation of proteins that are otherwise suppressed by specific miRNA-mRNA binding events. This process is particularly effective when a circRNA is highly abundant and contains several binding sites for a target miRNA. For example, the well-known circRNA *CDR1as* (also called *ciRS-7*) has >60 binding sites for miR-7 (Hansen et al., 2013). The sponging of miR-7 by *CDR1as* has been described in Parkinson's disease, Alzheimer's disease, and several cancers (Shao and Chen, 2016). CircRNAs can also bind RBPs and these interactions may interfere with or enhance the functions of several proteins and mRNAs. For example, *circPABPN1* suppressed cell proliferation by interacting with the RBP HuR, preventing HuR from binding to *PABPN1* mRNA, and thereby suppressing the translation of PABPN1, a protein critically involved in cell proliferation (Abdelmohsen et al., 2017). Here, we review the increasingly recognized roles of circRNAs in hematological malignancies (Bonizzato et al., 2016; Mei et al., 2019), with a particular focus on the binding and possible sponging of oncogenic or tumor-suppressive miRNAs. These circRNAs, their effectors, and impacts on hematologic diseases are summarized in **Table 1** and **Figure 1**.

circRNAs in AML

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, with an incidence of over 20,000 cases per year in the United States (De Kouchkovsky and Abdul-Hay, 2016). AML is characterized by the rapid growth of abnormal and immature white blood cells, inhibiting the production of normal hematopoietic cells in the bone marrow.

Many cytogenetic abnormalities causing AML have been characterized and include the large chromosomal translocations t(8;21), t(15;17), and t(9;11), which create the fusion proteins RUNX1-RUNX1T1, PML-RARA, and MLL-AF9, respectively (De Kouchkovsky and Abdul-Hay, 2016). Using patient samples, Guarnerio et al. (2016) found that the rearrangement of chromosomes led to the biogenesis of fusion-circRNAs (f-circRNAs) and identified two tumor-promoting f-circRNAs, *f-circPR*, and *f-circM9*, derived from the fusion transcripts *PML-RARA* and *MLL-MLLT3* (AF9), respectively. These f-circRNAs enhanced cell proliferation and promoted leukemogenesis *in vivo* in mice when co-expressed with their oncogenic fusion protein counterparts. Furthermore, f-circRNAs contributed to therapy resistance by conferring protection from apoptosis during treatment with the chemotherapeutic drugs arsenic trioxide (ATO) and cytarabine (Ara-C).

Cytogenetically normal AML (CN-AML) is not associated with chromosomal aberrations but is characterized by heterogeneous gene mutations with therapeutic and prognostic implications. For instance, mutations in *FLT3-IDT* (internal tandem duplication in the fms-related tyrosine kinase 3 gene) are associated with a higher risk of relapse, whereas mutations in the chaperone nucleophosmin gene (*NPM1*) are associated with a favorable prognosis in the absence of other mutations (De Kouchkovsky and Abdul-Hay, 2016). Hirsch et al. (2017) identified several *NPM1*-derived-circRNAs in CN-AML cell lines carrying either the normal or the mutated *NPM1* gene. The circRNA *hsa_circ_0075001* was elevated in AML cells independently of the *NPM1* mutational status. The levels of *hsa_circ_0075001* were higher in a cohort of 46 patients with undifferentiated blasts and correlated negatively with the expression of genes involved in Toll-like receptor (TLR) signaling, which is implicated in hematopoietic cell differentiation (Nagai et al., 2006; Okamoto et al., 2009; Eriksson et al., 2017). Moreover, in patients with high *hsa_circ_0075001* levels, the abundance of miR-181 target genes was reduced; the authors linked these two observations by noting that *NPM1* mRNA has miR-181 binding sites and circRNAs derived from *NPM1* may sequester miR-181 (Hirsch et al., 2017). Importantly, miR-181 is a critical regulator of cellular differentiation and hematological malignancies (Su et al., 2015).

Another circRNA upregulated in CN-AML primary samples, *circDLEU2* promoted tumor formation *in vivo* in mice. High levels of *circDLEU2* were proposed to reduce miR-496 function and to promote the expression of PRKACB (Protein Kinase, cAMP-dependent Catalytic β), leading to alterations in cell proliferation and apoptosis (Wu et al., 2018). In CN-AML patients, high levels of another circRNA, *circKLHL8*, correlated with better overall outcomes, and event-free survival, together with a lower percentage of malignant blasts in blood and bone marrow (Papaioannou et al., 2020). Two other circRNAs, *circFOXO3* and *circFBXW7*, were hypothesized to function as tumor suppressors in AML (Zhou et al., 2019; Papaioannou et al., 2020).

In recent years, additional circRNAs dysregulated in AML have been identified. Chen et al. (2018) found that *circANAPC7* (*circ_101141*) was upregulated in AML bone marrow samples and could play a role in the disease by sponging microRNAs in the miR-181 family, which regulate hematopoietic differentiation. Additional studies were performed on AML bone marrow samples; Ping et al. (2019a) identified *circ_0009910* as a circRNA that sponged the tumor-suppressor microRNA miR-20a-5p, thus promoting cancer growth, while Fan et al. (2018) reported that *circ_100290* promoted AML cell proliferation and inhibited apoptosis by sponging miR-293, ultimately increasing the expression of Rab10, a member of the oncogenic RAS family. Li et al. (2017) reported the dynamic expression of *circ_0004277* in AML patients: *circ_0004277* levels were low in newly diagnosed patients compared to healthy controls, but its expression was restored after complete response to induction therapy. Follow-up studies revealed that *circ_0004277* levels decreased again during relapse, confirming its potential value as a diagnostic and prognostic biomarker. Bioinformatic analysis predicted that *circ_0004277* might be part of a complex network including several miRNAs and mRNAs.

TABLE 1 | Circular RNAs implicated in hematological malignancies.

	circRNA	Levels	miRNAs, RBPs, and pathways targeted	Impact on hematologic disease	References
ACUTE MYELOID LEUKEMIA (AML)					
PML-RARA	***f-circPR (PML)***	*de novo*	Signaling through AKT	Increased cell proliferation Chemotherapy resistance	Guarnerio et al., 2016
MLL-AF9	***f-circM9***	*de novo*	Signaling through MAPK and AKT	Increased cell proliferation Chemotherapy resistance	Guarnerio et al., 2016
NPM1	***circNPM1 hsa_circ_0075001***	UP	miR-181 TLR signaling	Altered differentiation Promotion of leukemogenesis	Hirsch et al., 2017
DLEU2	***circDLEU2 hsa_circ_0000488***	UP	miR-496 and PRKACB	Increased cell proliferation Inhibition of apoptosis	Wu et al., 2018
KLHL8	***circKLHL8***	Associated with outcome	miR-155 and increased CDKN1, CDKN2, BCL6, TLR4, CEBP	Positive prognostic marker	Papaioannou et al., 2020
FBXW7	***circFBXW7***	DOWN	Signal transduction Leukocyte differentiation	Tumor suppression	Papaioannou et al., 2020
FOXO3	***circFOXO3***	DOWN	Apoptotic pathways	Induced apoptosis Diagnostic and prognostic biomarker	Zhou et al., 2019
ANAPC7	***circANAPC7 hsa_circRNA_101141***	UP	miR-181	Possible role in HSCs differentiation	Chen et al., 2018
MFN2	***circ_0009910 hsa_circRNA_100053***	UP	miR-20a-5p Proliferative pathways	Cancer growth	Ping et al., 2019a
SLC30A7	***circ_100290***	UP	miR-203/Rab10	Increased cell proliferation Inhibition of apoptosis	Fan et al., 2018
WDR7	***hsa_cir_0004277***	DOWN	Unconfirmed	Diagnostic and prognostic biomarker	Li et al., 2017
(Unknown)	***circ-0004136***	UP	miR-142, miR-29a	Increased cell proliferation Inhibition of apoptosis	Yuan et al., 2019
VIMENTIN	***circVIM***	UP	Unknown	Prognostic biomarker	Yi et al., 2019
HIPK2	***circHIPK2 (PML)***	DOWN	miR-124-3p CEBPA	Prognostic Biomarker Possible role in differentiation induced by ATRA treatment	Li et al., 2018a
(Unknown)	***hsa_circ_0004520***	UP	PLXNB2, VEGFA	Angiogenesis Prognostic biomarker	Lv et al., 2018
MYBL2	***circMYBL2 hsa_circ_0006332***	UP	Enhances FLT3 translation	Increased cell proliferation and chemoresistance Inhibition of apoptosis	Sun et al., 2019
PAN3	***circPAN3***	UP	miR-153-5p, miR-183-5p XIAP	Inhibitor of apoptosis Increased chemoresistance	Shang et al., 2019
CHRONIC MYELOID LEUKEMIA (CML)					
BCR-ABL1	***f-circBA9.3***	*de novo*	Apoptotic pathways	Negative prognostic factor Increase chemoresistance	Pan et al., 2018
MFN2	***circ_0009910 hsa_circRNA_100053***	UP	Unconfirmed in CML	Negative prognostic biomarker Imatinib resistance	Ping et al., 2019b
(Unknown)	***hsa_circ_0080145***	UP	miR-29b	Increased cell proliferation Inhibition of apoptosis	Liu et al., 2018
ACUTE LYMPHOID LEUKEMIA (ALL)					
AF4	***circAF4***	UP	miR-128-3p MLL-AF4	Promotes leukemogenesis *in vitro* and *in vivo*	Huang et al., 2019
PVT1	***circPVT1***	UP	miR-125, let-7	Increased cell proliferation Inhibition of apoptosis	Hu et al., 2018
PAX5	***circPAX5***	UP	miR-124 (unconfirmed)	May promote B cell maturation in pediatric patients	Gaffo et al., 2019
HIPK3	***circHIPK3***	UP	miR-124 (unconfirmed)	Unknown	Gaffo et al., 2019
ENL, AF6, AF9, AF10, GAS7	***circENL, circAF6, circAF9, circAF10, circGAS7***	Unknown	Unknown	Unknown	Huang et al., 2019
CHRONIC LYMPHOID LEUKEMIA (CLL)					
RPL15	***circRPL15 hsa_circ_0064574***	UP	miR-146b-3p RAF1	Increased cell proliferation Diagnostic biomarker	Wu et al., 2020

(Continued)

TABLE 1 | Continued

	circRNA	Levels	miRNA, RBPs, and pathways targeted	Impact on hematologic disease	References
CBFB	***circCBFB hsa_circ_0000707***	UP	miR-607 FZD3, Wnt/β-catenin pathway activation	Increased cell proliferation Inhibition of apoptosis Prognostic and diagnostic marker	Xia et al., 2018
MTO1	***circ_0132266***	DOWN	miR-337-3p PML	Tumor suppressor	Wu et al., 2019
LYMPHOMAS					
LAMP1	***circLAMP1 hsa_circRNA_101303***	UP	miR-615-5p DDR2	Increased cell proliferation Inhibition of apoptosis	Deng et al., 2019
APC	***circAPC hsa_circ_0127621***	DOWN	miR-888 APC	Tumor suppressor Diagnostic and prognostic marker	Hu et al., 2019
NPM1-ALK	***f-circNPM1-ALK***	*de novo*	Unknown	Possible diagnostic marker for ALCL	Babin et al., 2018
MULTIPLE MYELOMA (MM)					
Unknown	***circ_0000190***	DOWN	miR-767-5p MAPK4	Prognostic marker Tumor suppressor Potential therapeutic target	Feng et al., 2019

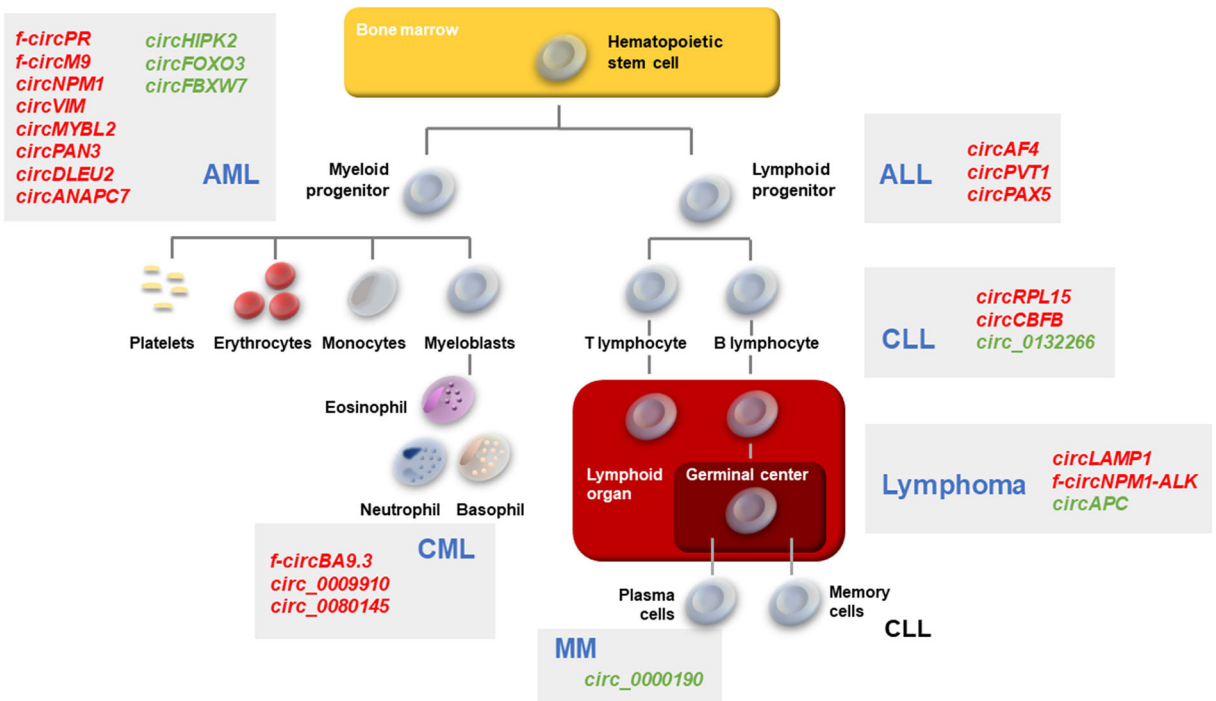

FIGURE 1 | Schematic of hematopoiesis depicting the developmental cell types giving rise to the major leukemias and lymphomas. AML, CML, ALL, CLL, Lymphomas, and MM described in the text are represented. Gray boxes, the main circRNAs associated with each malignancy are indicated in red (upregulated in malignancy) or green (downregulated in malignancy).

Yuan et al. (2019) reported a rise in *circ_0004136* in a pediatric AML cohort and proposed that *circ_0004136* promoted cell proliferation at least in part by binding and repressing miR-142, a microRNA known to be associated with pediatric AML. In a cohort of 113 AML patients, Yi et al. (2019) identified *circVIM*, derived from the *VIM* (vimentin) gene. Vimentin expression is known to be linked to tumor progression and can be a useful marker of the aggressiveness of certain cancers, such as gastric cancer (Fuyuhiro et al., 2010). Similarly, high levels of *circVIM* in AML were associated with shorter overall survival and leukemia-free survival, pointing to *circVIM* as a possible prognostic marker in AML (Yi et al., 2019).

Acute promyelocytic leukemia (APL), a less common form of AML, is characterized by the formation of the promyelocytic leukemia/retinoic acid receptor α (PML/RARα) fusion protein, which causes many of the features of the disease. APL has a

high responsiveness to all-*trans* retinoic acid (ATRA) treatment, which leads to further differentiation and maturation of the leukemic cells (Cicconi et al., 2018; Li et al., 2018a). (Li et al., 2018a) identified several circRNAs differentially expressed in APL-derived NB4 cells during ATRA treatment and validated in patient samples: *circHIPK2*, *circHIPK3*, *circPVT1*, *circRELL1*, and *circSMARCA5*. The authors focused on the levels of *circHIPK2*, which decline in newly diagnosed persons and are restored after complete remission. Further experiments proved a key relationship between this circRNA and cell maturation: *circHIPK2* sponged miR-124-3p and increased the expression of CEBPA, a transcription factor involved in hematopoiesis. Altogether, the results highlighted *circHIPK2* as a potential biomarker for APL.

Extramedullary Infiltration (EMI) is a poor prognostic indicator in AML characterized by the accumulation of blasts in extramedullary locations, including spleen, liver, skin, and central nervous system. Lv et al. (2018) found that most circRNAs upregulated in EMI bone marrow samples might be proposed to influence cell adhesion, migration, and signal transduction. Among them, *hsa_circ_0004520* is predicted to modulate the expression of VEGFA (vascular endothelial growth factor A), which could contribute to angiogenesis in AML-EMI.

The complexity of molecular and cytogenetic abnormalities is a challenge for the design of AML therapy. While induction therapy with cytarabine and an anthracycline remains a standard of care in AML, resistance may develop over time through different mechanisms (Dombret and Gardin, 2016). Specific tyrosine kinase inhibitors (TKI) have been developed to treat the aforementioned FLT3-ITD AML, but despite success in achieving remission in clinical trials, patients often relapsed or acquired resistance over time (De Kouchkovsky and Abdul-Hay, 2016; Sun et al., 2019). Sun et al. (2019) reported that *circMYBL2* silencing restored sensitivity of human FLT3-ITD$^+$ cells to the TKI quizartinib and inhibited cell proliferation in culture as well as in mice; mechanistically, *circMYBL2* enhanced FLT3 translation by facilitating the binding between *FLT3* mRNA and the RBP polypyrimidine tract-binding protein 1 (PTBP1).

Shang et al. (2019) identified *circPAN3* as a key factor in doxorubicin resistance in AML cell lines (33). *CircPAN3* was shown to bind miR-153-3p and miR-183-5p, in turn modulating the expression levels of XIAP (X-linked inhibitor of apoptosis protein), a key protein implicated in autophagy and apoptosis. In addition, *circPAN3* downregulation restored drug sensitivity, suggesting a role for this circRNA in AML resistance to conventional chemotherapies.

circRNAs in CML

Chronic myeloid leukemia (CML) is a rare clonal myeloproliferative malignancy with an annual incidence of one to two cases per 100,000 persons (Zhou and Xu, 2015). The cytogenetic hallmark of CML is the Philadelphia (Ph) chromosome, generated by the reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22) (q34;q11). The fusion gene *BCR-ABL1* arises as a consequence of the translocation and leads to the production of the oncogenic fusion protein BCR-ABL1, a tyrosine kinase that induces the phosphorylation, activation, and dysregulation of signaling molecules involved in the survival and growth of bone marrow progenitor cells (Kang et al., 2016). TKIs are the standard choice of treatment for CML, but their effectiveness depends on the phase of the disease and is challenged by the development of drug resistance over time (Litwinska and Machalinski, 2017; Patel et al., 2017). High levels of BCR-ABL1 kinase activity may arise as a consequence of gene amplification and Ph duplication, and may be sufficient to confer TKI resistance. In addition, specific mutations in the kinase domain (KD) of BCR-ABL1 may enhance drug resistance (Soverini et al., 2014; Patel et al., 2017).

Pan et al. (2018) identified f-circRNA *circBA9.3*, derived from the *BCR-ABL1* mRNA, as potentially involved in drug resistance to Imatinib, a second-generation TKI. *CircBA9.3* expression was higher in TKI-resistant patients compared to responsive controls. Moreover, *circBA9.3* transfection in BCR-ABL-negative cell lines enhanced proliferation and cancer progression. Further experiments confirmed that *circBA9.3* levels were positively correlated with the expression of BCR-ABL1. Regardless of whether first or second generation TKIs were administered, cells overexpressing *circBA9.3* displayed less apoptosis compared to controls.

Further studies investigated the role of circRNAs in CML drug resistance. Ping et al. (2019b) used circRNA microarrays to assess circRNA profiles in CML and found that *hsa_circ_100053* levels increased in both cells and serum of CML patients. In keeping with the proposal that *hsa_circ_100053* might be a potential biomarker in CML, higher expression of *hsa_circ_100053* was associated with advanced clinical stage, BCR/ABL1 mutational status and resistance to Imatinib. High levels of *hsa_circ_100053* were suggested as a negative prognostic factor in the overall survival of CML patients.

Through an RNA-sequencing screen, Liu et al. (2018) identified *hsa_circ_0080145* as being upregulated in cells from CML patients and in cell lines K562 and KU812. Moreover, *hsa_circ_0080145* silencing suppressed leukemic cell proliferation. In functional experiments, the authors found that *hsa_circ_0080145* was capable of sponging miR-29b, and the targets of miR-29b were predicted with the tool miRTarBase (http://miRTarBase.mbc.nctu.edu.tw/), which contains interactions validated experimentally. Gene ontology analysis revealed that these genes belong to three main groups: systemic lupus erythematosus pathway, cAMP signaling pathway, and heterocycle biosynthetic process. In agreement with these findings, a previous study reported that miR-29b was downregulated in CML and overexpression of miR-29b in K562 cells inhibited leukemic cell growth and promoted apoptosis through regulation of the BCR-ABL1 tyrosine kinase (Li et al., 2013). In addition, several other oncogenes could be potentially silenced by miR-29, including the antiapoptotic protein MCL1, upstream inhibitors of p53, DNA methyltransferases, and extracellular matrix proteins (Li et al., 2013).

Together, these findings highlight the importance of circRNAs as potential CML biomarkers with key roles in drug resistance and as targets for new therapeutic treatments.

circRNAs in ALL

Acute lymphoblastic leukemia (ALL) is the most common cancer among children in the USA and the most frequent cause of cancer death in young people (Hunger and Mullighan, 2015). ALL may arise from B-cell (B-ALL) or T-cell (T-ALL) precursors. Current medical treatments for ALL allow high survival rates, but relapse occurs in 15–20% of pediatric patients and is associated with a higher risk of treatment failure (Schrappe et al., 2012). Most somatic mutations acquired in ALL are chromosome rearrangements, such as translocations and hyperdiploidy. Translocation t(4;11)(q21;q23) resulting in the chimeric product MLL-AF4 is commonly identified in infant pro-B-ALL and has poor prognosis (Mrózek et al., 2009).

The first studies on circRNAs differentially expressed in ALL (Salzman et al., 2012) found that >10% of the transcripts encoded by hundreds of genes in naïve B cells (CD19+) and hematopoietic stem cells (CD34+) were circRNAs. Subsequently, Huang et al. (2019) identified different circRNAs derived from the MLL partner fusion gene *AF4*. The levels of *circAF4(ex3-4)* were higher in the leukemia cell line analyzed (RS4;11) and patients under 8 years of age. Moreover, *circAF4* levels correlated with the severity of disease, and *circAF4* silencing led to increased apoptosis in cells carrying the *MLL-AF4* translocation. In mice, *circAF4* knockdown improved survival and reduced spleen infiltration. By binding miR-128-3p, *circAF4* might sequester the microRNA away from the fusion *MLL-AF4* mRNA and enable MLL-AF4 expression. Simultaneous *circAF4* silencing and miR-128-3p overexpression *in vivo* supported this regulatory axis and suggested that *circAF4* acts as an oncogenic circRNA in leukemia. In a further study, Dal Molin et al. (2019) found that specific rearrangements leading to fusions between *MLL* and other genes not only generated alternative isoforms of circRNAs in different subtypes of leukemias, but may also contribute to the production of disease-associated aberrant circRNAs.

Hu et al. (2018) found that *circPVT1* dysregulation promoted cell proliferation and inhibited apoptosis in ALL cell lines by sponging miR-125 and let-7, ultimately increasing the expression of the oncogene MYC and the anti-apoptotic protein BCL2. Other studies showed that *circPVT1* regulated the function of let-7 family members which function as tumor suppressors (Panda et al., 2017a). *CircPVT1* had been previously described as a negative prognostic factor for gastric cancer (Chen et al., 2017a). In sum, by interfering with let-7 function, *circPVT1* was proposed to promote ALL leukemogenesis (Hu et al., 2018).

Gaffo et al. (2019) identified and quantified bioinformatically the circRNAs expressed in T cells, B cells and monocytes in physiological conditions; as expected, circRNA signatures varied with stage of differentiation and cell type. Further analysis of circRNAs differentially expressed in B-cell precursors of ALL pediatric patients found upregulated *circPVT1*, *circHIPK3*, and *circPAX5*. The *PAX5* (paired box protein five) gene encodes transcription factor BSAP (B-cell-lineage-specific activator protein), with a key role in defining and maintaining B-cell identity. The binding of *circPAX5* and *circHIPK3* to miR-124-5p was proposed to synergistically interfere with B cell maturation and promote disease progression.

circRNAs in CLL

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of small and mature $CD5^+$ B cells in blood, bone marrow and secondary lymphoid organs. CLL B-cells have a relatively low proliferation rate and high resistance to apoptosis (Kipps et al., 2017). The molecular classification of the disease relies on whether the neoplastic cells express a mutated or unmutated form of the immunoglobulin heavy chain variable region gene (*IGHV*), with the unmutated IGHV being a marker of poor prognosis and shorter survival. The variability in clinical behaviors reflects the underlying genetic heterogeneity (Kipps et al., 2017). To date, three main circRNAs have been studied in CLL: *circRPL15* (Wu et al., 2020), *circCBFB* (Xia et al., 2018), and *circ_0132266* (Wu et al., 2019).

CircRPL15 was evaluated as a potential biomarker for the diagnostic screening in plasma from patients with CLL, especially in cases without IGHV mutation (Wu et al., 2020). Upregulated *circRPL15* was proposed to sponge miR-146b-3p, thereby increasing RAF1 protein levels. As an effector of the proliferative RAS pathway, RAF1 could in turn phosphorylate and thereby activate MAPK (mitogen-activated protein kinase) signaling, promoting cell growth. In support of this paradigm, knockdown of *circRPL15* in human cell lines reduced the phosphorylation of mitogenic factors (Wu et al., 2020) and an earlier study by Wang et al. (2008) found that RAF1 was overexpressed in CLL.

After finding that *circCBFB*, derived from *CBFB* (core-binding factor subunit beta) pre-mRNA, was upregulated in untreated CLL cells from patients, Xia et al. (2018) proposed that it could serve as a prognostic and diagnostic marker in CLL patients. Mechanistically, *circCBFB* was found to activate the Wnt/ß-catenin pathway in human cell lines by binding miR-607 and thereby derepressing production of Frizzled (FZD3), a receptor for Wnt; the ensuing proliferative and anti-apoptotic phenotype could therefore be involved in CLL progression. Moreover, higher levels of *circCBFB* and *circRPL15* correlated with poor overall survival and shorter survival time.

Wu et al. (2019) proposed a tumor-suppressor role for *circ_0132266* in CLL based on the ability of *circ_0132266* to bind miR-337-3p in CLL cell lines. PML (promyelocytic leukemia protein), a broad regulator of gene expression and cell viability, is a key target of miR-337-3p. The authors linked the reduction in *circ_0132266* levels to the increased miR-337-3p levels and proposed that by sponging miR-337-3p, *circ_0132266* might be tumor-suppressive in CLL.

circRNAs in Lymphomas

Lymphomas originate from lymphocytes or their progenitor cells and tend to be localized in lymph nodes and the lymphatic system. Due to the high heterogeneity of lymphocytes, given the diverse lineages from which they arise, the stages of differentiation, and their specific functions, the classification of lymphomas is quite complex. Lymphomas are classified into Hodgkin and non-Hodgkin, the latter being more common; the non-Hodgkin's lymphoma can be divided into two major groups depending on the cell lineage: B-cell and T-cell lymphomas (Swerdlow et al., 2016).

In T-cell lymphoblastic lymphoma (T-LBL) cell lines, Deng et al. (2019) described a regulatory axis in which *circLAMP1* was proposed to sponge miR-615-5p, thereby increasing the levels of DDR2 (discoidin domain receptor tyrosine kinase 2). DDR2 encodes a member of the receptor tyrosine kinase (RTKs) protein family, is induced by collagen, and in turn activates signal transduction pathways involved in proliferation, extracellular matrix remodeling, wound repair, and tumor invasiveness. Gain- and loss-of function experiments supported a role for *circLAMP1* in promoting cell proliferation and repressing apoptosis in T-LBL cells.

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma worldwide (Li et al., 2018b). Morphologically, it is characterized by large B cells arranged in a diffuse pattern. RNA-seq analysis of DLBCL identified several genetic abnormalities and different mutation profiles. At present, the standard of care received by DLBCL patients is based on a combination of a monoclonal antibody (rituximab) and chemotherapy, a regimen called R-CHOP. The current 5-year overall survival rate is estimated around 60% (Li and Medeiros, 2018). *CircAPC*, downregulated in DLBCL cell lines and plasma from patients (Hu et al., 2019), originates from the backsplicing of the linear adenomatous polyposis coli (*APC*) gene, encoding a major regulator of cell proliferation. The tumor suppressor functions of the protein APC include regulation of cell adhesion, inhibition of Wnt/β-catenin pathway and control of cell cycle progression (Zhang and Shay, 2017). Importantly, DLBCL usually affects the gastrointestinal tract, where APC expression is most prominent (Li et al., 2018b). *CircAPC* was detected in both the cytoplasm and the nucleus of DLBCL cells (Hu et al., 2019). In the nucleus, *circAPC* bound the APC promoter and recruited DNA demethylase Ten-Eleven Translocation 1 (TET1), thereby reducing methylation and increasing *APC* transcription. In the cytoplasm, *circAPC* sequestered miR-888, enhancing APC translation and eventually inhibiting the proliferative Wnt/β-catenin pathway. In short, reduced levels of *circAPC* were proposed to promote DLBCL progression. Further analysis pointed to *circAPC* as a diagnostic and prognostic tool; for instance, low *circAPC* levels correlated with resistance to R-CHOP treatment, low International Prognostic Index (IPI) and advanced staging (formulated with the Ann-Harbor staging classification). Ectopic *circAPC* expression was shown to suppress DLBCL proliferation *in vitro* and *in vivo* in a mouse xenograft model.

ALCL (Anaplastic Large Cell Lymphoma) is a rare but highly aggressive T-cell non-Hodgkin lymphoma that originates from a chromosomal translocation that produces the fusion protein NPM1-ALK, a constitutively active tyrosine kinase (Fuchs et al., 2019). Babin et al. (2018) employed the CRISPR/Cas9 technology to generate the same translocation in different mouse and human cell lines and identified novel *f-circRNAs*, specifically *f-circRNA-mA* and *f-circRNA-hD* (collectively called *f-circRNA-NPM1-ALK*). This pool of circRNAs was also identified in some of the original tumors, thus representing a possible diagnostic marker for ALCL.

circRNAs in MM

Multiple myeloma (MM) is characterized by proliferation of immunoglobulin-secreting plasma cells within the bone marrow. It is the second most common hematological cancer, accounting for 10% of all blood malignancies (Walker et al., 2014), with a life expectancy ranging from a few months to >10 years and hampered by an extremely rate of metastasis and drug resistance (Palumbo et al., 2015). High-risk cytogenetic subgroups, including those with deletion of chromosome 17p or gain of chromosome 1q21, progress rapidly and are associated with shortened overall survival (Sherbenou et al., 2016).

Feng et al. (2019) found that *circ_0000190* was downregulated in bone marrow biopsies and peripheral blood derived from MM patients. The authors propose that the reduction in *circ_0000190* led to increased levels and function of miR-767-5p, which in turn lowered the levels of MAPK4, an inhibitor of MM progression. Analysis of 47 patients revealed that higher expression of *circ_0000190* was associated with longer progression-free disease and improved overall survival. In murine xenograft models, ectopic expression of *circ_0000190* reduced tumor progression by 60%, underscoring the potential therapeutic value of this circRNA. Interestingly, the tumor-suppressor role of *circ_0000190* was originally described in gastric cancer (Chen et al., 2017b).

CLOSING REMARKS AND CHALLENGES AHEAD

In this review, we have highlighted the progress made in identifying circRNAs implicated in hematological malignancies. Although many functions have been proposed for circRNAs (Panda et al., 2017b), most of the examples to-date underscore their ability to bind other molecules and sequester them away from miRNAs and RBPs. Through these specific interactions, circRNAs may influence the levels of proteins under the control of such miRNAs and RBPs. As uncovered in this review, many of these target proteins are implicated in controlling cell proliferation and survival. In the context of hematological diseases, dysregulation of RBPs and miRNAs has been linked to the initiation and progression of malignancy, chemotherapy resistance, and poor clinical outcome. To apply the emerging knowledge of circRNAs as potential interventions in the progression and treatment of leukemias and lymphomas, we have identified several challenges.

First, superior methods must be developed to identify and quantify circRNAs in all systems, including hematologic malignancies. More complete databases with comprehensive circRNA annotations are needed, particularly those that include information on tissue-specific circRNAs. In this regard, normalization of circRNA nomenclature will be particularly helpful to improve clarity across studies. Molecular methodologies must also improve to offer more sensitive and specific means of detecting circRNAs with diagnostic and prognostic value, given that they are often in quite low abundance.

Second, the field must adopt more rigorous methods of elucidating circRNA function and circRNA-interacting molecules. While the notion of circRNAs as "microRNA sponges" is certainly attractive, few studies have included the careful stoichiometric measurements and molecular biology interventions to demonstrate that a circRNA is indeed a sponge for an interacting microRNA. These careful analyses are particularly important, since many circRNAs exist in low copy numbers (often one or less per cell) while microRNAs often exist in hundreds or more copies per cell, so the sponging model may not be correct in every case.

Third, hematopoietic malignancies are particularly well suited for RNA-directed therapies such as antisense oligomers (ASOs), given their immediate access in the blood. However, the fact that the vast majority of circRNAs share most of their sequence with the parent linear RNA challenges these efforts. Improved bioinformatic and molecular approaches to optimize the targeting of the junction sequences of circRNAs will enable more accurate targeting of circRNAs for elimination in therapeutic settings. Conversely, for circRNAs with beneficial effects in hematopoietic malignancies, ectopic delivery methods could have therapeutic advantage; such delivery methods also await extensive development and optimization for use in the clinic.

In closing, we are gaining tools and knowledge of circRNAs implicated in hematologic cancers. The expansion and refinement of these tools and knowledge in the near future will enable the development of effective means of diagnosis, prognosis, and therapy directed at circRNAs in leukemias, lymphomas, and other hematologic malignancies.

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OP, MR, and MG wrote the paper.

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REVIEW
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Circular RNAs Are Regulators of Diverse Animal Transcriptomes: One Health Perspective

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Derived from linear (parental) precursor mRNA, circRNA are recycled exons and introns whose ends are ligated. By titrating microRNAs and RNA binding proteins, circRNA interconnect networks of competing endogenous RNAs. Without altering chromosomal DNA, circRNA regulates skeletal muscle development and proliferation, lactation, ovulation, brain development, and responses to infections and metabolic stress. This review integrates emerging knowledge of circRNA activity coming from genome-wide characterizations in many clades of animals. circRNA research addresses one of the main pillars of the One Health vision – to improve the health and productivity of food animals and generate translational knowledge in animal species.

Keywords: animal models, production animals, RNA biology, back-splicing, computational analysis, translational science, veterinary medicine, human health

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HISTORICAL PERSPECTIVE

Ubiquitous in Nature, Are RNA Circles Ancient Relics or Versatile Regulatory Molecules?

Linear polymers of DNA and RNA convey the flow of genetic information, whereas circularized forms of DNA and RNA serve regulatory roles in all domains of life. Given circularized DNA were identifiable before the advent of molecular biology and the current genomic-computational era, decades of study have established their significance.

The circularized DNA of mitochondria is necessary for energy generation, thereby governing growth, development and aging (Oldenburg and Bendich, 2015). Extrachromosomal circular DNA (EccDNA) are known to accumulate in the nuclei of healthy somatic cells (Møller et al., 2018). EccDNA structures encoding oncogenes accumulate in tumor cells and their abundant transcription drives oncogenesis (Von Hoff et al., 1992; Turner et al., 2017). Circular self-replicating plasmids and bacteriophages undergo autonomous rolling-circle replication and remodel host cell DNA through integration (Gilbert and Dressler, 1968; Dressler, 1970; Baas, 1985; Koonin and Ilyina, 1992; Ruiz-Masó et al., 2015; Wawrzyniak et al., 2017). DNA circles are significant in biological processes ranging from genome evolution to growth control and response to infection. This literature sets expectation circularized RNA (circRNA) are also highly significant.

CircRNA was first documented in viroids, infectious pathogens of higher plants (Sanger et al., 1976). The circular RNA structure was identified with biochemical evidence – viroid RNA was resistant to snake venom containing phosphodiesterase that degrades the phosphate on the 5′ end of linear molecules (Sanger et al., 1976). Evidence from phylogenetic studies posits viroid RNA replication is an ancient RNA relic of pre-cellular evolution (Diener, 1989). Viroids

generate circRNA by complementary base pairing between RNA motifs on the same strand and in association with host RNA polymerase II (Zhong et al., 2008).

Similar to viroids, the circRNA of hepatitis delta virus (HDV) undergoes recombination and is assisted by hepatitis delta antigen. HDV is a defective human pathogen that is dependent on hepatitis B virus for replication in liver. Because of its circRNA genome, HDV has been considered unique amongst all known animal viruses (Taylor, 2014). In archaea, circRNA biogenesis involves remnants rRNA, tRNA intron and Box C/D RNAs forming ribonucleoprotein with dimeric RNA ligase (Rnl3) (Becker et al., 2019). The function of circRNA and primordial RNA binding proteins (RBPs) set the stage for eukaryotic circRNA biogenesis involving spliceosome RNPs (more below).

Recently, two gamma herpesviruses, Epstein-Barr virus (Ungerleider et al., 2018) and Kaposi's sarcoma herpesvirus (Tagawa et al., 2018), were identified to generate circRNAs that are detectable in both the nucleus and cytoplasm of infected cells (Toptan et al., 2018). Levels of host circRNA decrease upon the viral infections, which may be due to reduced biogenesis, endonucleolytic decay by RNAse L (Li X. et al., 2017; Liu C. X. et al., 2019) or competition for host RBPs. Since host double-stranded (ds) RBPs are fundamental players in the host innate response to virus infection through binding of small viral RNA, virus-encoded circRNA may work to antagonize antiviral activity. Emerging evidence is viral circRNA prevent activation of protein kinase R, setting the stage to elucidate antagonism of antiviral response by exogenous circRNA (Chen et al., 2017; Liu C. X. et al., 2019).

A growing list of dsRBPs and splicing factors have been shown to mediate exon circularization and potentially may undergo sequestration and sorting by exogenous circRNA (for a recent review, see Huang et al., 2020). Future quantitative studies are speculated to reveal circRNAs are gene products of many animal viruses, which would raise the possibility for a novel diagnostic approach to detect these viruses. Significant primary sequence conservation has been discovered between circRNA of humans, old and new world monkeys, food animals, companion animals and murine species (Wang et al., 2014; Abdelmohsen et al., 2015; Chen et al., 2015; Ivanov et al., 2015; Venø et al., 2015; Zhang et al., 2016; Ouyang et al., 2018b; Qiu et al., 2018; Shangguan et al., 2018; Zhang F. et al., 2018). Sequence identity between circRNAs of mammals and flies indicate the fundamental steps in their biogenesis originated early in the evolution of life (Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2013). No doubt, circRNA are versatile regulatory molecules and their ubiquity in nature foretells important functions are yet-to-be discovered.

BIOGENESIS AND FUNCTION OF NASCENT CIRCULAR RNAs

Making the Most of Precursor mRNA Sequences

Eukaryotic circRNAs are generated during the co-transcriptional splicing of precursor mRNA (pre-mRNA) (**Figure 1**). Mature mRNA are decorated by 7-methyl guanosine (m7G) at the 5′ terminus and poly-adenosine residues at the 3′ terminus, modifications that are incompatible with covalent circularization of the linear RNA. The commitment to circRNA biogenesis involves poorly appreciated activity of spliceosome RNPs that is disengaged from canonical pre-mRNA processing. Functionally, the process has been characterized as mis-splicing or back-splicing (Jeck et al., 2013; Memczak et al., 2013; Yu and Kuo, 2019). The conserved patterns of back-splicing events reported in RNA from mouse, pig and human tissues supports the hypothesis that circRNA biogenesis is under positive selection (Venø et al., 2015; Yu and Kuo, 2019).

In canonical splicing of pre-mRNA, spliceosomes catalyze the excision of introns and ligation of consecutive exons (**Figure 1**). Alternative splicing engenders exon skipping to produce alternative open reading frames that encode unique protein isoforms (**Figure 1**). Spliceosomes activate 5′ splice site (ss) and 3′ ss for cleavage and ligation, while the intervening sequences between exons (introns) form lariat structures, which were long considered waste byproducts of linear splicing.

Non-canonical splicing has been characterized by delayed or aberrant spliceosome activity (Dvinge, 2018). Ligation of a 5′ ss with an upstream 3′ ss generates a covalently closed RNA molecule (**Figure 2**). The advanced computational analysis of RNAseq data has elucidated joints between 3′ and 5′ ss of non-consecutive exons and the joining of intron sequences at the branchpoint on a genome-wide scale (described in the next section). Computational analysis together with specialized protocols to enrich circRNAs in RNAseq samples, has fueled the explosion of circRNA research in the past 5 years. NCBI reported ∼5,000 new publications in the term 2015–2020 compared with 5,000 in the prior 50 years (1964–2014) (Pubmed July 20, 2020). As described in following sections, circRNA activity significantly expands the functional capacity of pre-mRNA, just as the protein coding capacity is amplified by alternative splicing.

A Prelude to Discovering a Full Repertoire of Functionalities: Nuclear Interactions Regulate the Fate of Natural circRNA

Although a majority of circRNA derive from protein-coding RNAs, these molecules are considered long non-coding RNAs (lncRNAs) (Yu and Kuo, 2019). Despite being considered non-coding RNAs, recent work has shown some circRNAs, once exported to the cytoplasm, can indeed be translated in a 5′-cap-independent manner (Pamudurti et al., 2017; Yang et al., 2017). While nuclear export mechanisms of mRNA are well-characterized, little is known about how circRNAs are exported from the nucleus to the cytoplasm as they lack many of the common signals defining the export pathway used for export of mRNAs and other RNA species. Recent research suggests that the length of mature circRNA is measured in both human and *Drosophila* cells and may play an important role (Huang C. et al., 2018; Li Z. et al., 2019). It was shown that human DExH/D-box helicase UAP56 (DDX39B) is required for efficient nuclear export of long circRNAs (>1200-nt), whereas URH49

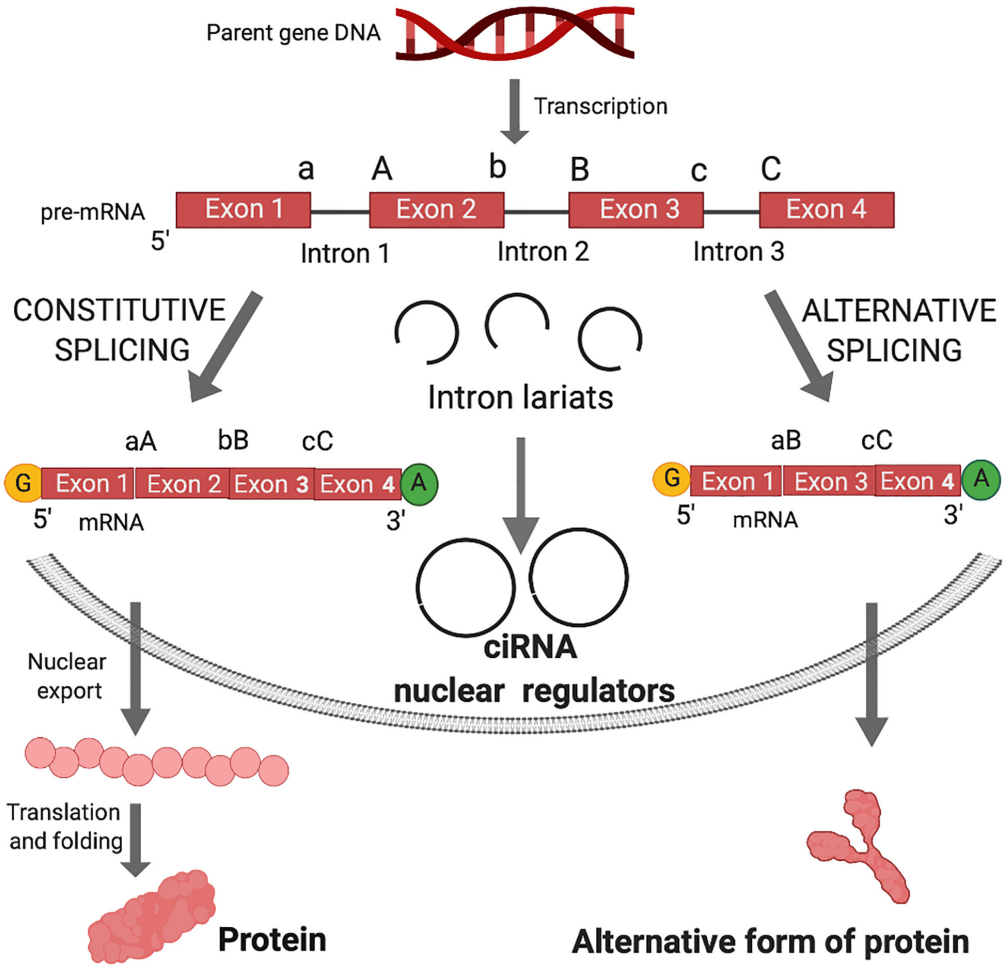

FIGURE 1 | Canonical splicing and the biogenesis of circular intronic RNA (ciRNA) and functional outcomes. Shortly after gene transcription, the nascent RNA (pre-mRNA) is processed by the addition of the 7-methyl guanosine cap (yellow G) and polyadenylate tail (green A). In the linear splicing process, introns are excised and exons are joined in a linear manner to form mRNA for translation into proteins. Exons are joined by ligation of 3′ splice sites (a, b, or c) and 5′ splice sites (A, B, or C). The intron sequences are excised from the pre-mRNA creating intron lariats. Lariats can be destroyed in the process of debranching after excision from the pre-mRNA. In case of debranching failure, ligation of lariats can generate circular intronic RNA (ciRNA). ciRNA are retained in the nucleus and can act as nuclear regulators of parental gene transcription. Alternative splicing occurs when an exon in the pre-mRNA is skipped, resulting in diverse mRNA isoforms that may be either degraded via nonsense mediated decay or undergo nuclear transport, translation and folding into an alternative form of protein.

(DDX39A) controls the localization of short circRNAs (<400-nt). Similar length-dependent export was observed in *Drosophila* and was mediated by Hel25E, a homolog to DDX39A and B, demonstrating interspecies conservation of this mechanism.

The deposition of nuclear factors to pre-mRNA is necessary for nucleo-cytoplasmic transport and subsequent commitment to decay, storage or efficient translation to protein (Le Hir et al., 2001; Singh et al., 2020). circRNAs that are derived from introns (circular intronic RNAs, ciRNAs), remain in the nucleus and may regulate transcription of parental genes (Zhang et al., 2013; Zang et al., 2020) (**Figure 1**). Those derived from exons undergo nucleocytoplasmic transport and post-transcriptionally regulate gene expression (**Figure 2**).

circRNA serve as microRNA (miR) decoys and harbor copies of miR response elements (MREs). Unlike mRNA, circRNAs bound to miR are completely resistant to miR-mediated target destabilization (Hansen et al., 2013). One miR may have hundreds of binding sites in mRNA- and circRNA-targets and the interplay between the circRNA, miR and target mRNA generates competing endogenous (ce) RNA crosstalk. Connecting the patterns of ceRNA crosstalk draws the ceRNA regulatory network (Lan et al., 2019). For a comprehensive review on circRNA-miR interactions, please refer to Chandra (2018).

Mounting evidence posits circRNA and miR function independently to significantly regulate posttranscriptional gene expression. The circRNA CDR1as (antisense to the cerebellar degeneration-related protein 1 transcript, also termed as ciRS-7), contains 70 MRE, and ciRS-7 binding does not activate miR-mediated destabilization (Hansen et al., 2013). The expression of CDR1as is upregulated by the tissue-specific transcription

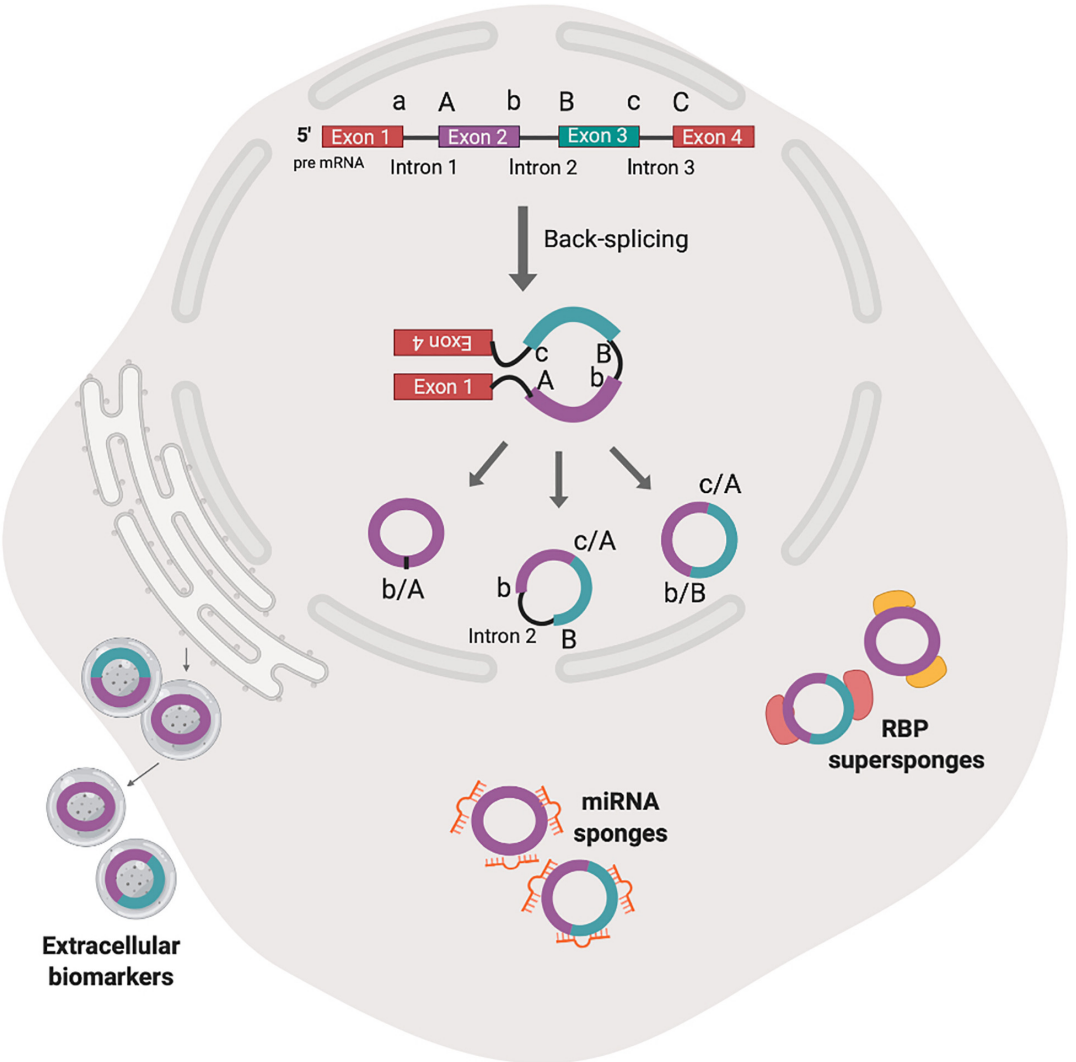

FIGURE 2 | The biogenesis by non-canonical splicing and activities of animal circRNAs. Back-splicing model shown here is hypothesized to be the predominant mechanism of animal circRNA biogenesis. The non-linear combination of 3′ splice donor (a, b) and proximal 5′ splice acceptor (A, B) generates single exon circRNA (b/A), backspliced exons (c/A, b/B) or exons and introns (c/A, b intron 2 B). After nuclear export, the circRNA have been shown to accumulate in exosomes and may serve as extracellular biomarkers. Cytosolic circRNAs have been shown to interact with cognate microRNA (miR) and RNA binding proteins (RBPs) (sponge) in competition with miR response element on target mRNAs or another protein partner.

factor *MyoD*, which has a critical role in muscle differentiation. By sequestering miR-7 and Argonaute, ciRS-7 was shown to derepresss miR-7 target mRNAs and activate embryonic muscle development in goat, e.g., insulin like growth factor 1 receptor (Li L. et al., 2019).

circRNAs also serve as protein decoys to regulate biological processes (**Figure 2**). For example, circ-Foxo3 can repress cell cycle progression by binding to G1 to S phase transition-related *CDK2* and *p21* (Zhang Z. et al., 2018). Also, circ-Foxo3 can retain the stress-associated proteins FAK and HIF1a in the cytoplasm to block their nuclear function and promote senescence of myocardial cells (Zang et al., 2020). The activity of circRNA ZNF609 in mouse and human myoblasts was shown to require nuclear protein binding and loading to polyribosomes (Legnini et al., 2017). For more on the association of circRNA with RBP and translation, please refer to Zang et al. (2020).

Emerging evidence indicates circRNAs are important during major biological processes and disease states, such as growth and development (Song et al., 2019); differential expression; neurodegenerative disease (Wang et al., 2018; Dube et al., 2019); malignancy (Li et al., 2015; Zhang Z. et al., 2018; Dube et al., 2019; Song et al., 2019). circRNA molecules are abundant in blood and saliva and are present in exosomes – secreted vesicles that serve as extracellular transport vehicles in the circulation (**Figure 2**) (Memczak et al., 2015). The confirmed prevalence and stability of circRNAs in body fluids and their spatial-temporal distribution indicates potential utility as diagnostic and prognostic biomarkers for diseases (Li et al., 2015).

Despite distinct patterns of tissue-specific expression (more below), some circRNA exhibit no difference in the abundance in plasma or serum between patients and healthy controls (Zhang Z. et al., 2018). More research is necessary to elucidate functions of circRNA before they find their way in clinical applied science. But, emerging evidence posits translational value of circRNAs to veterinary and biomedical sciences.

CONTEMPORARY METHODOLOGY FOR DETECTION OF, AND OBSTACLES TO, CHARACTERIZING circRNA

Experimental Methods

The expression of circRNAs was an almost completely uncharacterized component of eukaryotic gene expression until sample preparation protocols and deep sequencing were advanced (Lasda and Parker, 2019). Whereas enrichment of mRNAs by selection of poly-adenosine (polyA) RNA species has been a default step in the process of library preparation, this step depletes circRNAs (Barrett and Salzman, 2016). Primer selection is another common variable in RNA-seq protocols. The most commonly used primer, oligo deoxythymine (dT) hybridizes to the polyA residues concentrated at the 3′ terminus of most eukaryotic mRNAs. Since circRNAs lack the 3′ polyA tail, use of oligo (dT) primer imposes bias against detection of circRNA. Instead, random hexamer primers and paired-end sequencing reactions are prudent approaches to detect circRNAs in total RNA preparations (Panda and Gorospe, 2018).

Protocols to enrich circRNAs in RNA samples have been established. In transcriptome analysis, depletion of rRNA is desirable since 90% of cellular RNA preparations are ribosomal RNA and only 5% is non-coding RNA or mRNA, respectively (Salzman et al., 2012, 2013; Jeck and Sharpless, 2014; Barrett and Salzman, 2016). Following rRNA depletion, circRNAs are not depleted. The use of RNA exonucleases to digest linear RNA isoforms is now widely used for circRNA enrichment from a total RNA preparation (Du et al., 2017; Verduci et al., 2019). Digestion of mRNA samples with RNAse R, a processive exonuclease that digests linear RNA, has been widely adopted to deplete samples of lncRNA, mRNA and other linear RNA species prior to library preparation, a technique also known as CircleSeq (Jeck et al., 2013). In 34 paired samples of only rRNA being depleted (rRNA-) and a combination of rRNA depleted with RNAse R treatment (rRNA−/RNAseR+), most of circRNAs (50–80% of highly expressed circRNAs, respectively) detected in the rRNA- dataset were validated in the rRNA−/RNAseR+ dataset as well (Zhang et al., 2020). This shows that while CircleSeq technique certainly enriches circular species, it is not a mandatory step for successful detection of circRNAs in the sample. However, limitations of both approaches have been recognized. Zhang et al. (2020) evaluated RNA-seq datasets from four species (human, fly, worm, mouse) and observed high variance in the efficiency of rRNA sequence depletion. This discrepancy was attributed to the limited species specificity of current RiboMinus transcriptome isolation kits available. Caveats in the RNA sample processing significantly alter circRNA quantification and quality controls are critical to measure reproducibility within replicate sample analysis and between studies. CircleSeq, for instance, has the capacity to generate deep coverage of circular and lariat products, but this approach can introduce an unintentional bias. The digestion step enriches circRNA species that are RNAse R-resistant. Drawbacks to the use of RNAse R are that a single nicking event unleashes exonuclease sensitivity, and the possibility of contaminating RNA endonucleases (Jeck and Sharpless, 2014).

circRNA isolation methodology with high confidence standards was described by Panda et al. (2017). While RNAse R digests linear transcripts, activity is limited on fragmented linear RNA and highly structured species such as transfer RNA and small nuclear RNA and small non-coding RNA. Therefore, an innovative method termed RPAD (RNase R treatment followed by Polyadenylation and polyA+ RNA Depletion) prepares circRNA of the highest purity by a two-step elimination of linear transcripts in the sample. Briefly, the procedure is to treat the total RNA sample with RNAse R to digest linear transcripts, followed by polyadenylate polymerase, which labels undepleted linear molecules bearing free 3′-OH ends. Last is incubation with oligo-dT to deplete residual polyA-containing RNAs from the circRNA preparation.

As a group, circRNA generally are less abundant than their linear RNA counterparts. However, with the advancement of RNA library preparation, the reliability of RNA-seq technology and new computational strategies, results of quantitative investigations reveal certain human and chicken circular transcripts are more abundant than their homologous mRNAs (Salzman et al., 2012; Ji et al., 2019).

Bioinformatic Tools

Numerous algorithms have been developed to detect junction-spanning sequences in deep-sequencing data set (**Table 1**). Two approaches discover a back-splicing junction site specific for circRNAs: the pseudo-reference approach and the segmented-based computational analysis of non-linear exons and introns. All pipelines specify an external aligner, such as Bowtie or HiSat2. The aligner begins by filtering out reads that contiguously align to the genome and/or to the transcriptome (Gao and Zhao, 2018). Subsequent processing of the unaligned reads identifies those that align to a back-splice junction, which is identified by the algorithm employing either pseudo-reference or a split-alignment base. Multiple algorithm specific filters, including read counts, multiple sample detection and statistical scores, being the most common, are applied to limit the rate of false positive reads.

The first group of programs is a candidate-based approach (pseudo-reference), where a circRNA reference set of all possible combinations of scrambled exon–exon junctions is built from a gene annotation repository. The back-splice junction reads are aligned contiguously to the computational reference set by algorithms such as KNIFE and NCLscan (Li and Han, 2019) (**Table 1**). The efficiency of these algorithms to identify novel back-splice junctions is lower than algorithms employing the fragmented-based criteria.

The second group of programs, fragmented-based, is also referred to as segment- or split-alignment-based and relies on

TABLE 1 | Overview of the most common algorithms used to detect circular RNAs from RNA sequencing data.

Software	Mapper	Approach	Link and references
Pseudo-reference algorithms			
KNIFE	Bowtie Bowtie 2	Quantifies splicing events at both annotated and unannotated exon boundaries	Szabo et al., 2015
NCLscan	BWA Novoalign	Detects non-colinear transcripts: circRNA; *trans*-splicing events; fusion transcripts	Chuang et al., 2016
Segmented-based algorithms			
MapSplice	Bowtie	Finds splice junctions using approximate sequence similarity and without dependence on features or locations of the splice sites	Wang et al., 2010
Segemehl	*Per se*	Identifies fusion reads by implementing a matching strategy based on enhanced suffix arrays	Hoffmann et al., 2014
find_circ	Bowtie2	Performs *de novo* detection of back-splice junction	Venø et al., 2015
circRNA_finder	STAR	Predicts circRNA that are within 100 bases of splice sites	Hansen et al., 2016
CIRI	BWA-MEM	Uses maximum likelihood estimate to detect back-splice junctions using multiple-seed matching	Zhang et al., 2016
CircPRO	BWA-MEM, Bowtie2	Identifies circRNA with potential to be protein-coding or non-coding circRNAs	Meng et al., 2017
CIRC explorer2	TopHat/STAR	An annotating tool that parses mapping information from other aligners	Zhang F. et al., 2018

the identification of back-splicing junctions from the mapping information provided by aligning reads to the reference genome or transcriptome (Gao and Zhao, 2018; Li and Han, 2019) (**Table 1**). This group of programs has been used more frequently and the results provided have been comparable between different algorithms. CIRI, find_circ, circExplorer, circRNAseq and MapSplice have reproducibly identified circRNAs using reference exonic, intronic and intergenic sequences that have been deposited in specialized databases: circBase and CIRCpedia, among others (Aghaee-Bakhtiari, 2018) (**Table 2**). These algorithms provide versatility to input either single-end or paired-end sequencing data; the latter is considered to enhance the sensitivity and reliability of the data analysis. Consensus is needed to establish widely utilized quality control standards that measure the accuracy of circRNA expression levels to improve the validity of data comparison between studies (Szabo et al., 2017). The computational processing of circRNAs from populations of RNA sequences presents an opportunity to apply machine learning methods to large datasets.

Once detected by the algorithm, circRNAs in published data are deposited into one or more databases (**Table 2**). Some databases catalog results based on species of origin, tissue location and disease-association, whereas others focus in-depth on an assigned function, protein coding capacities and the scope of competing endogenous networks (**Table 2**). Questions we pose are: What is the specific threshold or the criteria for candidate circRNA results to be published in sanctioned databases? What are the research community standards for rigor and reproducibility and for the assignment of Digital Object Identifiers to ensure data remain discoverable, freely reusable and citable (Martone, 2014).

Computational reproducibility has become an integral issue in scientific research, particularly due to the rapid advancement of computer environments (Boettiger, 2015). Several initiatives aim to enhance science data reproducibility and data literacy, e.g., The Carpentries, Docker, and Snake Make. Data Carpentry provides hands-on workshops to equip scientists for successful big-data analysis, e.g., scripting and data management (Pugachev, 2019). Snake Make is a Python-based framework for formalizing data analysis that provides rules-guided workflow and portable execution environment (Köster and Rahmann, 2012). Docker Container shares code and dependencies necessary for smooth reproducibility of datasets made available with scientific publications (Boettiger, 2015). These tools are useful beyond the scope of circRNAs into all aspects of Omics and other research requiring extensive computational processing.

CIRCULAR RNAs IN ANIMAL HEALTH AND DISEASE

Emerging data indicate circRNA connect feed-back loops in all life processes, from embryonic development to growth, differentiation and response to infection. Aberration of ceRNA networks is consequential to infectious disease, neoplastic transformation, developmental and degenerative disease. In the following sections, perspectives coming from circRNA investigations in livestock, companion animals and wild species are discussed and considered in relation to food production and biomedical science. We emphasize emerging issues and promising avenues to utilize circRNA to improve health of people and non-rodent animal species.

Production Animals – Animal Protein Sourced From Meat, Eggs, and Dairy

Stunning advances have been made in the genetics of food-producing animals. Selection for certain production traits has created a very different livestock compared to the historical primitive breeds. For decades, breeding efforts have been undertaken to select for higher milk yield in dairy cows, faster muscle growth in cattle or larger eggs in poultry. With that in mind, genomic information alone explains only a part of the phenotypic variance in traits (Ibeagha-Awemu and Zhao, 2015).

TABLE 2 | Overview of publicly available circular RNA databases.

Name/Species	Description	Link and references
Databases containing solely human circular RNAs		
Circ2Traits	Categorizes circRNA by potential involvement in diseases and potential interaction with disease related miRs	Ghosal et al., 2013
CircInteractome	Predicts and maps binding sites for RNA binding proteins and miRs on reported circRNAs	Dudekula et al., 2016
CircNet	First database to collect tissue-specific circRNA profiles and proposed circRNA-miR regulatory networks	Liu et al., 2016
circRNADb	Collects annotated protein-coding human circRNAs	Chen et al., 2016
CSCD	Collects cancer-related circRNAs	Xia et al., 2018
Databases containing circRNAs from multiple species		
StarBase/ human, mouse, worm	Decodes various interaction networks, a component of starBase	Li et al., 2014
circBase/ human, mouse, fly, worm, fish	Compiles publicly available circRNA datasets and the python scripts to process RNA seq data for discovery of circRNAs	Glažar et al., 2014
CIRCpedia_v2/ human, mouse, rat, zebrafish, fly, worm	Allows users to search circRNAs with expression characteristics in various physiological and diseased tissues and cell types and provides conservation analysis of circRNAs between humans and mice	Dong et al., 2018
CircAtlas/ human, monkey, mouse	Collects expression patterns, genomic features, functional annotations and conservation of circ RNAs derived from 44 normal tissues	Ji et al., 2019
CircFunBase/ human, cattle, chicken, fly, monkey, mouse, pig, rat, rabbit, plants	Visualizes circRNA-miR interaction networks and the genome context of circRNAs	Meng et al., 2019
Database containing plant circular RNAs		
PlantcircBase	Collects circRNAs from plant RNA seq data and predicts miR-mRNA networks	Chu et al., 2017

Non-coding RNAs serve epigenetic mechanisms, in addition to DNA methylation, histone tail modification and chromatin remodeling, that have the ability to modify phenotype without altering chromosomal DNA (Waddington, 2012). Epigenetic modifications can be altered by external (climate, pathogens, nutrition) or internal (hormonal cues) environmental factors and have the ability to change gene expression and promote emergence of specific phenotypes in individuals. Understanding the epigenetic determinants of animal diseases and their role in pathogenesis, control, treatment and eradication represents a major opportunity to apply epigenetic markers for further improvement of animal productivity (Waddington, 2012). Skeletal muscle growth and differentiation (Luo et al., 2013; Nie et al., 2015), milk production (Yang et al., 2018) and egg laying (Adetula et al., 2018) are precisely regulated by hormonal and developmental cues with circRNA prominently serving as a rheostat to balance mRNA expression with non-coding RNA regulation.

Poultry – Meat

The genome-wide identification and function analysis of circRNAs in chicken skeletal muscle were characterized over the stages of embryonic development (Ouyang et al., 2018b). Thirteen-thousand circRNAs were identified in total RNA preparations of leg muscles of six female Xinghua chickens during day 11 (E11) and 16 (E16) of embryonic development and the 1st day post hatch (P1). circRNA were most abundant on day E16 followed by P1 (**Table 3**). Analysis of differentially expressed circRNA (DEcircRNA) reveals that not only do the circRNA accumulate over time, emblematic of the aging process (Westholm et al., 2015), but parental genes of the DEcircRNA are involved in the development of muscle cell structure and differentiation and activity.

By screening the circRNA sequences for MRE content, two miRs associated with skeletal muscle development and differentiation of muscle cells were identified: miR-206 and miR-1a. The linear *RBFOX* mRNA is template for the translation of RBFOX-splicing factors that are essential for the maintenance of skeletal muscle mass and proteostasis (Singh et al., 2018). Two circRNA isoforms derived from the *RBFOX* mRNA were identified that have MRE for miR-206 and miR-1a. The sequestration of miR-206 and/or miR-1a by RBFOX2 circRNA has the potential to downregulate the RBFOX-splicing factors. This would tilt the dynamic balance between muscle protein synthesis and tightly controlled protein degradation that maintains muscle mass. Results also revealed exonic circSVIL derived from the supervilin gene involved in myogenesis to be the most abundant and differentially expressed circRNA in all three developmental stages in the Xinghua chicken.

In their second study, the authors focused on molecular and biochemical experiments to explore in depth properties of circSVIL (Ouyang et al., 2018a). It was found to harbor binding sites for miR-203, a miR targeting *c-JUN* and *MEF2C* genes involved in muscle differentiation and proliferation in myoblasts (Lu et al., 2017). Expression of circSVI*L* increased from E11 to

TABLE 3 | Summary of variables in studies of circular RNAs in non-rodent animal models.

Tissue	Total RNA sample		Software	Condition/disease involvement	Year published
	rRNA-depleted	circRNA enriched			
***Gallus gallus* (Chicken)**					
Skeletal muscle	×	×	CIRI	Embryonic muscle development	Ouyang et al., 2018a,b
Skeletal muscle	×	×	N/A	Embryonic muscle development	Chen et al., 2019
Liver	×	×	find_circ	Avian leukosis virus pathogenesis	Zhang et al., 2017
Spleen	×		CIRI	Avian leukosis virus pathogenesis	Qiu et al., 2018
***Sus scrofa* (Pig)**					
Brain	×		find_circ	Embryonic brain development	Venø et al., 2015
Heart	×		find_circ	Postnatal development	Liang et al., 2017
Liver					
Spleen					
Lung					
Kidney					
Ovarium					
Testis					
Muscle					
Fat					
Skeletal muscle	×	×	CIRCexplorer2	Embryonic muscle development	Hong et al., 2019
Mammary gland	×		CIRCexplorer2	Impact of heat stress on milk production	Sun et al., 2020
***Bos taurus* (Cow)**					
Mammary gland	×		CIRI	Casein content in milk	Zhang et al., 2016
Skeletal muscle	×	×	N/A	Prenatal and postnatal muscle differentiation	Li et al., 2018
***Ovis aries* (Sheep)**					
Skeletal muscle	×	×	find_circ	Prenatal and postnatal muscle differentiation	Li C. et al., 2017
***Canis familiaris* (Dog)**					
Heart	×		find_circ	Rapid atrial pacing model of atrial fibrillation	Shangguan et al., 2018
***Oryctolagus cuniculus* (Rabbit)**					
Carotid artery	×		CIRCexplorer2	Atherosclerosis	Zhang F. et al., 2018
***Macaca mulatta* (Rhesus macaque)**					
Skeletal muscle	×	×	CIRCexplorer	*Aging*	Abdelmohsen et al., 2015

E14, suggesting it is active in muscle differentiation during late stage embryonic development. The high expression levels during late embryo development correspond to previously documented spatial-temporal patterns of circRNAs in human, fly and pig (Salzman et al., 2013; Venø et al., 2015; Westholm et al., 2015).

A most recent study by Ouyang et al. (2018b) and Chen et al. (2019) focused on experimental validation of their previous RNA sequencing data, where they observed *HIPK3* gene produced eleven circular isoforms. The qRT-PCR analysis confirmed circHIPK3 expression level in E16 was significantly higher than at E11 and P1. This circRNA was shown to bind miR miR-30a-3p and inhibit myoblast proliferation by targeting the *MEF2C* gene required for maintaining the differentiated state of muscle cells. By contrast, circHIPK3 was shown to promote proliferation and differentiation of chicken myoblasts. These results reaffirm the hypothesis circRNAs competitively bind miRs, upregulating targeted linear mRNA. Interestingly, both miR-30a-3p and circHIPK3 followed the same expression trend – the levels decreased sharply in the first 2 days the cells were cultured in the differentiation media and then increased to a steady state in the differentiated cells. Further studies are warranted to map these ceRNA networks and document other miRs regulated through these circRNAs.

As the US is the world's largest poultry meat producer and second largest poultry meat exporter, scientific knowledge of circRNAs regulating biological processes would be of practical value for US poultry producers nationwide. Given the significant progress that was made in the genetics of food-producing animals, more research into epigenetic activity and potential application of non-coding RNA technology has yet unrealized potential.

Swine – Meat

Similar to the observations in poultry (Ouyang et al., 2018a), an abundance of circRNA have been discovered in total RNA preparation from muscles of domestic pig (Liang et al., 2017). In skeletal muscle, circRNAs were differentially expressed in age matched controls between day 0, 30, and 240. Comparison of circRNA profiles at day 0 and day 30 implicated distinct temporal profiling related to postnatal growth and muscle development. CircRNAs generated from transcripts encoding

proteins associated with glycosaminoglycan metabolism or regulation of calcium channels were differentially expressed in the day 30 to day 240 comparison. The most abundant circRNAs originated from transcripts related to muscle hypertrophy, including the *myosin* gene family.

While Liang et al. (2017) profiled circRNAs in the postnatal pork muscle tissue, Hong et al. (2019) analyzed circRNA expression profiles during embryonic skeletal muscle development in Duroc pigs. Their analysis used RNAse R-enriched samples harvested at 33, 65, and 90 days before birth (Hong et al., 2019). Significantly fewer circRNAs were found to be expressed on day 90 when compared to the other two time points, an interesting find considering the main muscle fiber development in pigs occur at 33 and 65 days post-coitus, indicating circRNA may have crucial functions in the initiation stage of skeletal muscle development. When exploring the parental sequences of the circRNA, regardless of the time point, they identified several abundant circular candidates derive from *myosin* gene family, similar to the observations made by Liang et al. (2017) in postnatal muscle tissues. They constructed circRNA-miR-mRNA networks between several key genes in myogenesis and circRNAs that revealed *PITX2* and *FGF2* are regulated by multiple circRNAs. These findings resonate with previous results that translation control of myosin RNA determines the quality of pork by regulating total fiber number, intramuscular fat content, water holding capacity and meat color in Berkshire pigs, a porcine breed known for its outstanding meat quality (Lim et al., 2015). The utility of circRNAs in regulating *myosin* gene expression warrants more in-depth investigations to develop technologies useful to the porcine meat industry. Another significant impact of the study of porcine embryonic muscle tissue was the identification of over 7,000 circRNAs, which at the time, was far more that the entire existing database of porcine circRNAs. This important advance points out the value of using RNAse R treatment prior to RNA sequencing to enrich circular species.

The authors of a more recent study decided to look into the circRNA profiles in mammary gland of lactating sows under heat stress (Sun et al., 2020), since it was reported that circRNAs are expressed in bovine mammary glands and could regulate milk content (Zhang et al., 2016). Even though swine milk is not a primary product for human consumption, quality and yield of milk in lactating sows plays an important role for feeding viable offspring raised for meat. Several previous reports suggested a potential direct correlation between environmental temperature and milk yield (Black et al., 1993; West, 2003; Liu J. et al., 2019). Heat-stressed lactating sows reduce their feed intake and their declined milk production does negatively affect piglet growth and development during lactation. Thermal stress is a common occurrence in high-yielding swine and identifying approaches to manage milk production is important for the sustainable and profitable porcine industry.

RNA sequencing analysis by Sun et al. (2020) revealed heat stress also significantly decreased the levels of casein family genes, *CSN1S1, CSN1S2, CSN3* in swine. The analysis of circRNA transcriptomes between non-heat-stressed and heat-stressed sows revealed 50 DE circRNAs between the groups. By performing Pearson correlation analysis between DE circRNAs and DE mRNAs, authors identified significant interactions between DE circRNAs and four lactation-related coding genes (*CSN1S1, CSN1S2, CSN3, WAP*) that were annotated by GO enrichment analyses. One circRNA, circCSN1S1_2, specifically stood out and was positively associated with the expression of the *CSN1S1*, *CSN1S2*, *CSN3*, and *WAP* genes. Furthermore, the MRE identified in circCSN1S1_2 were predicted to competitively bind miR-204 to increase expression of parental gene, *CSN1S1*. These trends were similar to observations in dairy cows (more below) (Zhang et al., 2016).

Poultry – Eggs

Consistent egg production is a characteristic of healthy hens and depends on hormonal and developmental regulation. Infection is the major concern to avoid in the production of high quality and quantity of poultry meat and eggs. Avian leukosis virus (ALV) infection heightens mortality of hens, reduces egg laying performance and diminishes the quality of eggs by reducing size and increasing fragility of the shell (Zhang et al., 2017; Qiu et al., 2018). The retrovirus infection is a major pathogen of both broilers and layer-type chickens and leads to enormous economic losses in the developing world and global poultry industry (Zhang et al., 2017).

ALV strain J (ALV-J) was first isolated from commercial broilers in 1988 in the United Kingdom and has since spread to other countries (Payne et al., 1991). ALV-J was found to be associated with myeloid leukosis in meat-type and layer-type chickens (Payne et al., 1991; Fadly and Smith, 1999; Cheng et al., 2010). As a result of strict eradication programs, ALV-J reportedly has been eliminated from breeding flocks, but ALV still remains an issue in broiler flocks (Li et al., 2016). Even though the myeloid neoplasm progresses slowly in commercial poultry, the virus infection delays growth and enhances susceptibility to secondary infections. Considering that there is no vaccine for ALV yet, there is strong incentive to identify efficient strategies to promote resistance to ALV-J-induced tumor formation and to breed chickens naturally resistant to this pathogen. The potential to use circRNA as predictors of resistance to the viral infection or as biomarkers of ALV pathogenesis has been posited in recent studies.

Chickens vary in susceptibility or resistance to ALV infection through activity of divergent receptors (Plachý et al., 2017) and cell-endogenous miRs (Li et al., 2012). Four differentially expressed miRs with MREs in nearly 500 mRNAs were found significantly enriched in liver tumor tissues extracted from ALV-J positive chickens (Wang et al., 2013). The predicted target genes were found to be involved in tumorigenesis-related pathways, MAPK signaling pathway and Wnt signaling pathway. Zhang et al. (2017) postulated circRNAs are protective against ALV-J infection and tumorigenesis in chickens. To evaluate endogenous circRNAs as initiators of protective immune effects, circRNA sequencing on total RNA preparations was performed.

circRNA sequences were obtained from three liver tissue samples of ALV-J resistant chicken and three liver tissues of ALV-J susceptible chicken. The results identified 1,800 circRNAs. Thirty-two circRNA were differentially expressed, with 12

upregulated in ALV-J resistant chickens. Gene ontology identified the functionality of the parental transcripts in upregulated circRNAs to be immune-related pathways, such as antigen receptor signaling and B cell activation, which suggested involvement of the circRNA in antiviral immunity. Also, some of the predicted miR target genes, such as *eIF4E* and *PI3K*, are known to positively regulate the mTOR pathway, thus activating protein synthesis. mTOR activity is essential for cell growth and its dysregulation is linked to uncontrolled cell proliferation in cancer. Therefore, circRNA attenuation of mTOR activity by sequestering miRs was posited to be tumor-suppressive (Zhang et al., 2017).

Qiu et al. (2018) sequenced total RNA isolated from spleen of 20-weeks-old ALV-positive and ALV-negative chicken characterized as black-bone silky fowls. They reported the apparent loss of approximately 30% of circRNAs in ALV-infected chickens, aligning with the reported downregulation of circRNA in response to viral pathogens (Li X. et al., 2017; Liu C. X. et al., 2019). They observed correlation between genes deriving from differentially expressed circRNA, the parental mRNAs and complementary miRs, and the generalized upregulation of immune-related genes. By constructing ceRNA networks, authors speculated circRNAs contribute to ALV-mediated tumorigenesis. While the relationship between circRNAs and ALV pathogenesis has been based on computational predictions that remain to be validated experimentally, circRNAs have also been postulated to mediate bacterial and viral diseases in humans (Haque and Harries, 2017; Huang et al., 2017; Tagawa et al., 2018). Taken together, these findings provide inferences circRNAs regulate disease initiation and progression in poultry.

Cattle

Total RNA was isolated from rear quarters of the mammary glands of four lactating Holstein cows at a dairy farm during the lactation cycle at 90- and 250-days post-partum (Zhang et al., 2016). Three-thousand genes in the mammary gland were predicted to produce circular transcripts and many were related to genes encoding components of vesicles, endoplasmic reticulum, and mitochondrial lumen that contribute to milk protein biosynthesis and secretion. Four of the four casein-encoding genes (*CSN1S1*, *CSN1S2*, *CSN2,* and *CSN3)* were found to express circRNAs in the mammary gland. Expression levels were up to nine-fold increased on day 90 compared to day 250, correlating with the observed decrease in milk casein production as lactation wanes. Furthermore, cattle casein circRNAs had an abundance of MRE for miR predicted to target CSN1S1 and CSN2 mRNAs. The results support the hypothesis CSN circRNA regulate milk production through competitive binding of miR that upregulates translation of Casein proteins (**Figure 2**).

circRNAs also play a role in cattle muscle development. To identify circRNAs in skeletal muscle, three longissimus muscle samples were obtained from Qinchuan cattle at embryonic day 90 and adulthood at 24 months (Li et al., 2018). They identified circFGFR4 is a highly expressed circRNA with binding sites for miR-107 that decreased expression of established myogenic markers, *MyoD* and *myogenin* and the formation of myotubes. The validation experiments established circFGFR4 binds miR-107, positively influencing myoblast differentiation and development. Previously, abundant miR107 was identified in cattle skeletal muscle and shown to progressively decrease in quantity between embryonic and adult stage (Sun et al., 2013). It is possible increased expression and/or availability of circFGFR4 sequesters miR107 progressively.

Another report on circRNA in cattle skeletal muscle tissue profiled prenatal and postnatal *longissimus dorsi* muscle in sheep (Li C. et al., 2017). This study relied on computational prediction models to construct competing endogenous RNA networks to be tested in future validation studies. Nonetheless, the authors posited circRNAs have multiple target sites for miRs previously associated with muscle development, e.g. miR143, miR-133 and miR-23. Further analysis of mRNA targets and KEGG pathway analysis revealed the circRNA derived from parental genes in muscle growth and development signaling pathways. More data acquisition will be important to grasp the role of circRNA in sheep.

Animal Models for Translational Research

Whether naturally-occurring or experimentally-induced, study of circRNA in farm animals contributes richly to the fruits of biomedical research. Animals and people suffer from breast cancer, melanoma, obesity and even psychological disorders, like anxiety and depression. Comparative basic science investigation of pathogenesis and molecular genetics, as well as risk factor assessment have been invaluable resources to learn how to cure disease of animals and people (Morello et al., 2011; Makielski et al., 2019).

Swine

The biology of the brain is prominently regulated by non-coding RNAs (Nie et al., 2019). In the mammalian brain, circRNAs contribute to neurological development and neuronal tissue differentiation (Jeck et al., 2013; Rybak-Wolf et al., 2014; You et al., 2015; Venø et al., 2015; Zhao et al., 2016; Floris et al., 2017). There is strong bias for circRNA upregulation in neural tissues of adult mice that has suggested the disruption of ceRNA networks contributes to aging (Gruner et al., 2016). Indeed, there is significant association between the expression of certain circRNAs like circHOMER1 in the brain and Alzheimer's traits ($p = 10^{-12}$) (Zhao et al., 2016; Huang J. L. et al., 2018).

The stages of fetal brain development has been well-characterized in swine and Venø et al. (2015) used the swine model to profile developmentally regulated circRNAs. The expression of *Sus scrofa* circRNAs was measured in the cortex, hippocampus, cerebellum, brainstem and basal ganglia at several time-points from early embryonic development to the time of birth. The authors reported clear fluctuations in circRNA expression as the gestation progressed. But, in contrast to results in poultry (Ouyang et al., 2018a), porcine circRNAs increased as the gestation progressed, peaking at embryonic day E60, followed by a steep decline between E80 until birth. The spatial-temporal expression profile correlated with differentiation of the fetal cortex, specifically, neuronal migration of E60 brain to acquire the gyri and sulci characteristic of the gyrencephalic brain.

Remarkably, porcine circRNAs represented over 10% of all expressed genes (range 6 – 14%) (Venø et al., 2015). The introns flanking circularized exons frequently have complementary SINEs, suggesting these RNA motifs drive circRNA by facilitating base pairing between the repetitive sequence of flanking introns, similar to viroid RNA motifs that were discussed above. This study constitutes the first circRNA profiling of the brain development of a large animal (**Table 3**).

Liang et al. (2017) used the Guizhou miniature pig model to perform spatial-temporal profiling of nine different organs (heart, liver, spleen, lung, kidney, ovarium, testis, muscle, and fat) and skeletal muscle tissues. Sampling was performed at three post-natal developmental stages, day 0, 30, and 240 after birth. They identified dynamic expression changes and the high abundance of circRNAs in the testicular tissue. Notably, Sry circRNA, previously reported to be predominant over linear *Sry* transcript in mouse testis (Capel et al., 1993), was not found. Significant new findings were the identification of several porcine heart-specific circRNAs related to hypertrophic cardiomyopathy, recapitulating circRNAs in human heart development and pathogenesis of cardiac diseases (Sonnenschein et al., 2019). Results identified MREs in 30% of the porcine circRNAs, implicating functionalities beyond miR sponging for porcine circRNAs. Similar to other non-coding RNAs, circRNA have the potential to modulate the local free concentration of RBPs by direct binding and by occupying their binding sites in target RNAs. Given circRNA are abundant, stable and only recently appreciated, their activity may profoundly balance gene expression and response to metabolic stress and infection.

Approximately 90% of the porcine circRNA splicing regions aligned with the mouse genome and one-fourth of the splice sites were identical between these two species, showing significant conservation between the profiles of porcine and murine circRNAs (Venø et al., 2015). CircRNA of Guizhou miniature pig originated from orthologous loci identified in murine (25%) and human (87%) circRNA profiles, confirming circRNA sequence conservation is prevalent between these species (Liang et al., 2017). In addition, authors report almost half of the detected porcine circRNAs were flanked by long introns, matching the prior observations made in humans (Zhao et al., 2019). Perhaps an outlier, chicken exon-intron circHRH4 was also flanked by long partial introns (Qiu et al., 2018). These observations could support a theory that repetitive sequences in flanking introns and RBPs are brought together to facilitate back-splicing (**Figure 2**). However, bovine circRNAs appear to lack repetitive sequences in flanking introns to mediate circularization (Zhang et al., 2016). Future studies are warranted to define the intrinsic determinants of RNA circularization that are conserved among different clades of animals.

Companion Animals

Even though companion animals with a defined pedigree are a promising translational model (Nichols et al., 2009; Pinho et al., 2012; Hytönen and Lohi, 2016; Supsavhad et al., 2016; Hicks et al., 2017), to our knowledge only two studies have profiled circRNA expression (**Table 3**). Both studies investigated circRNAs in the context of cardiovascular diseases (Shangguan et al., 2018; Zhang F. et al., 2018).

Chronic rapid atrial pacing (RAP) in dogs is an established model to study atrial fibrillation (AF), a common arrhythmia, which in humans leads to heart failure (Kijtawornrat et al., 2008). RNA sequencing of atrial tissues from dogs that suffered RAP and healthy controls showed differences in the expression of circRNAs (Shangguan et al., 2018). Gene Ontogeny identified transcripts encoding cytoskeleton and ion channel activity that participate in the process of atrial structural and electronic remodeling. An extensive network of differentially expressed circRNAs and AF-related miR were associated with the development of AF.

The relationship between circRNAs and atherosclerosis was investigated after inducing atherosclerotic plaques on the right carotid arteries in New Zealand white male rabbits by endothelial injury and high fat diet (Zhang F. et al., 2018). Differential analysis of circRNA, miR and mRNA revealed significant differences between the treatment and control group that mapped to immune response, cell adhesion, T-cell activation and cytokine production pathways. The study found hundreds of circRNAs and mRNAs having similar MRE, setting up potential widespread competition for miRs in each subnetwork. Consistent with a role for circRNA, study of human circANRIL identified changes affect human smooth muscle vascular tissue preventing the development of changes leading atherosclerosis (Kohlmaier et al., 2016; Xu et al., 2019). Further studies are warranted to define whether generalized dysregulation of circRNA predisposes to atherosclerosis.

Non-human Primates

Numerous experiments have established that muscle in humans is one of the tissues that is enriched in circRNAs. The dystrophy gene is among the first genes identified to generate circRNAs in skeletal muscle (Surono, 1999; Zhang et al., 2019). It is thought that one of the proposed models for circRNA biogenesis, exon skipping, may provide a cure to Duchenne muscular dystrophy patients by generating circular isoforms that skip over a region in the gene that accounts for 60% of the cases exhibiting this disease phenotype (Zhang et al., 2019). As mentioned above, circANRIL in INK4 locus was shown to protect from atherosclerosis by preventing oxidative stress and proliferation of smooth muscle cells in the endothelial vascular wall, and inducing apoptosis by activating p53 (Holdt et al., 2016; Shi et al., 2020). The circ-ZNF609, which was found to act as miR sponge to promote myoblast differentiation, was the first reported protein coding circRNA in skeletal muscle (Zhang et al., 2019). For a comprehensive review on circRNAs as regulators of myogenesis, please refer to Zhang et al. (2019).

On the other hand, Abdelmohsen et al. (2015) profiled expression of circRNAs in a non-human primate muscle tissue. CircRNAs were computationally predicted in total RNA isolated from 24 samples of the quadriceps leg muscle (*vastus lateralis*) from young, middle-aged and elderly rhesus macaques (age range 0.003–41 years old). The majority of the detected circRNAs were similar among the different age groups, but 19 were significantly

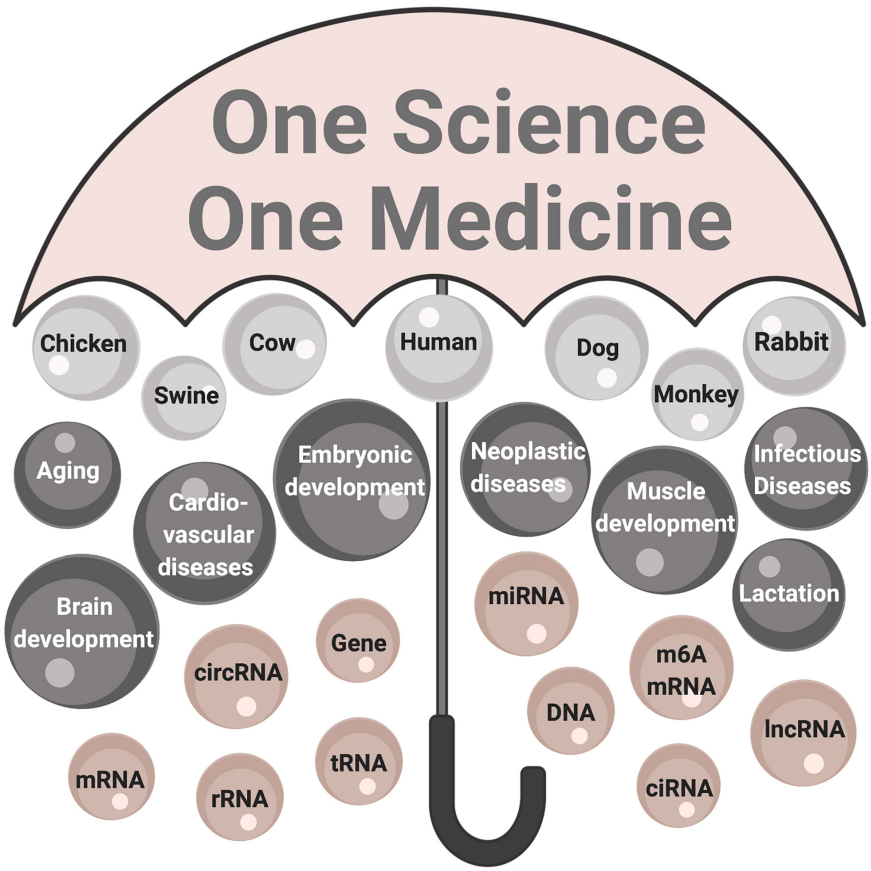

FIGURE 3 | The One Health perspective of circRNA research. The umbrella labeled One Science One Medicine encompasses circRNA research in human and animal species that is contextual to healthy people, animals, environments. The significance of circRNA research: organisms are top-level components (light gray); processes and conditions affected are mid-level components (dark gray); and fundamental components are molecules (mauve). mRNA, messenger RNA; circRNA, circularized RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; miR, microRNA, m^6A mRNA, mRNA modified by adenine modification; ciRNA, circularized intronic RNA; lncRNA, long non-coding RNA.

downregulated circRNA as individuals aged. The significance of circRNA to muscle development, musculoskeletal pathology and age-related changes in primates is likely to be informed by studies of swine and poultry skeletal muscle with high statistical power.

Expression Landscape of circRNA: Man, Macaque, and Mouse

In a landmark publication, Ji et al. (2019) explored the landscape of the human, macaque, and mouse transcriptomes across 19 primary tissues, identifying 70,186 evolutionarily conserved circRNAs between these species and confirming the highest prevalence in neural and testicular tissues. Sixty-seven percent of the circRNA were detected in only one tissue. By constructing long-insert RNAseq libraries (400–800 base), in combination with long reads and high sequencing depth, endogenous circRNA sequences could be reconstructed, thereby increasing reliability of circRNA annotation. Notable was the classification of comparable tissue expression patterns between circRNA and RBP, implicating coordinate activity of circRNAs and RBPs in modulating the local concentration of linear RNAs or their cognate binding sites. The expression variance of the circRNA was low across different individuals, suggesting that circRNA expression is under tight regulation rather than being stochastic.

CONCLUDING REMARKS

One Health is a systems biology vision that encompasses the health of people, animals and global environment under one large umbrella (Zinsstag et al., 2011). Under the One Health umbrella, circRNA align innovative basic research that has elucidated new feed-back loops interconnecting processes and conditions common between people and animals (**Figure 3**). Even though the existence of circRNA has been known for nearly 50 years, circRNA literature has doubled in the past 5 years – largely through computational biology and RNAseq technology accompanied by molecular biology tools. circRNAs are now known to regulate biological processes beginning at embryonic development, throughout growth cycles and the aging process. Investment is warranted to elucidate competing endogenous

RNA networks in animals and people and integrate the genetic and epigenetic expression patterns that once appeared stochastic to prevent disease. Implementing the One Health perspective over the next 5 years is to apply innovative basic research findings to the invention of diagnostic tools and therapeutic application of circRNAs in veterinary and human medicine.

AUTHOR CONTRIBUTIONS

KB-L outlined the manuscript, tables, and figures. DZ wrote the manuscript and created the figures. DZ and KB-L finalized the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Circular RNAs: Emerging Role in Cancer Diagnostics and Therapeutics

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Circular RNAs (circRNAs) are rapidly coming to the fore as major regulators of gene expression and cellular functions. They elicit their influence *via* a plethora of diverse molecular mechanisms. It is not surprising that aberrant circRNA expression is common in cancers and they have been implicated in multiple aspects of cancer pathophysiology such as apoptosis, invasion, migration, and proliferation. We summarize the emerging role of circRNAs as biomarkers and therapeutic targets in cancer.

Keywords: circRNAs, cancer, biomarkers, diagnostics, therapeutics

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INTRODUCTION

Our understanding of the human transcriptome has increased significantly by the discovery and understanding of the role of regulatory non-coding RNAs in physiology and diseases such as cancer (Vo et al., 2019). Among the non-coding regulatory transcripts, circRNAs have attracted intense research scrutiny in recent years (Chen, 2020). CircRNAs are single-stranded covalently closed continuous loop structures lacking free ends and a polyadenylate tail (Li X. et al., 2018b; Kristensen et al., 2019; Chen, 2020). Close to one-fifth of active genes in the human genome can potentially give rise to circRNAs (Salzman et al., 2012; Li X. et al., 2018b; Kristensen et al., 2019; Chen, 2020). CircRNAs are composed of exonic and/or intronic sequences and are primarily generated by back-splicing, a non-canonical alternative RNA splicing event mediated by the spliceosome and regulated by a combination of *cis*-elements and *trans*-factors (Chen and Yang, 2015; Li X. et al., 2018b; Kristensen et al., 2019; Chen, 2020). Due to the absence of free ends circRNAs are not susceptible to destruction by RNA degradation machinery and are more stable than linear RNAs (Lasda et al., 2014; Wang et al., 2015; Zhang Y. et al., 2016). The majority but not all circRNAs are non-coding and exhibit their biological functions by sequestration of miRNAs/proteins. Some circRNAs regulate transcription, splicing and may also be translated to polypeptides. CircRNAs are involved in the regulation of cancer hallmarks such as self-sustenance in growth signals, proliferation, angiogenesis, resistance to apoptosis, unlimited replicative potential, and metastasis (Shi, 2017; Bach et al., 2019; Vo et al., 2019). Here we summarize and catalog the advances in the use of circRNAs as biomarkers for cancer diagnosis and as therapeutic targets.

BIOGENESIS OF CIRCULAR RNAs

In eukaryotes, the generation of a mature mRNA is a result of interaction between transcription, splicing, capping, polyadenylation, export, and degradation (Black, 2003; Moore and Proudfoot, 2009; Nilsen and Graveley, 2010). CircRNAs are formed by a specialized non-conventional alternative splicing referred to as back-splicing (Zhang X. O. et al., 2016). In contrast to the classical

canonical splicing, during back-splicing, a downstream 5' splice-site is joined to an upstream 3' splice-site across a single or multiple exons leading to the formation of circRNA species (**Figure 1A**) (You et al., 2015; Li X. et al., 2018b; Kristensen et al., 2019; Chen, 2020).

Two models have been proposed to explain the coupling of back-splicing to canonical splicing for circRNAs biogenesis (i) "exon-skipping" or "lariat-intermediate" model and (ii) "direct back-splicing" model (Lasda et al., 2014). In the "lariat intermediate" model, canonical splicing occurs first and generates an intronless linear RNA, and an intron lariat bearing skipped exons which eventually undergoes back-splicing (Zaphiropoulos, 1996; Kelly et al., 2015) (**Figures 1B,C**). In the "direct back-splicing" model back-splicing occurs first leading to the formation of a circRNA followed by the creation of a linear RNA (Li Y. et al., 2017) (**Figure 1A**). Based on their origin, circRNAs fall into three major classes, exonic, intronic, and exon-intron circRNAs. Except for splice-sites, no particular sequences are necessary for circularization, however, a median exonic length is required for back-splicing involving either single or multiple exons (Ashwal-fluss et al., 2014; Zhang et al., 2014).

CircRNA synthesis by back-splicing occurs both co-transcriptionally and post-transcriptionally and is favored by a high rate of transcription elongation (Ashwal-fluss et al., 2014; Zhang Y. et al., 2016; Vo et al., 2019). The ligation of a downstream 5′ splice-site with an upstream 3′ splice-site during back-splicing is not favored sterically leading to lower efficiency of back-splicing as opposed to conventional linear splicing (Jeck

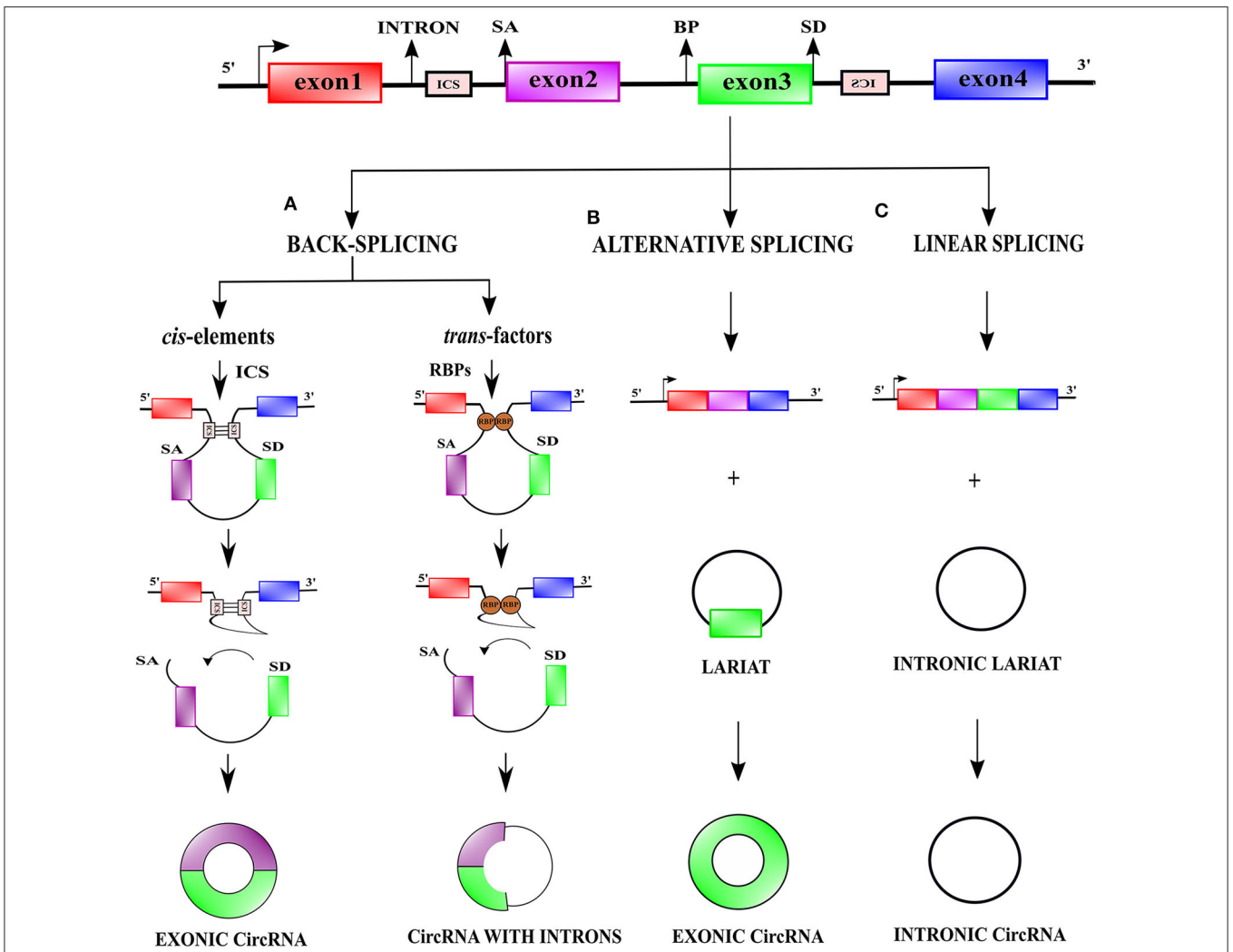

FIGURE 1 | Biogenesis of circular RNAs. **(A)** During transcription, linear and back-splicing of exons rival each other. Back-splicing is facilitated by long flanking introns, cis-elements i.e., intronic complementary elements (ICS), and trans-factors i.e., RNA-binding proteins (RBPs). To facilitate back-splicing, a downstream splice-donor (SD) site is brought in close vicinity with an upstream splice-acceptor (SA) site *via* base-pairing interactions between ICS or dimerization of RBPs. An upstream branch point (BP) nucleophilically attacks a downstream SD site, which thereafter nucleophilically attacks an upstream SA site, resulting in the creation of exonic circRNAs or exon-intron circRNAs. **(B)** Alternative splicing events like exon-skipping often generate skipped exon bearing lariat precursors, which can participate in the genesis of exonic circRNAs. **(C)** Intronic lariat precursors generated by linear canonical splicing can escape lariat debranching and serve as a source for intronic circRNAs.

et al., 2013; Zhang Y. et al., 2016). Interestingly, alternative back-splicing events can also occur and generate multiple circRNA isoforms (Gao et al., 2016; Zhang X. O. et al., 2016). Just like linear RNAs, circRNAs too are subjected to widespread reversible modification, in particular N6-methyladenosine (m6A) modification, which may influence their cellular fate (Zhou et al., 2017).

Role of Cis-Elements and Trans-Factors in Circular RNA Formation

CircRNA formation by back-splicing is facilitated by *cis*-elements such as intronic complementary sequences (ICS), flanking circRNA forming exons and *trans*-factors like RNA-binding proteins (RBPs) (**Figure 1A**) (Jeck et al., 2013; Ashwal-fluss et al., 2014; Liang and Wilusz, 2014; Zhang et al., 2014). ICS facilitate RNA pairing, by bringing distal splice-sites close to each other, which promotes circularization (Jeck et al., 2013). In humans, both complementary inverted-repeat *Alu* elements located in introns, as well as non-repetitive complementary sequences in introns, promote RNA pairing and subsequent back-splicing (Jeck et al., 2013; Liang and Wilusz, 2014; Zhang et al., 2014; Starke et al., 2015). *Trans*-factors contribute to circRNA biogenesis by modulating back-splicing: (i) by directly bridging distal splice-sites (ii) by binding to ICS. Some examples of *trans*-factors are RBPs such as Quaking (QKI), Heterogeneous-nuclear ribonucleoprotein L (HNRNPL), and RNA-binding motif protein 20 (RBM20) (**Figure 1A**) (Conn et al., 2015; Errichelli et al., 2017). RBPs which bind to ICS and regulate circRNA biogenesis bear double-stranded RNA-binding domains (dsRBDs) and can stabilize or destabilize the base-pairing between ICSs to promote or prevent back-splicing. The dsRBDs which promote back-splicing are nuclear factor 90 (NF90) and nuclear factor (NF110) (Patiño et al., 2015; Li et al., 2017) whereas dsRBDs which prevent back-splicing include DExH-Box Helicase 9 (DHX9) and adenosine deaminase 1 acting on RNA (ADAR1) (Ivanov et al., 2015; Aktaş et al., 2017).

MECHANISM OF ACTION OF CIRCULAR RNAs

Circular RNAs as miRNA Sponges

CircRNAs competitively bind and sponge miRNAs leading to the stabilization of their target transcripts. They can have single or multiple binding sites for single or several miRNAs (**Figure 2A**). For instance, the expression of miR-7 target genes is regulated by *CDR1as*, which harbors >70 conserved binding sites for miR-7 (Hansen et al., 2013). Some circRNAs acting as miRNA sponges have oncogenic and tumor-suppressive properties (Kristensen et al., 2019). For example, *circCCDC66* binds two miRNAs, miR-33b and miR-93, and promotes tumorigenesis in colorectal cancer by upregulation of c-MYC (Hsiao et al., 2017).

Circular RNAs as Protein Sponges

Interestingly, circRNAs can also bind to proteins and prevent their activity (**Figure 2B**) (Ashwal-fluss et al., 2014). For example, *circPABPN1* binds to the Hu-antigen R (HuR) and prevents its binding to the cognate linear mRNA resulting in its reduced translation (Abdelmohsen et al., 2017). Certain circRNAs bind to multiple proteins and hold them together as a scaffold to facilitate their interaction. For example, *circAMOTL1* simultaneously binds to both AKT1 and PDK1 in cardiac tissue serving as a scaffold to facilitate the phosphorylation of AKT1 (protein kinase B) by PDK1 (3-phosphoinositide-dependent protein kinase 1) (Zeng et al., 2017).

Circular RNAs as Regulators of Transcription and Splicing

Nuclear circRNAs also modulate transcription and splicing (**Figure 2C**). For example, the intronic *ci-ankrd52* assembles at the transcription sites of its cognate gene and positively regulates RNA polymerase II driven transcription (Yang Y. et al., 2017). *CircRNAs EIF3J* and *PAIP2* interact and form complexes with U1snRNP, which in turn interact with RNA polymerase II at the promoters of the parental genes, leading to transcriptional enhancement (Li Z. et al., 2015). Some circRNAs also regulate alternative splicing e.g., *circ-UBR5* modulates RNA splicing by binding to splicing regulators such as QKI, NOVA1, and U1snRNA (Qin et al., 2018; Chen, 2020).

Functions of Circular RNAs Encoded Microproteins

Most circRNAs are noncoding but a few circRNAs have short open reading frames (ORFs) which are translated into short peptides referred to as microproteins (**Figure 2D**). Generally, microproteins are <100 amino acids in length and possess distinct functions as compared to the protein coded by their cognate linear mRNA (Hanada et al., 2009; Andrews and Rothnagel, 2014). CircRNAs undergo cap-independent translation facilitated by internal ribosomal entry sites (IRESs) and m6A modification in the 5'untranslated region (5'UTR) (Abe et al., 2015). Examples of microproteins encoding circRNAs include *circFBXW7*, *circZNF609*, *circMbl*, *circPINTexon2*, and *circSHPRH* (Motegi et al., 2006; Akhoondi et al., 2007; Yang Y. et al., 2018; Zhang M. et al., 2018; Zhang et al., 2019).

CIRCULAR RNAs AS CANCER BIOMARKERS

CircRNAs have several attributes that make them potential biomarkers for cancer diagnosis and prognosis. They are more stable than linear RNAs due to lack of free 5′ and 3′ ends (Memczak et al., 2013; Li Z. et al., 2015; Zhang Z. et al., 2018; Vo et al., 2019), and often display tissue and developmental stage-specific expression pattern, and can be quantitatively detected by reverse transcription followed by real-time quantitative polymerase chain reaction (RT-qPCR) (Panda and Gorospe, 2018). Moreover, altered expression of circRNAs has been frequently observed in cancer tissues and/or in plasma, and

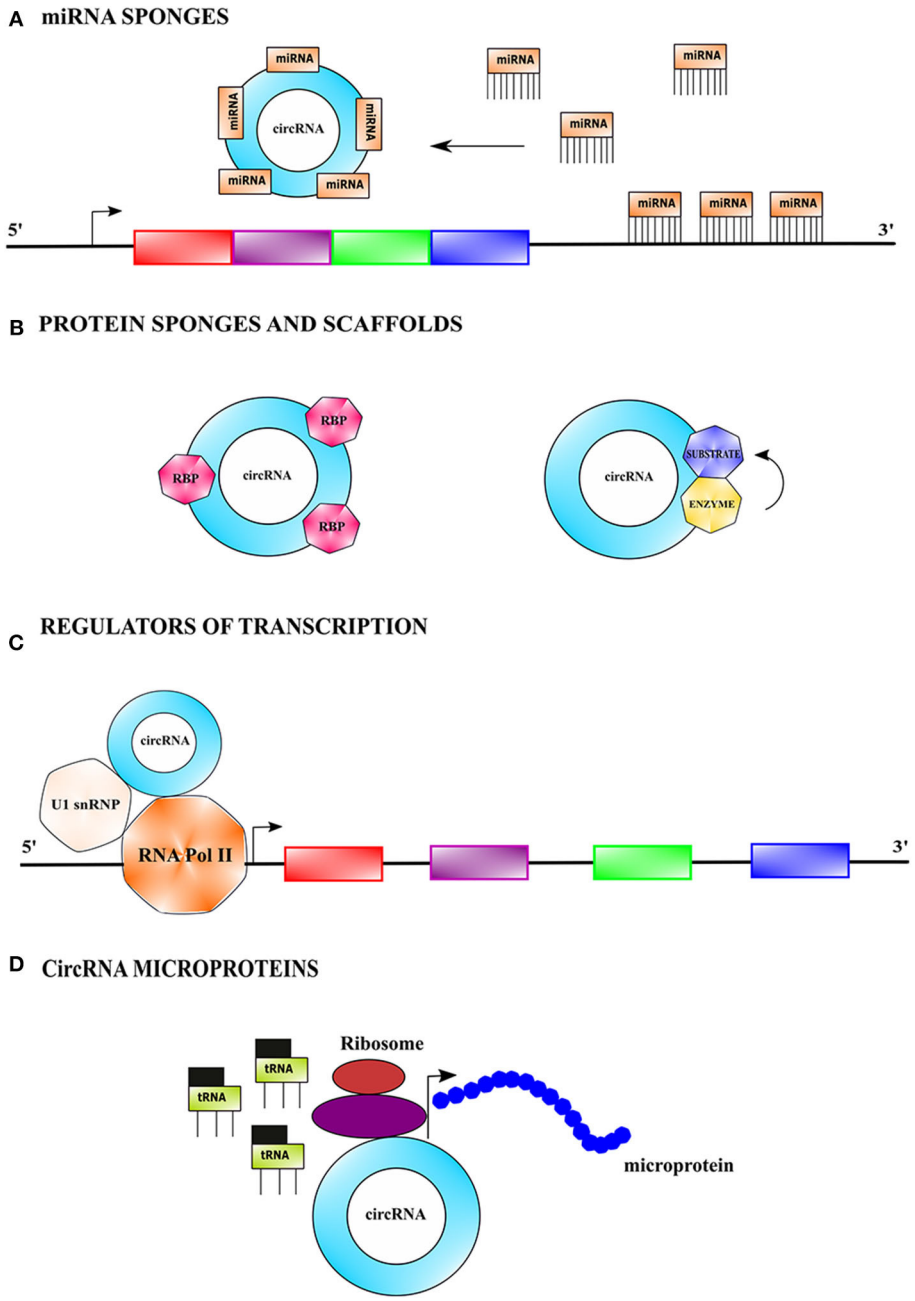

FIGURE 2 | Mechanistic action of circular RNAs. **(A)** CircRNAs can serve as miRNA sponges by competitively binding to miRNA(s) *via* base-pairing interactions, causing stabilization of target transcript(s) of the sequestered miRNA(s), and thus making it more available for translation. **(B)** CircRNAs can sponges protein by binding to them and thus indirectly regulating their functions. CircRNAs can also function as protein scaffolds by facilitating colocalization of an enzyme and its substrate to influence the kinetics of catalysis. **(C)** CircRNAs can modulate transcription by binding to RNA polymerase II complex bearing the U1 small nuclear ribonucleoprotein among other proteins and augment the function of certain proteins of the complex. **(D)** CircRNAs bearing internal ribosome entry site (IRES) elements and initiation codons can initiate translation in a cap-independent fashion and generate short polypeptides referred to as microproteins.

saliva from cancer patients (Memczak et al., 2013, 2015; Bahn et al., 2015; Li Z. et al., 2015; Panda and Gorospe, 2018; Zhang Z. et al., 2018; Vo et al., 2019). Li et al. first reported the presence of circRNAs in exosomes in serum of cancer patients and several cancer cell types and coined the term exo-circRNAs (Li Y. et al., 2015). The presence of exo-circRNAs in a variety of human bodily fluids that may be assessed easily without biopsy, makes exo-circRNAs a good choice for cancer diagnosis (Bai et al., 2019; Geng X. et al., 2020). Numerous circRNAs are dysregulated in cancer but few have the potential

TABLE 1 | Biological functions and roles of circular RNAs in cancer diagnostics and therapeutics.

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
circ_0009910	AML	↑	Sponges miR-20a-5p and inhibits apoptosis. Overexpressing miR-20a-5p counteracts chemoresistance *in vitro* and *in vivo* through targeting KIF26B by modulating the activities of the MAPK/ERK and cAMP/PKA signaling pathways	miR-20a-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – Mono-Mac-6, KG-1, AML2, AML5	+/–	Ping et al., 2019a
	GC	↑	Associated with distant metastasis and differentiation; downregulation reduces GC cell proliferation, migration and invasion. Acts as oncogene that acts by inducing the EMT. Its knockdown suppresses the migration invasion and EMT of GC cells *in vitro*.	–	Human; *in vitro*	Human cell lines – BGC823, SGC7901, AGS, MGC803, MKN45, GES1	+/+	Liu M. et al., 2018
	CML	↑	Promotes cell growth and imatinib resistance, reduces apoptosis and autophagic activation. It accelerates imatinib-resistance in cells by modulating ULK1-induced autophagy *via* targeting miR-34a-5p, providing a potential target in imatinib resistance of CML.	miR-34a-5p	Human; *in vitro*	Human cell lines – K562, K562/R	+/+	Cao et al., 2020
	OSC	↑	Sponges miR-449a which targets IL6R; prohibits cell-cycle arrest, promotes proliferation and inhibits apoptosis.	miR-449a	Human; *in vitro*	Human cell lines – MG63, Saos-2, U2OS, hFOB	+/+	Deng et al., 2018
circ-vimentin	AML	↑	Associated with poor overall survival (OS), leukemia-free survival and vimentin expression.	–	Human; *in vivo*	–	+/–	Yi and Lin, 2018
circ_0075001	AML	↑	Lowers expression of components of Toll-like receptor signaling pathway	–	Human; *in vitro*	Human cell lines – NB-4, KASUMI-1, OCI-AML5, OCI-AML3, ME-1, MV4-11, K562	+/–	Hirsch et al., 2017
circ_0004277	AML	↓	–	–	Human; *in vivo*	–	+/–	Li W. et al., 2017
circ_100053	CML	↑	Associated with clinical stage, BCR/ABL mutant status and imatinib resistance	–	Human; *in vivo*	–	+/–	Ping et al., 2019b
circ–RPL15	CLL	↑	Inhibits miR-146b-3p mediated suppression of the RAS/RAF1/MEK/ERK pathway	miR-146b-3p	Human; *in vitro*	Human cell lines – MEC-1 and JVM-3	+/+	Wu Z. et al., 2019
circ-CBFB	CLL	↑	Activates Wnt/β-catenin signaling pathway	miR-607	Human; *in vitro*	Human cell line - MEC-1	+/+	Xia L. et al., 2018
circ_0007841	MM	↑	Correlated with chromosomal aberrations such as gain 1q21, t (4:14), mutations in ATR and IRF4 genes; miR-199a-3p affects the multi-chemoresistance of OS *via* targeting AK4; overexpression correlates with osteolytic bone destruction in MM; overexpressed in BTZ-resistant MM cell lines	miR-199a-3p	Human; *in vitro*	Human cell lines – THP-1, KM3, U266, RPMI-8226, KM3/BTZ, 146 U266/BTZ, RPMI-8226/BTZ	+/–	Gao et al., 2019
circ_0000190	MM	↓	Correlates with prognosis survival rates of MM patients, inhibits MM progression *via* modulating miR-767-5p/MAPK4 pathway	miR-767-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – MM.1S, NCI-H929	+/+	Feng et al., 2019
	GC	↓	Associated with tumor diameter, lymphatic metastasis, distal metastasis, TNM stage and CA19-9 level	–	Human; *in vivo*	–	+/–	Chen et al., 2017
	OSC	↓	Correlated with bigger tumor size, advanced staging (IIB/III) and distant metastasis	miR-767-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines –hFOB1.19, SAOS-2, MG63, U2OS, SJSA1, and HOS	+/–	Li et al., 2020
circ-SMARCA5	MM	↓	Higher expression is correlated with lower β2-MG level and less advanced ISS stage; native resistance to drugs is common due to multiple chromosomal abnormities in the pathogenesis of MM	miR-767-5p	Human; *in vitro*	Human cell lines – NCI-H929, RPMI8226, U226, OPM2, JJN3	+/+	Liu H. et al., 2019

(Continued)

TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
	GC	↓	Correlates with differentiation, lymph node metastasis, vascular invasion	–	Human; *in vitro*	Human cell lines – GES-1, MGC803, MKN45, AGS, MKN74, BGC-823, SGC-7901	+/–	Cai et al., 2019
	HCC	↓	Promotes apoptosis and expression of tumor suppressor TIMP3, inhibits proliferation, invasion and metastasis; reverses tumor growth along with decreased expression of MMP9 and MMP7	miR-17-3p miR-181b-5p	Human; *in vitro*	Human cell lines – Huh7, HCCLM9, HepG2	+/+	Li Z. et al., 2019
circRNA_101237	MM	↑	Increases significantly in bortezomib-resistant cell lines; overexpression is associated with a poor response to chemotherapy in MM patients	–	Human; *in vitro*	Human cell lines – THP-1, MM.1S, H929, MM.1S/BTZ, H929/BTZ	+/–	Liu and Wang, 2020
	HCC	↑	Associated with tumor size, lymph node metastasis, distant metastasis and TNM stage; cisplatin resistance associated with loss of Runt-associated transcription factor 3 and upregulation of cyclophilin B	–	Human; *in vitro*	Human cell lines – HCCLM3, Hep3B, MHCC97-H, cisplatin-resistant Huh7/DDP cells	+/–	Zhou et al., 2020
circ-APC	BCL	↓(DLBCL)	Inhibits Wnt/β-catenin signaling	miR-888	Human, Mouse; *in vitro/in vivo*	Human cell lines – SUDHL-3, U2932, TMD8, OCI-Ly3, L428, GM12878	+/+	Hu et al., 2019
CDR1as	CRC	↑	Enhances EGFR/RAF1/MAPK pathway, induces cell growth, resistance to apoptosis and cell cycle arrest	miR-7	Human; *in vitro*	Human cell lines –HCT-116, DLD-1, NCM460, CCD841CoN	+/+	Weng et al., 2017
	HCC	↑	Promotes HCC progression by activating PI3K/AKT/mTOR pathway	miR-7	Human; *in vivo*	–	+/+	Xu et al., 2017
	GC	↑	Activates PTEN/PI3K/AKT pathway	miR-7	Human, Mouse; *in vitro/in vivo*	Human cell lines – MGC-803, HGC-27, GES-1	+/+	Pan et al., 2018
circCCDC66	CRC	↑	Promote CRC growth and metastasis by stabilizing MYC mRNA	miRNA-33b, miR-93	Human; *in vitro/in vivo*	Human cell lines – HCT-116, HT-29	+/–	Hsiao et al., 2017
circ_0004585	CRC	↑	Associated with increased patient's tumor size	–	Human; *in vivo*	–	+/–	Tian et al., 2019
circ_0007142	CRC	↑	Regulates proliferation and invasion of CRC; upregulation is associated with lymphatic metastasis	miR-103a-2-5p	Human; *in vitro*	Human cell lines – HCT-116, HT-29, LoVo, HCO	+/–	Zhu et al., 2019
circHUEW1	CRC	↑	Associated with lympho-vascular invasion, lymph node metastasis, distant metastasis, and TNM stage; affects IGF2/β-catenin signaling pathway	miR-486	Human; *in vivo*, *in vitro*	Human cell lines – HCT116, SW480	+/+	Chen H. Y. et al., 2020
circ_0001178	CRC	↑	Metastatic clinical features, advanced TNM stage and adverse prognosis; induces EMT through increasing ZEB1 expression	miR-382, miR-587 and miR-616	Human, Mouse; *in vitro/in vivo*	Human cell lines – NCM460 cells, CRC LoVo, SW620	+/+	Ren et al., 2020
circ_0005075	CRC	↑	Downregulation modulated Wnt/β-catenin pathways and reduced cell proliferation and metastasis; Knockdown suppresses EMT progression by decreasing the levels of Vimentin and N-cadherin	–	Human; *in vivo*, *in vitro*	Human cell lines – HCO, SW480, SW620, HT29, HCT116, SW1116, LOVO	+/+	Jin et al., 2019
	HCC	↑	Contributes to HCC proliferation, invasion, and metastasis	miR-23b-5p, miR-93-3p, miR581,miR-23a-5p	Human; *in vivo*	–	+/–	Shang et al., 2016
circHIPK3	CRC	↑	Promotes CRC progression,increases expression of downstream oncogenic target genes, FAK, IGF1R, EGFR, and YY1 that activate PI3K/AKT and MEK/ERK signaling pathways to promote cancer progression and drug resistance	miR-7	Human, Mouse; *in vitro/in vivo*	Human cell lines – FHC, HCT116, HT29, SW480, SW620, DLD1	+/+	Zeng et al., 2018
	OSC	↓	Correlates with Enneking stage and lung metastasis.	–	Human; *in vitro*	Human cell lines – SaoS2, HOS, KH-OS, MG63, 143B, U2OS	+/–	Kun-peng et al., 2018b

(Continued)

TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
	BCa	↓	Reduces aggressiveness and metastasis by targeting the miR-558/heparanase axis	miR-558	Human, Mouse; *in vitro/in vivo*	Human cell lines –T24T, UMUC3, SV-HUC-1, HUVEC,	+/+	Li Y. et al., 2017
circ_0001649	CRC	↓	–	–	Human; *in vitro*	Human cell line - H116	+/–	Ji et al., 2018
	HCC	↓	Associated with tumor size, occurrence of tumor embolus; correlates with metastasis,	–	Human; *in vitro*	Human cell lines – HCC–LM3, MHCC-97L	+/–	Qin et al., 2016
	LC	↓(NSCLC)	associated with shorter OS, positive lymph node, and differentiation grade	miR-331-3p miR-338-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – A549, H358, H1299, H1581, 16HBE	+/–	Liu H. et al., 2018
circITGA7	CRC	↓(CRC)	Inactivates Ras signaling pathway; associated with tumor size, lymph metastasis, distant metastasis, and TNM stage	miR-370-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – SW480, RKO, Caco-2, SW620, LoVo, HCT116, DLD1, FHC	+/–	Li X. et al., 2018a
circ_0000711	CRC	↓	–	–	Human; *in vitro*	Human cell lines – NCM460, HCT116, COLO205, HT29	+/–	Li J. et al., 2018b
circ_0014717	CRC	↓	Overexpression promotes G0/G1 phase arrest, reduces growth, invasion and distal metastasis	–	Human, Mouse; *in vitro/in vivo*	Human cell lines – HCT116, HT29, SW480, FHC	+/–	Wang F. et al., 2018
circFBXW7	BC	↓(TNBC)	Inhibits tumor progression; up-regulates FBXW7 and degrades c-Myc, negatively correlated with metastasis	miR-197-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – MCF-10A, MCF-7, T47D, BT474, SKBR-3, MDA-MB-453, MDA-MB-468, MDAMB-231, BT549, HCC38, 4T1, MA-891	+/+	Ye F. et al., 2019
circSEPT9	BC	↑(TNBC)	Activates LIF/Stat3 signaling pathway, correlates with lymph node metastasis	miR-637	Human, Mouse; *in vitro/in vivo*	Human cell lines – MDA-MB-231, BT-549, MDA-MB-468, MDA-MB-453, SUM-159, MCF-10A	+/–	Zheng et al., 2020
circ_0001785	BC	↑(BC)	Associated with histological grade, TNM stage and distant metastasis	–	Human; *in vivo*	–	+/–	Yin et al., 2018
circCDYL	BC	↑	Regulates miR-1275-ATG7/ULK1 axis; downregulates the expression of autophagy associated genes AKT and ULK1; enhances malignant progression	miR-1275	Human, Mouse; *in vitro/in vivo*	Human cell lines – MDA-MB231, MCF-7	+/+	Liang et al., 2020
	MM	↑	Promotes MM growth by targeting YAP; inhibits apoptosis	miR-1180	Human, Mouse; *in vitro/in vivo*	Human cell lines – MM1.S, NCI-H929	+/+	Chen F. et al., 2020
circKIF4A	BC	↑(TNBC)	Induces TNBC cell proliferation and migration regulating the expression of KIF4A; miR-375 can sensitize resistant cells to tamoxifen and partly reverse EMT	miR-375	Human, Mouse; *in vitro/in vivo*	Human cell lines – MCF10A, MCF-7, T47D, BT474, KBR3, MDA-MB-453, MDA-MB-468, MDA-MB-231, BT549, HCC38	+/–	Tang et al., 2019
circPLK1	BC	↑(TNBC)	Promotes TNBC cell proliferation and metastasis by regulating PLK1	miR-296-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – MCF10A, MDAMB-468, MDA-MB-453, MDA-MB-231, HCC38, BT549	+/–	Kong Y. et al., 2019
circHMCU	BC	↑(BC)	Enhanced proliferation and metastasis; can modify EMT pathway, promotes mesenchymal phenotypes and inhibits epithelial phenotypes; stable and resistant to ActD treatment	let-7 family	Human, Mouse; *in vitro/in vivo*	Human cell lines –MDA-MB-231, MDA-MB-468, MCF7	+/–	Song et al., 2020
circ_0068033	BC	↓(BC)	Overexpression induces apoptosis	miR-659	Human, Mouse; *in vitro/in vivo*	Human cell lines – MCF10A, MCF-7, T47D, MDA-MB-468	+/–	Yuan et al., 2020
circ_0005075	HCC	↑	Contributes proliferation, invasion, and metastasis	miR-23b-5p, miR-93-3p, miR581 and miR-23a-5p	Human; *in vivo*	–	+/–	Shang et al., 2016

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TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
circ_100338	HCC	↑	Increased cell metastasis progression; regulates the MTOR signaling pathway	miR-141-3p	Human; *in vitro*	Human cell lines –Hep3B, BEL7402, MHCC97H, HCCLM6	+/+	Huang X. Y. et al., 2017
circRHOT1	HCC	↑	Promotes HCC progression, recruits TIP60,enhances invasion, inhibits apoptosis, and promotes metastasis	–	Human, Mouse; *in vitro/in vivo*	Human cell lines –Hep3B, Huh7	+/+	Wang L. et al., 2019
circ_0091579	HCC	↑	Associated with poor OS	–	Human; *in vivo*		+/–	Zhang C. et al., 2018
circ-HOMER1	HCC	↑	Increases the expression of CXCL6; associated with larger tumor size, higher TNM stage, and worse prognosis	miR-1322	Human; *in vitro*	Human cell lines – Sk-Hep-1, SMMC-7721, HCCLM3, Huh-7, HepG2 cells, L02	+/–	Zhao M. et al., 2020
circ_0016788	HCC	↑	Downregulates miR-486/CDK4 expression; associated with poor OS	miR-486	Human; *in vivo*	-	+/+	Cheng et al., 2020
circ_0078602	HCC	↓	Associated with a poor prognosis	-	Human; *in vivo*	–	+/–	Kou et al., 2019
circC3P1	HCC	↓	Associated with TNM stage, tumor size and vascular invasion, overexpression decreased metastatic nodules	miR-4641	Human, Mouse; *in vitro/in vivo*	Human cell lines –BEL7402, Hep3B, HuH7, MHCC97-L, HL-7702	+/-	Zhong et al., 2018
circ-ITCH	HCC	↓	Correlated with poor OS	-	Human; *in vivo*	–	+/–	Guo et al., 2017
	BCa	↓	Inhibits cell proliferation, migration and invasion through circ-ITCH/miR-17,miR-224/p21,PTEN signaling axis	miR-17,miR-224	Human, Mouse; *in vitro/in vivo*	Human cell lines – EJ, T24, 253 J, RT4, TCC-SUP, UMUC, J82, 5637, SV-HUC	+/+	Yang C. et al., 2018
	CRC	↓	Overexpression reduces cell proliferation by downregulating c-Myc and cyclinD1	miR-7, miR-20a	Human; *in vitro*	Human cell lines – HCT116, SW480	+/+	Huang et al., 2015
circMTO1	HCC	↓	Promotes expression of a tumor suppressor p21 resulting in reduced tumor cell proliferation, metastasis and invasion	miR-9	Human, Mouse; *in vitro/in vivo*	Human cell lines – HepG2, SMMC-7721, QGY-7701, SK-Hep1	+/+	Han et al., 2017
circ_0013520	GBM	↑	Correlated with tumor size, TNM and worse OS	–	Human; *in vitro*	Human cell lines – SHG-44, U251, HEB	+/–	Zhou and Fan, 2020
circ_0004379	GBM	↑	Correlated with tumor size, TNM and worse OS	–	Human; *in vitro*	Human cell lines – SHG-44, U251, HEB	+/–	Zhou and Fan, 2020
circ-CDC45	GBM	↑	Associated with larger tumor size, higher grade, and worse survival	miR-516b, miR-527	Human; *in vitro*	Human cell lines – U87MG, U118, U251, LN229	+/-	Liu J. et al., 2019
circNFIX	GBM	↑	Predicts poor prognosis; promotes cell propagation and migration; knockdown enhances TMZ sensitivity in resistant cells; regulates NOTCH pathway	miR-132, miR-34a-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – HA1800, SF-539, SHG-44, U87	+/+	Xu et al., 2018; Ding et al., 2020
circ_0013958	LC	↑(LAC)	Associated with the TNM stage and lymphatic metastasis	miR-134	Human; *in vitro*	Human cell lines – A549, H1299, BEAS-2B	+/–	Zhu X. et al., 2017
circFARSA	LC	↑(NSCLC)	Promotes cell migration and invasion; upregulates FASN	miR-330-5p, miR-326	Human; *in vitro/in vivo*	Human cell line – A549	+/–	Hang et al., 2018
	CRC	↑	Promotes proliferation, migration, and invasion;regulates miR-330-5p/LASP1 axis	miR-330-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – FHC, LS174T, RKO, HT29, HCT116, SW480	+/+	Lu C. et al., 2020
circ_0014130	LC	↑(NSCLC)	Associated with tumor volume, distant metastasis;upregulates Bcl2	miR-136-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – PC-9, A549	+/–	Geng Y. et al., 2020
circ_0000792	LC	↑(LAD)	Overexpression is correlated with T stage, distant metastasis	–	Human; *in vivo*	–	+/–	Li, 2018
circ_100876	LC	↑(NSCLC)	Related to carcinogenesis of NSCLC and it might serve as a potential prognostic biomarker and therapeutic target	–	Human; *in vivo*	–	+/+	Yao J. T. et al., 2017
circFADS2	LC	↑	Associated with advanced TNM stage, lymph node metastasis, poor differentiation, and shorter OS; induces progression, invasion and proliferation	miR-498	Human; *in vitro*	Human cell line – HepG2	+/+	Zhao F. et al., 2018

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TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
	CRC	↑	Associated with distant metastasis	-	Human; *in vivo*	–	+/–	Xiao et al., 2020
circPVT1	LC	↑(NSCLC)	Associated with distant metastasis; promotes cell proliferation, migration and invasion, and inhibits apoptosis through upregulated E2F2 and E2F2-related protein expression	miR-125b	Human, Mouse; *in vitro/in vivo*	Human cell lines – A549, H292, SPC-A1, H1299, H1650, H1975, SK-MES-1, HBE	+/+	Li X. et al., 2018c
	OSC	↑	Associated to chemoresistance and lung metastasis; knockdown decreases ABCB1 expression, promotes chemoresistance; knockdown partly reverses the doxorubicin and cisplatin resistance	–	Human; *in vitro*	Human cell lines – SaoS2, KHOS, U2OS, MG63	+/+	Kun-peng et al., 2018b
	ALL	↑	Promotes cell proliferation and inhibits apoptosis	let-7,miR-125	Human; *in vivo*, *in vitro*	Human ALL cell lines	+/+	Hu et al., 2018
circ_0067934	LC	↑(NSCLC)	Tumor-promoting circRNA; induces cell proliferation, metastasis and invasion	–	Human; *in vitro*	Human cell lines – A549, H1299, SK-MES-1, PC-9, BEAS-2B	+/-	Wang and Li, 2018
	ESCC	↑	Promotes proliferation and migration	–	Human; *in vitro*	Human cell lines – TE-13, ECA-109	+/+	Zong et al., 2018b
	LSCC	↑	Promote cell proliferation and metastasis	miR-1324	Human; *in vitro*	Human cell lines – TU212, TU686, 16HBE	+/–	Chu, 2020
	HCC	↑	Enhances migration, invasion and proliferation of cells; regulates Wnt/β-catenin signaling pathway	miR-1324	Human; *in vitro*	Human cell lines – BEL7402, Hep3B, HuH7, MHCC97-L, HL-7702	+/+	Zhu et al., 2018
circPRKCI	LC	↑	Upregulation increased proliferation and tumorigenesis	miR-545, miR-589	Human, Mouse; *in vitro/in vivo*	Human cell lines – A549, NCI-H1975, NCI-H1703, NCI-H226, NCI-H46, PC9, NCI-H1299, SPC-A1, HCC827, HBE	+/–	Qiu et al., 2018
circ_0000064	LC	↑	Promotes cell proliferation and inhibits cell apoptosis, enhances expression of bcl-2; overexpression is correlated with TNM stage, lymph node metastasis,	–	Human; *in vitro*	Human cell lines – A549, H1229	+/+	Luo et al., 2017
circ_0016760	LC	↑(NSCLC)	miR-1287 directly targets GAGE1, higher expression associated with shorter OS, correlated with lymph node metastasis	miR-1287	Human, Mouse; *in vitro/in vivo*	Human cell lines – A549, H358, H1299, H1975	+/–	Li Y. et al., 2018
circ_102231	LC	↑(LAC)	Promotes lung cancer cells proliferation, migration and invasion	–	Cell line model (*in vitro* exp)	Human cell lines – BEAS-2B, A549	+/+	Zong et al., 2018a
circRNA_103809	LC	↑	Regulates miR-4302/ZNF121/MYC loop; promotes tumor growth, cell proliferation and invasion, associated with tumor stage and lymph node metastasis	miR-4302	Human, Mouse; *in vitro/in vivo*	Human cell lines – A549, H125, 95D, NCI-H292, H1975, HBE	+/+	Liu W. et al., 2018
	CRC	↓	Promote apoptosis through FOXO4 activity	miR-532-3p	Human; *in vitro*	Human cell lines – SW620, HCT116, COCA-2, HT29, FHC	+/+	Bian et al., 2018
circ_0005962	LC	↑(LAC)	–	–	Human; *in vivo*	–	+/–	Liu X. X. et al., 2019
circ_0086414	LC	↓(LAC)	Associated with EGFR mutations	–	Human; *in vivo*	–	+/–	Liu X. X. et al., 2019
circ-PRMT5	LC	↑(NSCLC)	Correlated with larger tumor, LNM, poor OS and progression free survival; upregulates EZH2	–	Human, Mouse; *in vitro/in vivo*	Human cell lines – HBE, A549, 95-D, HCC827, H1299, SK-MES-1	+/–	Wang Y. et al., 2019
	GC	↑	Promotes GC cell growth, clone formation, migration and invasion and inhibits apoptosis	miR-145, miR-1304	Human; *in vitro*	Human cell lines – AGS, MKN-28, MKN45, BGC823, MGC803, SGC7901, GES-1	+/+	Du et al., 2019
circ-RAD23B	LC	↑(NSCLC)	Regulates miR-593e3p/CCND2 axis; increases cell invasion *via* miR-653e5p/TIAM1 pathway	miR-593e3p, miR-653e5p	Human; *in vitro*	Human cell lines – 16HBE, H1299, H1581, H358, A549	+/+	Han et al., 2019
circ_0102533	LC	↑(NSCLC)	Associated with tumor type, TNM stages, lymph nodes metastasis and distant metastasis or recurrence	–	Human; *in vitro*	Human cell lines – A549, H1299, H1792, SK-MES-1, SPC-A1	+/–	Zhou X. et al., 2018

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TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
circ_0079530	LC	↑	Enhances cell proliferation and invasion	–	Human; *in vitro*	Human cell lines – A549, H1299, H460, Calu1, BEAS-2B	+/–	Li J. et al., 2018a
circFGFR3	LC	↑(NSCLC)	Increases cell invasion and proliferation, regulates Gal-1, pAKT, and p-ERK1/2	miR-22-3p	Human; *in vitro*	Human cell lines – 95C, 95D, A549, H460	+/–	Qiu B. Q. et al., 2019
circ_000984	LC	↑(NSCLC)	Promotes cell proliferation and metastasis; regulates Wnt/β-catenin signaling	–	Human; *in vitro*	Human cell lines – H1975, SPC-A1, H1299, HCC827, PC 9, A549, BEAS-2B	+/–	Li X. et al., 2019
circ_0001946	LC	↑(LAC)	Regulates SIRT1 that activates Wnt/β-catenin signaling pathway	miR-135a-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – H1299, A549, Calu3, SPC-A1, BEAS-2B	+/–	Yao et al., 2019
	GBM	↓	Reduces the migration, invasion, and proliferation of GBM cells	miR-671-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – U87, U251, HM	+/+	Li, 2019a
circ_0037515	LC	↓(NSCLC)	–	–	Human; *in vivo*	–	+/–	Zhao D. et al., 2020
circ_0037516	LC	↓(NSCLC)	–	–	Human; *in vivo*	–	+/-	Zhao D. et al., 2020
circ_0033155	LC	↓(NSCLC)	Reduces cell proliferation, colony formation and migration, correlated with lymphatic metastasis	–	Human; *in vitro*	Human cell lines – HCC827, H1975	+/+	Gu et al., 2018
circ_100395	LC	↓	Promotes LC malignancy regulating miR-1228/TCF21 axis	miR-1228	Human; *in vitro*	Human cell lines – A549, H460, Beas-2B	+/+	Chen D. et al., 2018
circ-FOXO3	LC	↓(NSCLC)	Promotes NSCLC development; releases FOXO3; miR-155 and FOXO transcription factors affect chemoresistance	miR-155	Human; *in vitro*	Human cell lines – A549, SPC-A1, NCI-H1299, NCI-H1650, SK-MES-1	+/+	Zhang Y. et al., 2018
circ_0056616	LC	↑(LAD)	Upregulation is correlated with TNM stage and lymph node metastasis	–	Human; *in vitro*	Human cell lines – PC9, PC14, HEK293T	+/–	He Y. et al., 2020
circ_0010882	GC	↑	Contributes to the proliferation of GC cells, migration, invasion, and apoptosis through modulating PI3K/Akt/mTOR pathway	–	Human; *in vitro*	Human cell lines – HGC-27, MKN-45, SGC-7901, BGC-823, GES-1	+/–	Peng et al., 2020
circ-DCAF6	GC	↑	Enhances GC progression	miR-1231, miR-1256	Human; *in vitro*	Human cell lines – AGS, BGC823, MGC803, GES1	+/–	Wu L. et al., 2019
circ_0000419	GC	↓	Associated with tumor stage, lymphatic and distal metastasis, venous and perineural invasion	hsa-miR-141-5p, hsa-miR-589-3p	Human; *in vitro*	Human cell lines – BGC-823, HGC-27, MGC-803, SGC-7901, GES-1	+/–	Tao et al., 2020
circ_0006156	GC	↓	Associated with lymph node metastasis, nerve invasion and degree of tumor differentiation	–	Human; *in vivo*	–	+/–	He F. et al., 2020
circ_0001821	GC	↓	Negatively associated with tumor depth and lymph node metastasis	–	Human; *in vitro*	Human cell lines – SGC-7901, HGC-27, BGC-823, AGS, MKN-1	+/-	Kong S. et al., 2019
circCCDC9	GC	↓	Upregulation sponges miR-6792-3p that targets CAV1, a tumor suppressor gene	miR-6792-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – GES-1, AGS, BGC-823, HGC-27, MGC-803, MKN-28, MKN-45, SGC-7901	+/+	Luo Z. et al., 2020
circRHOBTB3	GC	↓	Prevents the growth of cells, promotes expression of p21	miR-654-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – AGS, HGC27, MKN45	+/+	Deng et al., 2020
circ_100269	GC	↓	Resists GC development	miR-630	Human; *in vitro*	Human cell lines – AGS, MKN28, MKN45, BGC823, MGC803, SGC7901, GES1	+/+	Zhang Y. et al., 2017
circ_0000745	GC	↓	Associated with tumor differentiation	–	Human; *in vivo*	–	+/–	Huang M. et al., 2017
circPSMC3	GC	↓	Contributes to GC progression by regulating PTEN/miRNA-296-5p axis; PTEN regulates chemoresistance	miRNA-296-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – BGC823, MGC803, SGC7901, AGS, MKN45	+/-	Rong et al., 2019
circ-KIAA1244	GC	↓	Associated with TNM stage, lymphatic metastasis	–	Human; *in vivo*	–	+/–	Tang et al., 2018

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TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
circYAP1	GC	↓	Decreases GC cell growth and invasion; regulates miR-367-5p/p27 Kip1 axis	miR-367-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – GES-1, HGC-27	+/–	Liu H. et al., 2018
circ_0006848	GC	↓	Correlates with tumor differentiation and tumor size	–	–	–	+/–	Lu et al., 2019a
circ_0000520	GC	↓	Associated with TNM stage	–	Human; *in vitro*	Human cell lines – MKN-45, BGC-823, MGC- 80 803, AGS	+/–	Sun et al., 2017
circ_0001895	GC	↓	Associated with cell differentiation, Borrmann type, and tissue CEA expression	–	Human; *in vitro*	Human cell lines – GES-1, AGS, BGC-823, HGC27, MGC-803, SGC-7901	+/–	Shao et al., 2017
circ_0005556	GC	↓	Downregulation correlated with differentiation, TNM stage and lymphatic metastasis	–	Human; *in vivo*	–	+/–	Yang L. et al., 2019
circ_0067582	GC	↓	–	–	Human; *in vivo*	–	+/–	Yu et al., 2020
circ_0000467	GC	↑	Promotes proliferation, migration, and invasion of GC cells,inhibits tumor apoptosis	–	Human; *in vitro*	Human cell lines – HGC-27, MGC-803, AGS, NUGC-3, GES-1	+/–	Lu et al., 2019b
circ_102958	GC	↑	Overexpression is correlated with TNM stage	–	Human; *in vivo*	–	+/–	Wei et al., 2019
circFUT8	BCa	↓	Inhibits migration and invasion of Bca cells through silencing KLF10-mediated Slug signaling, inhibitory effect on lymphatic metastasis	miR-570-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – SV-HUC-1, T24, UM-UC-3	+/–	He Y. et al., 2020
circ_0071662	BCa	↓	Inhibits cell proliferation and invasion; upregulates HPGD and NF2	miR-146b-3p	Human; *in vivo*	–	+/+	Abulizi et al., 2019
circ_0018289	CC	↑	Associated with poor disease free survival	–	Human; *in vivo*	–	+/–	He Q. et al., 2020
circ_0001038	CC	↑	Promotes cell metastasis; suppresses inhibition of oncogenic targets like CNNM3 and MACC1	miR-337-3p	Human; *in vivo*	–	+/–	Wang Y. et al., 2020
circEIF4G2	CC	↑	Induce cell growth and migration	miR-218	Human; *in vitro*	Human cell lines – HeLa, CasKi, C33A, SiHa cells	+/–	Mao et al., 2019
circCLK3	CC	↑	Promotes cell proliferation, EMT, migration and invasion	miR-320a	Human, Mouse; *in vitro/in vivo*	Human cell lines – SiHa, HeLa, CaSki, C-33A, MS751	+/–	Hong et al., 2019
circ_0000388	CC	↑	Induces proliferation, migration, invasion,inhibit apoptosis; regulates miR-377-3p/TCF12 axis	miR-377-3p	Human; *in vitro*	Human cell lines – HeLa, SiHa	+/–	Meng et al., 2020
circ_0101996	CC	↑	–	–	Human; *in vivo*	–	+/–	Wang Y-M. et al., 2017
circ_0101119	CC	↑	–	–	Human; *in vivo*	–	+/–	Wang Y-M. et al., 2017
circ_0104649	CC	↑	–	–	Human; *in vivo*	–	–	Wang Y-M. et al., 2017
circ_0104443	CC	↑	–	–	Human; *in vivo*	–	–	Wang Y-M. et al., 2017
circFoxO3a	CC	↓	Correlates with stromal invasion, positive lymph node metastasis and poor prognosis	–	Human; *in vivo*	–	+/–	Tang et al., 2020
circ_0081001	OSC	↑	Overexpression was associated with poor prognosis	–	Human; *in vitro*	Human cell lines – MG63,KHOS,U2OS	+/–	Kun-peng et al., 2018a
circ_0002052	OSC	↓	Overexpression suppresses OS cell proliferation, migration and invasion while promoting apoptosis; regulates *circ_0002052*/miR-1205/APC2/Wnt/b-catenin pathway	miR-1205	Human; *in vitro*	Human cell lines – hFOB 1.19, 293T	+/+	Wu Z. et al., 2018
circ-SLC7A5	ESCC	↑	Overexpression correlated with TNM stage and poor OS	–	Human; *in vitro*	Human cell lines – K30, K70, K140, K180, K150, K450, T10, T12w	+/–	Wang Q. et al., 2020
circ_0004771	ESCC	↑	Increases the expression of CDC25	miR-339-5p	Human; *in vitro*	Human cell lines – FHC, HCT-116, SW480	+/–	Huang E. et al., 2020

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TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
	CRC	↑	–	–	Human; *in vitro*	Human cell lines – FHC, HCT-116, SW480	+/–	Pan et al., 2019
circ_0092125	OSCC	↓	Correlated with tumor size, TNM stage, and lymph node metastasis	-	Human; *in vitro*	Human cell lines – SCC15, SCC25, CAL27	+/–	Gao et al., 2020
circ_0001874	OSCC	↑	Associated with TNM stage and tumor grade	–	Human; *in vivo*	–	+/–	Zhao S. Y. et al., 2018
circ_0001971	OSCC	↑	Upregulation is associated with TNM stage	–	Human; *in vivo*	–	+/–	Zhao S. Y. et al., 2018
circ-CCND1	LSCC	↑	Improve the stability of CCND1 mRNA; increases LSCC growth	HuR; miR-646	Human, Mouse; *in vitro/in vivo*	Human cell lines –AMC-HN-8, Hep-2, LSC-1, TU212, TU177, TU686, SCC10A, NP-69	+/–	Zang et al., 2020
circFLNA	LSCC	↑	Induces migration of LSCC cells by targeting miR486-3p/FLNA axis; high level of FLNA implicates poor survival and drug resistance	miR486-3p	Human; *in vitro*	Human cell lines –Tu212, SCC-2, SCC40	+/+	Wang J. X. et al., 2019
circMATR3	*HSCC*	↑	Upregulation of oncogene USP28 that contributes to MYC stability	miR-188-5p, miR-448	Human; *in vitro*	Human cell line - FaDu	+/–	Wang Z. et al., 2020
circMORC3	*HSCC*	↓	Associated with T stages and tumor sizes	–	–	–	+/–	Zheng and Chen, 2020
circMYBL2	AML	↑	Increases the translational efficiency of FLT3 kinase; knockdown impairs the cytoactivity of FLT3-ITD AML cells, including quizartinib-resistant cells	–	Human, Mouse; *in vitro/in vivo*	Human cell lines – MV4-11, MOLM-13,THP-1, HL60, NB4 and ML-2,U937	+/+	Sun et al., 2019
Circ-DLEU2	AML	↑	Induces cell proliferation and reduces apoptosis	miR-496	Human, Mouse; *in vitro/in vivo*	Human cell lines – MOLM-13, HL-60, MV-4-11	+/+	Wu D. M. et al., 2018
f-circPR	AML	↑	Promoted cell proliferation	–	–	–	+/+	Guarnerio et al., 2016
f-circM9	AML	↑	Favors leukemia progression	–	–	–	+/+	Guarnerio et al., 2016
circ_001569	CRC	↑	Promotes cell proliferation and invasion	miR-145	Human; *in vitro*	Human cell lines – SW480, HCT116, SW620, LOVO	+/+	Xie et al., 2016
circ_0007534	CRC	↑	Increase in the Bcl2/Bax ratio in CRC cells and inhibits apoptosis	–	Human; *in vitro*	Human cell lines – SW620, HCT116, LoVo, SW480, HT29	+/+	Zhang R. et al., 2018
circ_0000069	CRC	↑	Knockdown induces G0/G1 arrest and inhibits cancer progression	–	Human; *in vitro*	Human cell lines – HT29, LoVo, HCT-116, SW480	+/+	Guo et al., 2016
circ_0020397	CRC	↑	Upregulates TERT and thereby induces cell proliferation	miR-138	Human; *in vitro*	Human cell lines – LoVo, HCT116, SW480, SW620	+/+	Zhang X. et al., 2017
circBANP	CRC	↑	Promotes CRC cell proliferation; induces p-Akt protein expression	–	Human; *in vitro*	Human cell lines – HT29, HCT116	+/+	Zhu M. et al., 2017
circ5615	CRC	↑	promotes CRC progression through miR-149-5p/TNKS axis	miR-149-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – HCT 116, LoVo, HT-29, SW480, NCM460	+/+	Ma et al., 2020
circPTK2	CRC	↑	Promotes EMT of CRC cells *via* expression of mesenchymal marker vimentin	–	Human, Mouse; *in vitro/in vivo*	Human cell lines – HCT15, SW620, SW480, LOVO	+/+	Yang H. et al., 2020
	LC	↓(NSCLC)	Overexpression augments T1F1γ expression, reduces TGF β induced EMT	miR-429/miR-200b3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – BEAS-2B, A549, H1299, H1650, SPC-A1, Calu3,H226, H520, SK-MES-1	+/+	Wang L. et al., 2018
circ_0060745	CRC	↑	Promotes CSE1L-mediated CRC cell proliferation and metastasis	miR-4736	Human; *in vitro*	Human cell lines – NCM460, HT29, LOVO, PKO, SW480	+/+	Wang and Wang, 2020
circ_0008285	CRC	↓	Inhibits CRC cell proliferation and migration; regulates PI3K/AKT pathway	miR-382-5p	Human; *in vitro*	Human cell lines – SW480, RKO, HCT8, SW620, HCT116, DLD1,FHC	+/+	Wang et al., 2020
circ-0001313	CRC	↑	Inhibits apoptosis regulating PI3K/Akt signaling pathway	miRNA-510-5p	Human; *in vitro*	Human cell lines – SW620, HCT116, SW480, HT-29, LoVo, NCM460	+/+	Tu et al., 2020

(Continued)

TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
circDDX17	CRC	↓	inhibits cell proliferation, migration, invasion, and promotes apoptosis	hsa-miR-21-5p	Human; *in vitro*	Human cell lines – SW480, SW620, HT29, LoVo, HCT116, RKO	+/+	Li X-N et al., 2018
circ-ABCB10	BC	↑	Knockdown suppresses proliferation and induces apoptosis	miR-1271	Human; *in vitro*	Human cell lines – MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-453	+/+	Liang et al., 2017
circEHMT1	BC	↓	Inhibits metastasis by regulating circEHMT1/miR-1233-3p/KLF4 axis	miR-1233-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – ZR-75-1, MCF-7, MB-468, T47D, SK-BR3, MDA-MB-231, BT-549, HMEpC	+/+	Lu M. et al., 2020
circ_0011946	BC	↑	Promotes migration and invasion	miR26a/b	Human; *in vitro*	Human cell lines – HS-578T, T47D, MCF-7, BT549, MDA-MB-231, SKBR-3	+/+	Zhou J. et al., 2018
circGFRA1	BC	↑(TNBC)	Promotes cell proliferation and inhibits apoptosis; regulates GFRA1 expression	miR-34a	Human, Mouse; *in vitro/in vivo*	Human cell lines – MCF10A, SKBR3, T47D, BT474, MCF-7, BT-483, BT-20, BT549, MDA-MB-468, MDA-MB-231	+/+	He et al., 2017
circ_0001982	BC	↑	Promotes tumorigenesis	miR-143	Human; *in vitro*	Human cell lines – MDA-MB-231, MCF-7, MDAMB-468, MDA-MB-435s	+/+	Tang et al., 2017
circTADA2A	BC	↓	Possesses tumor-suppressor capability, restores the expression of SOCS3, suppressed cell proliferation, migration, invasion, clonogenicity	miR-203a-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – MCF-7 MDA-MB-231	+/+	Xu et al., 2019
circ-10720	HCC	↑	Promotes migration, invasion and EMT by stabilizing vimentin	miR-1246, miR-578, miR-490-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – PLC-PRF-5, SMMC-7721, HEK-293T	+/+	Meng et al., 2018
circPTGR1	HCC	↑	Knockdown promotes expression of epithelial markers and reduces the levels of mesenchymal markers	miR-449a	Human, Mouse; *in vitro/in vivo*	Human cell lines – HepG2, L-O2, SMCC7721, HEP3B, HUH7, MHCC97-L, MHCC 97H, HCC-LM3	+/+	Wang G. et al., 2019
circTRIM33–12	HCC	↓	Upregulates TET1 expression; suppresses tumor proliferation, migration, invasion	miR-191	Human, Mouse; *in vitro/in vivo*	Human cell lines – MHCC97-L, HCC97-H, HCCLM3, SMMC-7721	+/+	Zhang P. F. et al., 2019
circ-BIRC6	HCC	↑	Knockdown reduces Bcl2 mRNA and protein levels	miR3918	Human, Mouse; *in vitro/in vivo*	Human cell lines – SKHEP-1, Huh-7 MHCC97H	+/+	Tang et al., 2015
circ_0070269	HCC	↓	Increases expression of NPTX1,that inhibits aggressive tumor behavior	miR182	Human, Mouse; *in vitro/in vivo*	Human cell lines – Hep3B, SMMC-7721, HepG2, PLC, Huh-7,LO2	+/+	Zhang P. F. et al., 2019
circADAMTS13	HCC	↓	Acts as a tumor suppressant; inhibits HCC proliferation	miR-484	Human; *in vitro*	Human cell lines – PLC/PRF/5, SK-Hep-1, Hep3B, HepG2	+/+	Qiu L. et al., 2019
cZNF292	GBM	↑	Promotes angiogenesis; regulates STAT3/5/β-catenin pathway	–	Human; *in vitro*	Human cell lines – U87MG and U251	+/+	Yang P. et al., 2019
circ_0037251	GBM	↑	Enhances GBM progression, upregulates mTOR; inhibits cell apoptosis and G1 phase arrest	miR-1229-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – U373, U251, HEK293T	+/+	Cao et al., 2019
circMAPK4	GBM	↑	Suppress apoptosis through decreased phosphorylation of p38/MAPK	miR-125a-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – U138, U373, U87	+/+	He et al., 2020a

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TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
circ-U2AF1	GBM	↑	Enhances cell proliferation, migration, and invasion, increases expression of NOVA2	miR-7-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – U87MG, U251, U87, HEB	+/+	Li, 2019a
circNT5E	GBM	↑	Upregulates NT5E, SOX4, PI3KCA; promotes cell proliferation, migration, and invasion	miR-422a	Human, Mouse; *in vitro/in vivo*	Human cell lines – U87, U251	+/+	Wang R. et al., 2018a
circ_0029426	GBM	↑	Promotes cell proliferation, migration and invasion, and inhibits cell apoptosis	miR-197	Human; *in vitro*	Human cell lines – U87, U251, LN229, U87MG, A172, NHA	+/+	Zhang G. et al., 2019
circ-TTBK2	GBM	↑	Regulates *circ-TTBK2*/miR-217/HNF1β/Derlin-1 axis; promotes cell proliferation, migration, and invasion, while inhibiting apoptosis	miR-217	Human, Mouse; *in vitro/in vivo*	Human cell lines – U87, U251, HEK293T	+/+	Zheng et al., 2017
circMMP9	GBM	↑	Upregulates the expression of (CDK4) and aurora kinase A; promotes proliferation, migration and invasion abilities	miR-124	Human, Mouse; *in vitro/in vivo*	–	+/+	Wang R. et al., 2018b
circ_0020123	LC	↑(NSCLC)	Upregulates ZEB1 and EZH2 for tumor growth and EMT	miR-144	Human, Mouse; *in vitro/in vivo*	Human cell lines – PC9, H1573, A549, SK-MES-1, H1299, Calu-3	+/+	Qu et al., 2018
f-circEA-4a	LC	↑(NSCLC)	Induces cell proliferation, metastasis and invasion	–	Human; *in vitro*	Human cell lines – A549, HT1299	+/+	Tan et al., 2018
f-circEA-2a	LC	↑(NSCLC)	Promotes cell migration and invasion	–	Human; *in vitro*	Human cell lines – A549, HT1299	+/+	Tan et al., 2018
circ_104916	GC	↓	Inhibits cell proliferation, migration and EMT	–	Human, Mouse; *in vitro/in vivo*	Human cell lines – AGS, MKN-28, NCI-N87, MKN-45,GES1	+/+	Li J. et al., 2017
circPDSS1	GC	↑	Enhances expression of NEK2 leading to cell migration and proliferation	miR-186-5p	Human; *in vitro*	Human cell lines – MGC-803, HGC-27, BGC-823, GES-1	+/+	Ouyang et al., 2019
circ_0023642	GC	↑	Promotes cell proliferation and metastasis; upregulates N-cadherin, Vimentin and Snail expression	–	Human; *in vitro*	Human cell lines – MGC-803, MNK-45, SGC-7901, HGC-27, GES1	+/+	Zhou L. H. et al., 2018
circATAD1	GC	↑	Increases cell progression, upregulates YY1	miR-140-3p	Human; *in vivo*, *in vitro*	Human cell lines – GES1, SGC7901, BGC-823, AGS, MGS-803	+/+	Zhang L. et al., 2020
circFN1	GC	↑	Promotes viability and inhibits apoptosis; facilitates CDDP resistance *in vitro*	miR-182-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – SGC7901CDDP, BGC823C DDP, SGC7901, SGC823)	+/+	Huang X. X. et al., 2020
circCACTIN	GC	↑	Induces EMT and regulates Smad signaling, promote metastatic conversion, angiogenesis	miR-331-3p	Human, Mouse; *in vitro/in vivo*	Human cell lines – GES1, BGC-823, MGC-803, SGC-7901c	+/+	Zhang L. et al., 2019
circ-CEP85L	GC	↓	Inhibits tumor growth, proliferation and invasion of GC cells	miR-942-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – MGC-803, AGS, KATOIII, BGC-823, HGC-27, MKN-45	+/+	Lu J. et al., 2020
circMYLK	BCa	↑	Augments proliferation, migration, the tube formation of HUVEC, and EMT; stabilizes VEGFA	miR-29a	Human, Mouse; *in vitro/in vivo*	Human cell lines – EJ, T24, 5673, BIU-87	+/+	Zhong et al., 2017
circACVR2A	BCa	↓	Upregulates EYA4 expression; suppresses proliferation, migration and invasion and metastasis through miR-626/EYA4 axis	miR-626	Human, Mouse; *in vitro/in vivo*	Human cell lines – T24, UM-UC-3, RT4, J82, 5637, HT-1376, TCCSUP, SV-HUC-1	+/+	Dong et al., 2019
circ_0061140	Ovarian cancer	↑	Regulates miR370/FOXM1 pathway; promotes cell proliferation, migration, and the EMT	miR-370	Human, Mouse; *in vitro/in vivo*	Human cell lines – SKOV3, A2780, OV2008, IGROV1, ES-2	+/+	Chen Q. et al., 2018
circUBAP2	OSC	↑	Stable expression of Bcl-2; promotes OS growth and inhibits apoptosis	miR-143	Human, Mouse; *in vitro/in vivo*	Human cell lines – hFOB 1.19, MG63, U2OS	+/+	Zhang H. et al., 2017
circ_001564	OSC	↑	Inhibits cell cycle arrest in G0/G1 phase and apoptosis	miR-29c-3p	Human; *in vitro*	Human cell lines – U2OS, Saos-2, HOS, MG-63	+/+	Song and Li, 2018

(Continued)

TABLE 1 | Continued

circRNA	Associated Cancer (s)	Regulation (Up ↑, Down↓)	Biological Function	Target (s)	Type of model	Type of cell line (s)	Biomarker/ therapeutics	References
circNASP	OSC	↑	Augments FOXF1 expression that leads to proliferation and invasion of OS cells, positively correlates with the tumor size and metastasis	miR-1253	Human; *in vitro*	Human cell lines – 143B and MG63	+/+	Huang et al., 2018
circ_0000337	ESCC	↑	Promotes cell proliferation, migration, and invasion	miR-670-5p	Human; *in vitro*	Human cell lines – KYSE-150, TE-1, HET-1A	+/+	Song et al., 2019
circUHRF1	OSCC	↑	Modulates the transcription factor c-Myc; regulates circUHRF1/miR-526b-5p/c-Myc/TGF-β1/ESRP1 axis	miR-526b-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – SCC25, CAL27, SCC15, TSCCA	+/+	Zhao W. et al., 2020
circ_0082182	CRC	↑	Correlated with lymph node metastasis	–	Human; *in vitro*	Human cell lines – HCT116, SW480, SW620, NCM460	+/–	Ye D. et al., 2019
circ_0000370	CRC	↑	Correlated with lymph node metastasis	–	Human; *in vitro*	Human cell lines – HCT116, SW480, SW620, NCM460	+/–	Ye D. et al., 2019
circ_0035445	CRC	↓	Correlated with TNM stage	–	Human; *in vitro*	Human cell lines – HCT116, SW480, SW620 and normal cell line-NCM460	+/–	Ye D. et al., 2019
circ5615	CRC	↑	Promotes CRC progression through miR-149-5p/TNKS axis	miR-149-5p	Human, Mouse; *in vitro/in vivo*	Human cell lines – HCT 116, LoVo, HT-29, and SW480, NCM460 and HEK-293T	+/+	Ma et al., 2020
circ_0060745	CRC	↑	Regulates miR-4736/SCE1L	miR-4736	Human; *in vitro*	Human cell lines – human colon epithelial cell line (NCM460) and human CRC cell lines (HT29, LoVo, PKO, and SW480)	+/–	Wang and Wang, 2020
circ_0026344	CRC	↓	Correlated with metastasis	miR-21,miR-31	Human, Mouse; *in vitro/in vivo*	Human cell lines – HCT116, SW480, HT29, SW620, NCM460, FHC, HEK293T	+/–	Yuan et al., 2018
circ_0000567	CRC	↓	Correlated with lymph metastasis, distal metastasis and TNM stage	–	Human; *in vitro*	Human cell lines – FHC, SW480, RKO, CACO2, SW620, HCT116	+/–	Wang J. et al., 2018
circ_0003906	CRC	↓	Correlated with poor differentiation and lymphatic metastasis	–	Human; *in vitro*	Human cell lines: NCM460, SW480, SW620, HCT8, HCT116, HT29, LoVo	+/–	Zhuo et al., 2017

AML, Acute Myeloid Leukemia; CML, Chronic Myeloid Leukemia; CLL, Chronic Lymphocytic Leukemia; MM, Multiple Myeloma; BCL, B cell Lymphoma; CRC, Colorectal Cancer; BC, Breast Cancer; HCC, Hepatocellular Carcinoma; GBM, Glioblastoma; LC, Lung Cancer; NSCLC, Non-Small Cell Lung carcinoma; LAC, Lung Adenocarcinoma; GC, Gastric Cancer; BCa, Bladder Cancer; CC, Cervical Cancer; OSC, Osteosarcoma; ESCC, Esophageal Squamous Cell Cancer; OSCC, Oral Squamous Cell Carcinoma; LSCC, Laryngeal Squamous Cell Cancer; HSCC, Hypopharyngeal Squamous Cell Carcinoma.

to serve as biomarkers for cancer and are summarized and cataloged in **Table 1**.

Hematological Malignancies

Acute Myeloid Leukemia (AML)

Circ_0009910 is overexpressed in the bone marrow of AML patients, which correlates with poor overall survival (OS) (Ping et al., 2019a). It sponges miR-20a-5p and knocking it down induces apoptosis in AML cells (Ping et al., 2019a). *Circ-vimentin* is upregulated in AML patients and its elevated expression is an independent poor prognostic factor for OS and leukemia-free survival (LFS) in AML patients (Yi and Lin, 2018). Hirsh et al. examined the expression of *circ_0075001* in a cohort of NPM1 wild-type and mutated AML patients and found it to be positively correlated with expression of the cognate linear RNA but independent of the NPM1 mutational status (Hirsch et al., 2017). However, high *circ_0075001* expression levels defined patient subgroups characterized by lower expression of components of the Toll-like receptor (TLR) signaling pathway which is associated with a more immature AML phenotype (Hirsch et al., 2017). C*irc_0004277* was downregulated in AML patients expression, however, its expression is restored in AML patients subjected to chemotherapy indicating it as a potential diagnostic marker and treatment target in AML (Li W. et al., 2017).

Chronic Myeloid Leukemia (CML)

C*irc_100053* was upregulated in peripheral blood mononuclear cells and serum of CML patients and was associated with clinical stage and BCR/ABL mutation status (Ping et al., 2019b).

Elevated *CircRNA_100053* levels predicted a poor outcome in CML patients and imatinib resistance (Ping et al., 2019b).

Chronic Lymphocytic Leukemia (CLL)

Circ-RPL15 was upregulated in CLL patients and correlated with poor OS and immunoglobulin heavy-chain variable region (IGHV) mutation used in the validation of CLL prognosis (Wu Z. et al., 2019). *Circ-RPL15* sequesters miR-146b-3p and activates RAS/RAF1/MEK/ERK pathway to promote CLL development (Wu Z. et al., 2019). *Circ-CBFB* levels are also elevated in CLL patients and its expression can distinguish CLL patients from healthy controls (Xia L. et al., 2018). It sponges miR-607, which targets FZD3, an activator of Wnt/β-catenin signaling in CLL (Xia L. et al., 2018). Higher expression of *circ-CBFB* predicted reduced OS in CLL patients and may serve as a prognostic marker for CLL (Xia L. et al., 2018).

Multiple Myeloma (MM)

Elevated expression of *circ_0007841* in MM correlated with chromosomal aberrations such as gain 1q21, t (4:14), mutations in ATR, and IRF4 genes, however, its function in MM needs further investigation (Gao et al., 2019). Feng et al. observed lower levels of *circ_0000190* in MM tissues and peripheral blood, which correlated with the prognosis and OS of MM patients (Feng et al., 2019). C*irc-SMARCA5* is downregulated in MM and its higher expression correlates with lower β2-microglobulin (MG) level and less advanced International Staging System stage (Liu H. et al., 2019). C*irc-SMARCA5* downregulation correlates with reduced OS, progression-free survival (PFS), and treatment response in MM patients (Liu H. et al., 2019). *CircRNA_101237* level was upregulated in MM patients and has high diagnostic accuracy for MM (Liu and Wang, 2020). Its expression was elevated in patients with 13q14 deletion, 1q21 amplification, p53 deletion, and t(4,14) and t(14,16) gene mutations, but was decreased in those with t(11,14) gene mutations; also the upregulation was associated with a poor response to chemotherapy (Liu and Wang, 2020).

B Cell Lymphoma (BCL)

Circ-APC (circ_0127621) was downregulated in diffuse large B-cell lymphoma (DLBCL) and its levels in plasma can distinguish patients from healthy controls (Hu et al., 2019). Moreover, DLBCL patients with lower *circ-APC* levels were more likely to exhibit an advanced Ann Arbor stage, shorter OS, resist chemotherapy and display a low International Prognostic Index (Hu et al., 2019).

Solid Tumors

Colorectal Cancer (CRC)

CDR1as was upregulated in CRC tissues and its overexpression correlated with poor survival (Weng et al., 2017). Its upregulation is an independent risk factor for OS and enhanced EGFR/RAF1/MAPK pathway by inhibiting miR-7 tumor-suppressor activity (Weng et al., 2017). Similarly, *circCCDC66* was also elevated in CRC patients and its high expression correlated with poor prognosis (Hsiao et al., 2017). It sponges miRNA-33b and miR-93 to promote CRC growth and metastasis by stabilizing MYC mRNA (Hsiao et al., 2017). Increased expression of *circ_0004585* in the CRC tissues was associated with increased tumor size (Tian et al., 2019). *Circ_0007142* (Zhu et al., 2019). *Circ_0007142* regulates invasion of CRC by sponging miR-103a-2-5p and its upregulation, associated with poor differentiation and lymphatic metastasis (Zhu et al., 2019). Upregulation of *circ-HUEW1* in CRC tissues was associated with lymphovascular invasion, lymph node and distant metastasis, and Tumor, Node, and Metastasis (TNM) stage (Chen H. Y. et al., 2020). It sponges miR-486 and regulates the IGF2/β-catenin signaling pathway by targeting PLAGL2 (Chen H. Y. et al., 2020). CRC patients with higher *circ_0001178* were more likely to have metastatic features, advanced TNM stage, and adverse prognosis (Ren et al., 2020). It sponges miR-382, miR-587, and miR-616, all of which target ZEB1 (Ren et al., 2020). *Circ_0005075* was also highly expressed in CRC and is associated with depth of invasion and advanced TNM stage, and is a prognostic factor affecting both OS and disease-free survival (DFS) in CRC patients (Jin et al., 2019). *CircHIPK3* is upregulated in CRC tissues and serves as an independent prognostic factor of poor OS and positively correlates with metastasis and advanced clinical stage (Zeng et al., 2018). *Circhipk3* sponges miR-7 and promotes CRC progression by increasing the expression of its target genes FAK, IGF1R, EGFR, and YY1 (Zeng et al., 2018). Ye et al. discovered a 3-circRNA signature as a non-invasive biomarker for CRC diagnosis, they observed elevated expression of *circ_0082182* and *circ_0000370*, and downregulated *circ_0035445* levels in the plasma of CRC patients (Ye D. et al., 2019). The upregulation of *circ_0082182* and *circ_0000370* was strongly associated with lymph node metastasis, while the *circ_0035445* downregulation was connected with the TNM stage (Ye D. et al., 2019). Also, *circ_0082182* and *circ_0035445* showed a difference between preoperative and postoperative stages, while *circ_0000370* had no significant difference between these two stages (Ye D. et al., 2019). *Circ_0007534* was upregulated in plasma of CRC patients, which correlated with the progression of clinical classifications, metastatic phenotype, poor differentiation, and poor prognosis in CRC patients (Zhang W. et al., 2018). Increased *circ_0007534* expression was associated with poor prognosis in CRC patients (Zhang W. et al., 2018). Overexpression of *circFADS2* was closely related to the size, differentiation, infiltration depth, lymphatic and distant metastasis, and TNM stage of CRC patients (Xiao et al., 2020). Patients with increased *circFADS2* levels had a poor OS and had a better predictive value when combined with the TNM stage (Xiao et al., 2020). *Circ5615* was upregulated in CRC was an independent prognosis factor for CRC, and it was associated with a higher T stage and poor OS in CRC patients (Ma et al., 2020). Patients with elevated expression of *circ-FARSA* had a poor OS and the circRNA promotes CRC progression by regulating the miR-330-5p/LASP1 axis (Lu C. et al., 2020). Overexpression of *circ_0060745* in CRC tissues was significantly associated with shorter OS, advanced clinical stage, nodal classification, metastasis classification, and liver metastasis (Wang and Wang, 2020). It promoted CRC progression by sponging miR-4736 and regulating SCE1L expression (Wang and Wang, 2020). Downregulation of *circ_0001649* in CRC tissues and patient serum was negatively associated with CRC

differentiation (Ji et al., 2018). *CircITGA7* was downregulated in CRC tissues and was negatively associated with tumor size, lymph metastasis, distant metastasis, and TNM stage (Li X. et al., 2018a). It sponges miR-370-3p and inactivates Ras signaling pathway by upregulating neurofibromin 1 (NF1) (Li X. et al., 2018a). *Circ_0000711* was downregulated in CRC tissues and could act as a diagnostic marker for CRC (Li J. et al., 2018b). Downregulation of *circ_0014717* in CRC tissues was associated with distant metastasis, TNM stage, and poor OS (Wang F. et al., 2018). Its overexpression promoted cell-cycle arrest by increasing p16 expression leading to reduced growth and invasion of CRC cells (Wang F. et al., 2018). *Circ_0004771* was upregulated in serum exosomes of CRC patients and had good diagnostic potential (Pan et al., 2019). *Circ_0026344* was downregulated in CRC samples of stage III/IV as compared to tissues with stage I/II (Yuan et al., 2018). Lower *circ_0026344* expression correlated with metastasis and predicted poor prognosis in CRC patients (Yuan et al., 2018). *Circ_0000567* expression was downregulated in CRC tissues and was associated with tumor size, lymph metastasis, distal metastasis, and TNM stage (Wang J. et al., 2018). *Circ_0003906* was downregulated in CRC tumors and its lower expression was associated with a higher incidence of poor differentiation, lymphatic metastasis, and is an independent risk factor for survival of CRC patients (Zhuo et al., 2017). Analysis of serum from CRC patients revealed overexpression of *exo-circ-PNN* and it can serve as a potential non-invasive biomarker for CRC detection (Xie Y. et al., 2020).

Breast Cancer (BC)

C*ircFBXW7* was downregulated in triple-negative breast cancer (TNBC) and was correlated with poor clinical outcomes (Ye F. et al., 2019). Its expression was also negatively associated with tumor size, lymph node metastasis, and is an independent prognostic factor for TNBC (Ye F. et al., 2019). *CircFBXW7* sponges miR-197-3p and also encodes for a microprotein FBXW7-185aa that upregulates the tumor-suppressor, FBXW7 (Ye F. et al., 2019). The upregulation of *circSEPT9* in TNBC tissues was associated with advanced clinical stage and poor prognosis (Zheng et al., 2020). It decoys miR-637 and modulates, leukemia inhibitory factor (LIF) expression to activate TNBC progression (Zheng et al., 2020). *Circ_0001785* was upregulated in BC plasma samples and correlated with histological grade, TNM stage, and distant metastasis (Yin et al., 2018). Autophagy-associated *circCDYL* was upregulated in tissues and serum from BC patients (Liang et al., 2020). Higher *circCDYL* levels were associated with estrogen receptor (ER) negative status, higher Ki67 index, larger tumor size, and more lymphatic metastasis (Liang et al., 2020). Moreover, BC patients with high serum *circCDYL* had a poorer OS compared to early BC and benign patients (Liang et al., 2020). *CircKIF4A* was overexpressed in TNBC tissues, which correlated with tumor size, lymph node metastasis, and TNM stage (Tang et al., 2019). It induced TNBC cell proliferation and migration by sponging miR-375 and regulating KIF4A expression (Tang et al., 2019). The higher expression levels of *circKIF4A* correlated with poor OS in TNBC patients (Tang et al., 2019). *CircPLK1* was also upregulated in TNBC tissues and correlated with larger tumor size, lymph node positivity, advanced TNM stage and poor OS (Kong Y. et al., 2019). It promotes TNBC metastasis by sponging miR-296-5p and regulating PLK1 expression (Kong Y. et al., 2019). Song et al. observed a higher expression of cytoplasmic *circHMCU* in BC tissues (Song et al., 2020). *CircHMCU* sequestered members of let-7 family and enhanced proliferation and metastasis in BC (Song et al., 2020). Its upregulation was associated with histological grade, lymph node metastasis, TNM stage, and poor prognosis (Song et al., 2020). Downregulation of *circ_0068033* in BC tissues was associated with tumor size and TNM stage (Yuan et al., 2020). It sequesters miR-659 and its overexpression induces apoptosis (Yuan et al., 2020). Overexpression of *circGFRA1* in TNBC tissues was associated with tumor size, TNM staging, lymph node metastasis, and histological grade (He et al., 2017). Patients with upregulated *circGFRA1* had shorter OS and DFS (He et al., 2017). *CircGFRA1* increases proliferation and inhibit apoptosis by regulating the expression of its cognate gene GFRA1 by sponging miR-34a (He et al., 2017). *CircTADA2A-E6* and *circTADA2A-E5/E6* generated from the *TADA2A* gene were downregulated in BC (Xu et al., 2019). Lower expression of *circTADA2A-E6* was associated with increased lymphatic metastasis and advanced clinical stage (Xu et al., 2019). BC patients with downregulated *circTADA2A-E6* had a poor prognosis with shorter DFS and OS, whereas no association was identified between DFS or OS and *circTADA2A-E5/E6* levels (Xu et al., 2019).

Hepatocellular Carcinoma (HCC)

The upregulation of *circ_0005075* in HCC tissues was associated with tumor size and had good diagnostic potential (Shang et al., 2016). It decoys miR-23b-5p, miR-93-3p, miR581, and miR-23a-5p and contributes to HCC proliferation, invasion, and metastasis (Shang et al., 2016). Overexpression of *circ_100338* was related to low OS and metastatic progression in HCC patients with HBV infection (Huang X. Y. et al., 2017). It decoys miR-141-3p and increases metastatic progression in HCC (Huang X. Y. et al., 2017). *CircRHOT1* was upregulated in HCC, its expression was higher in stage III HCC tissues than in stage I/II (Wang L. et al., 2019). HCC patients with higher *circRHOT1* expression had poor prognosis (Wang L. et al., 2019). *Circ_0091579* was overexpressed in HCC tissues and its upregulation was associated with poor OS of HCC patients (Zhang C. et al., 2018). Interestingly, exposure of HCC samples to cisplatin upregulated *circRNA_101237*, and its expression correlated with tumor size, lymph node metastasis, distant metastasis, and TNM stage (Zhang C. et al., 2018). *CircRNA_101237* was upregulated in HCC tissues and serum samples, and was correlated with tumor size, lymph node metastasis, distant metastasis, and TNM stage (Zhou et al., 2020). Elevated serum *circRNA_101237* levels was an independent predictor of poor OS and prognosis in HCC patients (Zhou et al., 2020). *Circ-HOMER1* was also upregulated in HCC tissues and associated with larger tumor size, higher TNM stage, and poor prognosis (Zhao M. et al., 2020). It decoys miR-1322 and upregulates CXCL6 (Zhao M. et al., 2020). *Circ_0016788* was upregulated in HCC tissues and was associated with poor OS, higher performance status score, larger tumor size, increased Barcelona clinic liver cancer (BCLC) stage, abnormal

aspartate aminotransferase, abnormal alpha-fetoprotein and abnormal carbohydrate antigen 199 levels (Cheng et al., 2020). *CDR1as* was upregulated in HCC samples and was one of the independent factors of hepatic microvascular invasion and had the potential predictive ability (Xu et al., 2017). *CDR1as* promotes HCC progression by activating PI3K/AKT/mTOR pathway by sponging miR-7 (Xu et al., 2017). *Circ_0078602* was downregulated in HCC tissues and was associated with poor prognosis (Kou et al., 2019). *CircC3P1* was also downregulated in HCC and was negatively correlated with TNM stage, tumor size, vascular invasion, and lower OS in HCC patients (Zhong et al., 2018). It sponges miR-4641 which targets PCK1 (Zhong et al., 2018). *Circ_0001649* was downregulated in HCC and the decreased expression associated with tumor size and occurrence of tumor embolus (Qin et al., 2016). Guo et al. observed the downregulation of *circ-ITCH* in HCC tissues, which correlated with the poor OS, whereas upregulated *circ-ITCH* associated with favorable survival in HCC patients (Guo et al., 2017). *CircMTO1* was downregulated in HCC tissues and its decreased expression was associated with poor prognosis of HCC patients (Han et al., 2017). Downregulation of *circTRIM33-12* was observed in HCC tissues and associated with tumor proliferation, migration, invasion, and immune evasion, and it also served as an independent risk factor for OS and recurrence-free survival (RFS) of HCC patients after surgery (Zhang P. F. et al., 2019). *CircTRIM33-12* reduces HCC metastasis and immune evasion by upregulating TET1 expression by sponging miR-191 (Zhang P. F. et al., 2019). *CircADAMTS13* was downregulated in HCC tissues and this correlated with the absence of liver cirrhosis, larger tumor size, more severe BCLC stage, and poor patient prognosis (Qiu L. et al., 2019). *CircADAMTS13* serves as a tumor-suppressor by sponging miR-484 (Qiu L. et al., 2019). *Circ_0070269* was downregulated in HCC tissues and its low expression was correlated with advanced TNM stage, large tumor size, lymph node metastasis, poor OS, and metastasis-free survival of HCC patients (Xiaotong et al., 2019). *Circ_0070269* inhibits HCC progression by regulating the miR-182/NPTX1 axis (Xiaotong et al., 2019). Lower expression levels of *circSMARCA5* in tissues and plasma samples of HCC patients has good diagnostic potential (Li Z. et al., 2019). Downregulation of *circSMARCA5* was associated with tumor differentiation, TNM stage, cancer invasion, and cancer diameter (Li Z. et al., 2019). Adipose-secreted exo-circ-deubiquitination (*circ-DB*) was upregulated in HCC patients with higher body fat ratios (Zhang H. et al., 2019). It promotes HCC growth and reduces DNA damage by suppression of miR-34a and the activation of USP7 (Zhang H. et al., 2019). Depletion of *circ-DB* suppressed HCC growth and metastasis *in vivo* (Zhang H. et al., 2019).

Glioblastoma (GBM)

Lyu et al. using circRNA microarrays identified several differentially expressed circRNAs in GBM (Zhou and Fan, 2020). Enhanced expression of *circ_0013520* and *circ_0004379* correlated with tumor size, TNM stage, and worse OS in GBM patients (Zhou and Fan, 2020). *Circ-CDC45* was also elevated in GBM and associated with larger tumor size, higher grade, and poor OS in glioma (Liu J. et al., 2019). *Circ-CDC45* serves as a sponge for miR-516b and miR-527 which functions as tumor-suppressor in GBM (Liu J. et al., 2019). Exosomal *circNFIX* was upregulated in the serum of temozolomide (TMZ) resistant patients and predicted poor prognosis (Ding et al., 2020). It sequesters miR-132 in GBM cells and its knockdown enhanced TMZ-sensitivity (Ding et al., 2020). *Circ_0029426* was upregulated in GBM tissues and this was associated with tumor size and World Health Organization grading (Zhang G. et al., 2019). C*irc_0029426* was an independent prognostic factor for GBM and correlated with the poor OS (Zhang G. et al., 2019). It promotes GBM progression by sequestering miR-197 (Zhang G. et al., 2019).

Lung Cancer (LC)

Circ_0013958 was upregulated in lung adenocarcinoma (LUAD) tissues and plasma of patients and was associated with TNM stage and lymphatic metastasis (Zhu X. et al., 2017). *Circ_0013958* decoys miR-134 and upregulates CCND1 in LUAD (Zhu X. et al., 2017). *CircFARSA* was upregulated in tissues and plasma of non-small-cell lung carcinoma (NSCLC) patients (Hang et al., 2018). High expression of *circFARSA* correlated with cell migration and invasion (Hang et al., 2018). *CircFARSA* sequesters miR-330-5p and miR-326, leading to the upregulation of the oncogene fatty acid synthase (FASN) (Hang et al., 2018). *Circ_0014130* was also overexpressed in NSCLC tissues and correlated with tumor volume, distant metastasis, and poor prognosis (Geng Y. et al., 2020). Li et al. observed the upregulation of *circ_0000792* in LUAD tissues, which correlated with T stage, distant metastasis, and smoking status (Li, 2018). Overexpression of *circ_100876* in NSCLC tissues was correlated with tumor stage, lymph node metastasis, and reduced OS in NSCLC patients (Yao J. T. et al., 2017). *Circ_100876* acts by sequestering miR-136 which targets MMP13 (Yao J. T. et al., 2017). Microarray analysis revealed the upregulation of *circFADS2* in LC tissues and this correlated with advanced TNM stage, lymph node metastasis, poor differentiation, and shorter OS of NSCLC patients (Zhao F. et al., 2018). *CircFADS2* induces NSCLC progression by sponging miR-498 (Zhao F. et al., 2018). Overexpression of *circPVT1* in NSCLC tissues and serum samples was associated with distant metastasis (Li X. et al., 2018c). *Circ_0067934* was upregulated in NSCLC tissues and its overexpression was correlated with TNM stage, lymph node status, and distant metastasis (Wang and Li, 2018). Overexpression of *circ_0067934* is associated with poorer OS and is an independent poor prognostic factor for NSCLC patients (Wang and Li, 2018). *CircPRKCI* was upregulated in LUAD tissues and associated with tumor size, TNM stage, poor prognosis, and shorter OS (Qiu et al., 2018). Higher *circPRKCI* increased proliferation and tumorigenesis of LUAD by sponging miR-545 and miR-589 and upregulating E2F7 (Qiu et al., 2018). *Circ_0000064* was upregulated in LUAD tissues and its higher expression levels correlated with T stage, lymphatic metastasis and TNM stage (Luo et al., 2017). Increased *circ_0000064* inhibited Caspase-3, Caspase-9, and Bax, and enhanced Bcl-2 expression in LUAD (Luo et al., 2017). Overexpression of *circ_0016760* in LUAD tissues correlated with TNM stage, lymph node metastasis, smoking status, differentiation grade and shorter OS; promotes

NSCLC development by sponging miR-1287 that targets G-antigen 1 (GAGE1) and is an independent predictor for the survival of NSCLC patients after surgery (Li J. et al., 2018a). Yao et al. reported the upregulation of *circRNA_100876* in NSCLC tissues and it was associated with lymph node metastasis, tumor staging, and shorter OS (Yao J. T. et al., 2017). *CircRNA_102231* overexpression in LUAD tissues correlated with advanced TNM stage (III-IV), lymph node metastasis, and poor OS (Zong et al., 2018a). C*ircRNA_103809* was significantly overexpressed in LUAD tissues and its higher expression correlated with a poor OS (Liu W. et al., 2018). *CircRNA_103809* enhanced LUAD progression by regulating the miR-4302/ZNF121/MYC loop (Liu W. et al., 2018). *Circ_0005962* was appreciably upregulated and *circ_0086414* was downregulated in early-stage LUAD and this 2-circRNA signature is a promising diagnostic biomarker for early LUAD (Liu X. X. et al., 2019). Higher plasma levels of *circ_0086414* were associated with EGFR mutations (Liu X. X. et al., 2019). Upregulation of *circ-PRMT5* were observed in NSCLC tissues and they were associated with larger tumors, lymph node metastasis, later clinical TNM stage, poor OS and PFS in NSCLC patients and is an independent prognostic factor for NSCLC patients (Wang Y. et al., 2019). *Circ-RAD23B* overexpression in NSCLC tissues was associated with lymph node metastasis, lower differentiation grade, and poor OS (Han et al., 2019). *Circ-RAD23B* enhanced cell growth by regulating the miR-593-3p/CCND2 axis and increased cell invasion by regulating the miR-653e5p/TIAM1 pathway (Han et al., 2019). *Circ_0102533* was elevated in NSCLC tissues and whole blood samples up and its regulation was significantly associated with tumor type, TNM stages, lymph nodes metastasis, and distant metastasis or recurrence (Zhou X. et al., 2018). *Circ_0102533* was useful in the detection of stage I-II NSCLC patients and elevated *circ_0102533* levels in whole blood was acceptable as a blood-based tumor marker for NSCLC screening (Zhou X. et al., 2018). *Circ_0079530* functions as an oncogene in NSCLC by enhancing cell proliferation and invasion and its overexpression was associated with tumor size and lymph node metastasis (Li J. et al., 2018a). *CircFGFR3* was significantly upregulated in LC tissues and its overexpression was closely associated with poor prognosis and reduced OS after surgery (Qiu B. Q. et al., 2019). *CircFGFR3* increased NSCLC cell invasion and proliferation by regulating Gal-1, pAKT, and p-ERK1/2 by sponging miR-22-3p (Qiu B. Q. et al., 2019). Elevated *circ_000984* levels in NSCLC tissues correlated with advanced TNM stage, lymph nodes metastasis, poor OS, and lower DFS in NSCLC patients (Li X. et al., 2019). *Circ_000984* activated Wnt/β-catenin signaling and its overexpression is an independent prognostic indicator for NSCLC patients (Li X. et al., 2019). Overexpression of *circ_0001946* in LUAD tissues was associated with a higher TNM stage, tumor size, and low OS (Yao et al., 2019). *Circ_0001946* enhanced LUAD progression by sponging miR-135a-5p and stabilizing its target SIRT1, which activates Wnt/β-catenin signaling pathway (Yao et al., 2019). *Circ_0037515* and *circ_0037516* were significantly downregulated in NSCLC tissues and have the potential for diagnosis (Zhao D. et al., 2020). Reduced levels of *circ_0033155* in NSCLC tissue was associated with lymphatic metastasis, and its overexpression reduced cell proliferation, colony formation and migration, and increased the level of PTEN in NSCLC (Gu et al., 2018). Downregulation of *circ_100395* in LC tissues was associated with metastasis and poor prognosis (Chen D. et al., 2018). Overexpression of *circ_100395* reduced malignancy by regulating the miR-1228/TCF21 axis (Chen D. et al., 2018). Downregulation of *circ_0001649* in NSCLC tissues was associated with positive lymph node, smoking status, and differentiation grade (Liu T. et al., 2018). Patients with downregulated *circ_0001649* had shorter OS and it could be a prognostic biomarker for NSCLC (Liu T. et al., 2018). *CircRNA_0056616* was upregulated in tissues and plasma of LUAD patients and it was correlated with TNM stage and lymph node metastasis (He F. et al., 2020). *Circ_0000190* and *circ_000164* were overexpressed in plasma and tissues from LC patients and expression of *circ_0000190* was associated with late-stage, extra-thoracic metastasis, poor survival, and prognosis (Luo Y. H. et al., 2020). These exosomal circRNAs are easily detectable in liquid biopsy and may serve as potential biomarkers for LC (Luo Y. H. et al., 2020).

Gastric Cancer (GC)

Deregulation of circRNAs has been reported in many gastric cancers and they can potentially serve as useful prognostic markers and therapeutic targets (Naeli et al., 2020). Elevated *CDR1as* levels in GC tissues was an independent risk factor and linked to the poor OS in GC patients (Pan et al., 2018). *CDR1as* enhances the development of GC by activating PTEN/PI3K/AKT pathway by sponging miR-7 (Pan et al., 2018). Overexpression of *circ_0010882* in the plasma of GC patients was a prognostic factor for OS and correlated with the poor OS (Peng et al., 2020). *Circ_0010882* contributes to GC cells proliferation, migration, invasion, and apoptosis by modulating PI3K/AKT/mTOR pathway (Peng et al., 2020). The upregulation of *circ-DCAF6* was associated with depth of invasion, lymph node invasion, and TNM stage in GC patients and is an independent risk factor for OS (Wu L. et al., 2019). *Circ-PRMT5* was upregulated in GC tissues and it was associated with tumor size, TNM stages, degree of differentiation, lymph node metastasis, and distant metastasis (Wu L. et al., 2019). GC patients with reduced *circPRMT5* expression had better prognosis and OS than those with increased levels (Wu L. et al., 2019). *Circ-PRMT5* promoted GC cell growth, migration, and invasion by sponging miR-145 and miR-1304 and upregulating MYC expression (Du et al., 2019). *Circ_0009910* expression was significantly increased in GC tissues and correlated with clinical stage, distant metastasis, and differentiation (Liu M. et al., 2018). Patients with elevated *circ_0009910* had a poor OS compared to patients with decreased expression (Liu M. et al., 2018). *Circ_0000419* was downregulated in GC plasma and exosomes and this negatively correlated with tumor stage, lymphatic and distal metastasis, venous, and perineural invasion (Tao et al., 2020). *Circ_0000419* is predicted to sponge miR-141-5p and miR-589-3p and its downregulation significantly correlate with Borrmann type and differentiation grade (Tao et al., 2020). Patients with downregulated *circ_0000419* had a poor OS and DFS (Tao et al., 2020). Downregulation of *circ_0006156* in GC tissues was associated with lymph node metastasis, nerve invasion,

and degree of tumor differentiation, besides low expression of *circ_0006156* correlated with progression-free survival, and OS of GC patients (He Y. et al., 2020). *Circ_0001821* was significantly downregulated in GC tissues, and whole-blood specimens of GC patients (Kong S. et al., 2019). Downregulation of *circ_0001821* was negatively associated with tumor depth and lymph node metastasis (Kong S. et al., 2019). The combined use of circulating *circ_0001821* with the existing tumor markers yielded good diagnostic potential in GC (Kong S. et al., 2019). Downregulation of *circCCDC9* in GC tissues was negatively associated with tumor size, lymph node invasion, advanced clinical stage, and OS (Luo Z. et al., 2020). *CircCCDC9* sponges miR-6792-3p which targets CAV1 a tumor-suppressor gene in GC (Luo Z. et al., 2020). Downregulation of *circRHOBTB3* in GC tissues was associated with poor differentiation and unfavorable prognosis in GC patients (Deng et al., 2020). *CircRHOBTB3* has a tumor-suppressor activity and inhibits growth of GC cells by sponging miR-654-3p and promoting the expression of its target p21 (Deng et al., 2020). *CircRNA_100269* was downregulated in GC tissues and its lower expression was associated with histological subtypes and node invasion (Zhang Y. et al., 2017). GC patients with low *circRNA_100269* levels had poor OS than patients with higher levels (Zhang Y. et al., 2017). Downregulation of *circRNA_100269* promoted GC development by releasing its inhibitory effect on oncogenic miR-630 (Zhang Y. et al., 2017). *Circ_0000745* was downregulated in GC tissues and plasma samples of GC patients and was associated with tumor differentiation and TNM stage (Huang M. et al., 2017). The use of *circ_0000745* in plasma combined with carcinoembryogenic antigen showed potential for use as a diagnostic marker for GC (Huang M. et al., 2017). Downregulation of *circPSMC3* was observed in plasma and tissue samples from GC patients and is negatively correlated with TNM stage, lymphatic metastasis, and reduced OS in GC patients (Rong et al., 2019). *CircPSMC3* contributed to GC progression by regulating PTEN by sponging miRNA-296-5p (Rong et al., 2019). *Circ-KIAA1244* was downregulated in plasma and tissues from GC patients and was negatively associated with the TNM stage, lymphatic metastasis, and reduced OS (Tang et al., 2018). Downregulation of *circ-KIAA1244* was an independent prognostic indicator of OS for GC patients (Tang et al., 2018). Downregulation of *circ_0000190* was observed in tissues and plasma samples of GC patients and is correlated with tumor diameter, lymphatic metastasis, distal metastasis, TNM stage, and CA19-9 levels (Chen et al., 2017). Chen et al. observed downregulation of *circSMARCA5* in GC tissues and it correlated with differentiation, lymph node metastasis, vascular invasion, poor OS and DFS in GC patients, moreover, low *circSMARCA5* expression was an independent prognostic factor for survival of GC patients (Cai et al., 2019). *CircYAP1* was downregulated in GC tissues and was correlated with poor prognosis and reduced OS in GC patients (Liu H. et al., 2018). *CircYAP1* expression was higher in early-stage GC patients and such patients were more sensitive to chemotherapy (Liu H. et al., 2018). *CircYAP1* decreased cell growth and invasion by sponging miR-367-5p to upregulate $p27^{Kip1}$ (Liu H. et al., 2018). Lower expression of *circ_0006848* in GC tissues correlated with tumor differentiation and tumor size (Lu et al., 2019a). Levels of *circ_0000520* were also decreased in tissues and plasma of GC patients and correlated negatively with the TNM stage in tissues and with CEA expression in plasma (Sun et al., 2017). *Circ_0001895* was significantly downregulated in GC tissues and its lower expression was associated with cell differentiation, Borrmann type, and CEA expression (Shao et al., 2017). *Circ_0005556* was downregulated in GC tissues and its low expression closely correlated with poor differentiation, TNM stage, and lymphatic metastasis (Yang L. et al., 2019). GC patients with decreased *circ_0005556* levels had a shorter OS than those with higher levels (Yang L. et al., 2019). *Circ_0067582* was downregulated in GC tissues and is correlated with increased tumor diameter and high CA19-9 (Yu et al., 2020). *Circ_0067582* downregulation was associated with a better prognosis after surgery (Yu et al., 2020). *Circ_0000467* was overexpressed in GC tissue and plasma and this was correlated with the TNM stage (Lu et al., 2019b). Diagnostic potential of *circ_0000467* was found to be superior to other common plasma biomarkers such as CEA and carbohydrate antigens-724 (CA-724) (Lu et al., 2019b). Elevated *circRNA_102958* levels were observed in GC tissues and it was significantly correlated with the TNM stage (Wei et al., 2019). Overexpression of *circ-ATAD1* was observed in GC and associated with deeper invasion, positive lymph node metastasis, advanced TNM stages, and adverse prognosis (Zhang L. et al., 2020). It promotes GC tumorigenesis by regulating the miR-140-3p/YY1 signaling axis (Zhang L. et al., 2020). *CircSHKBP1* was overexpressed in tumors and serum exosomes of GC patients, and it correlated with advanced pathological staging and poor OS (Xie M. et al., 2020). *CircSHKBP1* promotes GC progression by sponging miR-582-3p to increase HuR levels and promoting VEGF stability, and also by binding HSP90 to prevent its interaction with STUB1 (Xie M. et al., 2020).

Bladder Cancer (BCa)

Downregulation of *circFUT8* in BCa tissues was correlated with poor prognosis, high histological grade, lymph node metastasis, and poor survival rate (He Q. et al., 2020). C*irc_0071662* was downregulated in BCa tissues and this correlated with poor prognosis, lymph node invasion and distal metastasis, and poor OS (Abulizi et al., 2019). Overexpression of *circ_0071662* inhibited cell proliferation and invasion by sponging miR-146b-3p and upregulating its targets, hydroxy prostaglandin dehydrogenase (HPGD) and neurofibromin 2 (NF2) (Abulizi et al., 2019). *Circ-ITCH* was downregulated in BCa and this was associated with the histological grade of BCa patients (Yang C. et al., 2018). BCa patients with decreased *circ-ITCH* expression had poor OS than those with higher levels (Yang C. et al., 2018). Upregulation of *circ-ITCH* inhibited cell proliferation, migration, and invasion through *circ-ITCH*/miR-17, miR-224/p21, PTEN signaling axis (Yang C. et al., 2018). *CircACVR2A* was downregulated in BCa tissues and cell lines and its downregulation was correlated with advanced pathological stage, high grade, lymphatic metastasis, and poor OS (Dong et al., 2019). C*ircACVR2A* reduces proliferation, migration, and invasion of BCa cells by sponging miR-626 to regulate EYA4 expression (Dong et al., 2019).

Cervical Cancer (CC)

Circ_0018289 was upregulated in CC tissues and this correlated with tumor size and lymph node metastasis and poor DFS in CC patients (He et al., 2020b). Overexpression of *circ_0001038* in CC tissues was associated with lymph node invasion, myometrial invasion, and unfavorable outcome (Wang Y. et al., 2020). It promotes metastasis by sequestering miR-337-3p and upregulating it targets, Cyclin A, CBS Domain Divalent Metal Cation Transport Mediator 3 (CNNM3), and Metastasis Associated In Colon Cancer 1 (MACC1) (Wang Y. et al., 2020). *CircEIF4G2* was upregulated in CC tissues and this correlated with tumor size and lymph node metastasis (Mao et al., 2019). Elevated expression of *circEIF4G2* was correlated with worse prognosis in CC patients and induced cell growth and migration by sponging miR-218 and increasing the expression of its target HOXA1 (Mao et al., 2019). Increased expression of *circCLK3* in CC tissues was associated with poor tumor differentiation, advanced International Federation of Gynecology and Obstetrics (FIGO) stages and depth of stromal invasion, and indicated poor OS and DFS (Hong et al., 2019). It decoys miR-320a to remove its suppressive effects on FoxM1 and promotes cell proliferation, EMT, migration, and invasion (Hong et al., 2019). Higher *circ_0000388* levels in CC patients were significantly associated with FIGO stage, lymph node metastasis, and depth of invasion (Meng et al., 2020). *Circ_0000388* increased the proliferation, migration, and invasion, and reduced apoptosis of CC through regulating the miR-377-3p/ TCF12 axis (Meng et al., 2020). Wang et al. observed that 4 circRNAs namely, *circ_0101996*, *circ_0104649*, *circ_0104443*, and *circ_0101119* were significantly upregulated in peripheral whole blood from CC patients (Wang Y-M. et al., 2017). Combined detection of *circ_0101996* and *circ_0101119* could easily distinguish CC patients from healthy controls (Wang Y-M. et al., 2017). *CircFoxO3a* was significantly downregulated in the serum of CC patients and correlated with deep stromal invasion, positive lymph node metastasis, and poor prognosis (Tang et al., 2020). C*ircFoxO3a* downregulation is a poor prognostic factor for both OS and recurrence-free survival, independent of positive lymph node metastasis in CC patients (Tang et al., 2020).

Osteosarcoma (OSC)

C*irc_0081001* was overexpressed in OSC tissues and serums samples and was associated with poor prognosis, and may serve as an independent prognostic factor and biomarker for OSC diagnosis and prognosis (Kun-peng et al., 2018a). *Circ_0002052* was also upregulated in OSC tissues and associated with advanced stage, tumor size, metastasis, and poor survival rate in OSC patients (Jing et al., 2020). *Circ-0002052* promotes OSC development by activating Wnt/β-catenin signaling by sponging miR-382 (Jing et al., 2020). *CircPVT1* was significantly upregulated in OSC tissues and serum samples (Kun-peng et al., 2018b). Moreover, levels of *circPVT1* were higher in patients with lung metastasis or chemoresistance (Kun-peng et al., 2018b). Increased expression of *circPVT1* correlated with advance Enneking stage, chemoresistance, and lung metastasis, and was found to be a better diagnostic marker than alkaline phosphatase (ALP) for OSC (Kun-peng et al., 2018a). *CircHIPK3* was downregulated in OSC tissues and plasma samples (Xiao-Long et al., 2018). Lower *circHIPK3* levels correlated with Enneking stage, lung metastasis, lower OS, and poor prognosis in OSC patients (Xiao-Long et al., 2018). *Circ_0000190* was found in the extracellular nanovesicles and transmitted from healthy cells to OSC cells to impede cancer development (Li et al., 2020). Reduced expression of *circ_0000190* correlated with bigger tumor size, advanced staging (IIB/III), and distant metastasis and is a potential biomarker for OSC (Li et al., 2020).

Head and Neck Squamous Cell Cancer (HNSCC)

Esophageal Squamous Cell Cancer (ESCC)

Overexpression of *circ-SLC7A5* in ESCC plasma samples was correlated with TNM stage and poor OS (Wang Q. et al., 2020). Elevated *circ-0004771* levels were associated with heavier tumor burden and poor prognosis (Huang E. et al., 2020). *Circ_0067934* was upregulated in ESCC tissues and its increased expression correlated with poor differentiation, I-II T stage, and I-II TNM stage (Zong et al., 2018b).

Oral Squamous Cell Carcinoma (OSCC)

Lower expression of *circ_0092125* in OSCC correlated with tumor size, TNM stage, and lymph node metastasis in OSCC patients (Gao et al., 2020). Downregulation of *circ_0092125* was associated with shorter OS and was an independent risk factor for OSCC prognosis (Gao et al., 2020). Zhao et al. compared circRNAs levels in the saliva between OSCC patients and healthy donors, and observed upregulation of *circ_0001874* and *circ_0001971* in the saliva of OSCC patients and this correlated with tumor stage and TNM (Zhao S. Y. et al., 2018).

Laryngeal Squamous Cell Carcinoma (LSCC)

Circ_0067934 was upregulated in LSCC tissues and its overexpression was associated with larger tumor size, stronger lymph node metastasis, distant metastasis, and poor prognosis with lower OS rate (Chu, 2020). Upregulated *circ-CCND1* levels in LSCC correlated with tumor size, poor differentiation, advanced TNM stage, and poor prognosis (Zang et al., 2020). It binds to HuR and miR-646 to enhance the stability of CCND1 mRNA (Zang et al., 2020). *CircFLNA* upregulation in LSCC was associated with lymph node metastasis (Wang J. X. et al., 2019). *CircFLNA* increased the migration of LSCC cells by targeting the miR486-3p/FLNA axis (Wang J. X. et al., 2019).

Hypopharyngeal Squamous Cell Carcinoma (HSCC)

CircMATR3 was upregulated in HSCC tissues and was associated with advanced clinical stage, poor lymph node metastasis, and poor survival of HSCC patients (Wang Z. et al., 2020). *CircMATR3* binds to miR-188-5p and miR-448, both having a common target, USP28 (Wang Z. et al., 2020). *CircMORC3* downregulation in HSCC tissues and plasma samples was associated with tumor stage and tumor size (Zheng and Chen, 2020).

CIRCULAR RNAs IN CANCER THERAPEUTICS

Recent advances in RNA-based therapeutics coupled with aberrant expression of circRNAs in cancers makes them attractive therapeutic tools (Liu et al., 2017; Yang Z. et al., 2017; Lei et al., 2019). For example, circRNAs with multiple binding sites for oncogenic proteins or miRNAs can be introduced exogenously to restore the normal regulatory network to control proliferation and apoptosis in cancer (Tay et al., 2015). To facilitate this, multiple strategies to manipulate circRNA levels are currently under investigation and have good prospect for being developed into circRNA-based therapeutic strategies in near future.

The easiest approach to inhibit circRNA expression is RNA interference, using small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) or by employing chemically modified antisense oligonucleotides (ASOs) complementary to the back-splice junction, latter is preferred for *in vivo* applications (Cortés-López and Miura, 2016; Santer et al., 2019). Furthermore, complete knockdown of circRNA, *CDR1as*, by CRISPR/Cas9 genome-editing has been achieved and *CDR1as* loss-of-function mutant mice were generated (Piwecka et al., 2017). Another possibility is use of the CRISPR/Cas13 RNA knockdown system, wherein circRNA silencing is attained by targeting the CRISPR/Cas13 guide RNA to the back-splice junction of the circRNA (Santer et al., 2019). CircRNA overexpression is usually achieved by retroviral, lentiviral, adenoviral, or adeno-associated virus (AAV) vector constructs bearing circRNA sequence flanked by introns containing intronic complementary sequences (ICS) and splicing signals (Wang K. et al., 2017; Bai et al., 2018; Xia P. et al., 2018). Additionally, antisense oligonucleotides (ASOs) can also be used to enhance circRNA expression, by targeting splice-sites or splice-enhancers to increase the efficiency of back-splicing (Zhang et al., 2014). Apart from this, non-viral systems for circRNA overexpression have also been explored, most notable being *in vitro* synthesis of circRNAs followed by their *in vivo* delivery. Exogenous circRNA production first involves the synthesis of linear RNA by *in vitro* transcription, followed by circularization by employing self-splicing introns or by T4 RNA ligase (Santer et al., 2019). CircRNAs with therapeutic potential are discussed below and summarized in **Table 1**.

Hematological Malignancies

Acute Myeloid Leukemia (AML)

CircMYBL2 is significantly upregulated in AML patients with FLT3-ITD mutations and it increases the translational efficiency of FLT3 transcript, by facilitating binding of polypyrimidine tract binding protein 1 (PTBP1) to FLT3 transcript (Sun et al., 2019). Downregulation of *circMYBL2* reduced levels of FLT3 kinase and inhibited proliferation and promoted differentiation of FLT3-ITD AML (Sun et al., 2019). Overexpression of *circ-DLEU2* promoted AML by sponging miR-496 and increasing levels of its target, PRKACB (Wu D. M. et al., 2018). Tumor growth due to overexpression of *circ-DLEU2 in vivo* was reversed by its knockdown (Wu D. M. et al., 2018). Guarnerio et al. demonstrated that the well-established oncogenic chromosomal translocations such as *PML/RARα* and *MLL/AF9* give rise to fusion circRNAs (f-circRNA), *f-circPR,* and *f-circM9,* respectively (Guarnerio et al., 2016). Expression of these f-circRNAs in mouse embryonic fibroblasts promoted cell proliferation and transformed foci-forming capability (Guarnerio et al., 2016). Consistent with its role in promoting cell proliferation, knockdown of *f-circM9* increased apoptosis in AML cells (Guarnerio et al., 2016). Presence of *f-circM9* conferred protection to leukemic cells in a mice model upon treatment by arsenic trioxide and cytarabine (Guarnerio et al., 2016).

Acute Lymphoid Leukemia (ALL)

Higher expression of *circ-PVT1* contributes to ALL progression by sponging let-7 and miR-125 (Hu et al., 2018). Knockdown of *circ-PVT1* inhibits cell proliferation and induces apoptosis by reducing expression of c-Myc and Bcl-2, which are targets of let-7 and miR-125 respectively (Hu et al., 2018).

Chronic Myeloid Leukemia (CML)

Circ_0009910 was upregulated in CML and promotes imatinib resistance by sequestering miR-34a-5p which targets ULK1 (Cao et al., 2020). Knockdown of *circ_0009910* reduced cell growth and imatinib resistance, along with increased apoptosis and autophagic activation (Cao et al., 2020).

Multiple Myeloma (MM)

Circ-CDYL facilitated MM growth by sponging miR-1180 and increasing the expression of its target YAP (Chen F. et al., 2020). Downregulation of *circ-CDYL* induces apoptosis by downregulating YAP (Chen F. et al., 2020).

B-Cell Lymphoma (BCL)

Circ-APC was significantly downregulated in DLBCL (Hu et al., 2019). In cytoplasm *circ-APC* sponges miR-888 leading to an increase in levels of its target APC, whereas in nucleus it binds to APC promoter and recruits the DNA demethylase TET1 to transcriptionally upregulate APC (Hu et al., 2019). Ectopically expressed *circ-APC* acts as a tumor-suppressor and acts by inhibiting Wnt/β-catenin signaling in DLBCL (Hu et al., 2019).

Solid Tumors

Colorectal Cancer (CRC)

CDR1as which acts as miR-7 RNA sponge is overexpressed in CRC and confers an aggressive oncogenic phenotype (Weng et al., 2017). *CDR1as* downregulation resulted in inhibition of CRC progression (Weng et al., 2017). EGFR expression is regulated by *circHIPK3* which is upregulated in CRC tissues (Zeng et al., 2018). Similar to *CDR1as*, *circHIPK3* also functions as a sponge for miR-7. The knockdown of *circHIPK3* inhibited cell proliferation, migration, invasion, and metastasis (Zeng et al., 2018). *Circ_001569* was significantly upregulated in CRC tissues and promoted cell proliferation and invasion (Xie et al., 2016). Mechanistically, *circ_001569* performs a tumor-promoting function by sponging miR-145 and upregulating its targets E2F5, BAG4, and FMNL2 (Xie et al., 2016). Downregulation of *circ_001569* resulted in reduced cell invasion and migration (Xie et al., 2016). *Circ_0007534* upregulation was associated with a metastatic phenotype and evasion of apoptosis in CRC (Zhang R. et al., 2018). Silencing of

circ_0007534 reduced Bcl2/Bax ratio in CRC cells and induced apoptosis (Zhang R. et al., 2018). Levels of *circ_0000069* were also elevated in CRC tissues and its knockdown induced cell-cycle arrest and inhibited cancer progression (Guo et al., 2016). *Circ_ITCH* acts as a sponge for miR-7 and miR-20a and is significantly downregulated in CRC tissues (Huang et al., 2015). Overexpression of *circ_ITCH* reduced cell proliferation in CRC by downregulating c-Myc and cyclinD1 (Huang et al., 2015). *CircRNA_103809* is also downregulated in CRC patients and its silencing promotes cell proliferation and migration *via* miR-532-3p/FOXO4 axis (Bian et al., 2018). Interestingly, telomerase reverse transcriptase (TERT) is one of the targets of the tumor-suppressor miR-138 which is sponged by *circ_0020397*(Zhang X. et al., 2017). *Circ_0020397* is upregulated in CRC tissues and its downregulation resulted in lower TERT levels and reduced cell proliferation (Zhang X. et al., 2017). *CircBANP* was significantly upregulated in CRC tissues and cell lines and its silencing suppressed CRC cell proliferation and reduced p-Akt protein expression (Zhu M. et al., 2017). *Circ5615* is upregulated in CRC tissues and functions by sequestering miR-149-5p which targets tankyrase (TNKS), an activator of Wnt/β-catenin stabilization (Ma et al., 2020). Downregulation of *circ5615* inhibited proliferation and promoted cell-cycle arrest (Ma et al., 2020). *CircFARSA* is upregulated in CRC tissues and sequesters miR-330-5p, leading to the upregulation of LASP1 (LIM and SH3 protein 1) (Lu C. et al., 2020). The silencing of *circFARSA* inhibited proliferation, migration, and invasion of CRC cells (Lu C. et al., 2020). *CircPTK2* is elevated in CRC tissues and functions by promoting EMT of CRC cells by binding to vimentin protein at Serine 38, 55, and 82 residues (Yang H. et al., 2020). *CircPTK2* knockdown reduced tumorigenicity and metastatic potential of CRC cells (Yang H. et al., 2020). *Circ_0060745* promotes CRC metastasis by sequestering miR-4736 and stabilizing its target CSE1L (chromosome segregation 1-like) (Wang and Wang, 2020). The knockdown of *circ_0060745* suppressed CRC cell migration and invasion (Wang and Wang, 2020). *Circ_0008285* is downregulated in CRC tissues and cell lines (Wang and Wang, 2020). It acts by suppressing PI3K/AKT signaling *via* miR-382-5p/PTEN axis, leading to inhibition of cell proliferation and migration in CRC (Wang and Wang, 2020). *Circ_0001313* is highly expressed in CRC tissues and modulates tumorigenesis by sponging miR-510-5p to elevate AKT2 expression (Tu et al., 2020). Depletion of *circ-0001313* decreased proliferation and induced apoptosis in CRC cells (Tu et al., 2020). *CircDDX17* is significantly downregulated in CRC tissues and its silencing promoted CRC cell proliferation, migration, invasion, and inhibited apoptosis (Li X-N et al., 2018).

Breast Cancer (BC)

Circ-ABCB10 sponges miR-1271 in BC, its depletion suppresses proliferation and induces apoptosis in BC cells (Liang et al., 2017). *CircEHMT1* was downregulated in BC tissues and promotes metastasis by upregulating MMP2 through *circEHMT1*/miR-1233-3p/KLF4 axis (Lu M. et al., 2020). Overexpression of *circEHMT1* inhibited migration and invasion of BC cells by reducing MMP2 expression (Lu M. et al., 2020). *Circ_0011946* functions by regulating the expression of replication factor C subunit 3 (RFC3), and silencing it suppressed migration and invasion of BC cells (Zhou J. et al., 2018). *CircGFRA1* was upregulated in TNBC cells and functions by regulating the expression of its cognate GFRA1 (GDNF Family Receptor Alpha 1) transcript by sequestering miR-34a, and its knockdown induces apoptosis (He et al., 2017). *Circ_0001982* was overexpressed in BC tissues and promotes BC tumorigenesis by sponging miR-143 (Tang et al., 2017). Silencing of *circ_0001982* suppressed cell proliferation, invasion, and induced apoptosis in BC cells (Tang et al., 2017). Interestingly, *circTADA2A-E6* and *circTADA2A-E5/E6*, originating from the TADA2A gene, were significantly downregulated in TNBC patients (Xu et al., 2019). *CircTADA2A-E6* displays tumor-suppressor properties and functions as a miR-203a-3p sponge and restores the expression of its target *SOCS3* (Xu et al., 2019). The knockdown of *circTADA2A-E6* promotes proliferation, clonogenicity, migration, and invasion in BC cells (Xu et al., 2019). *CircFBXW7* is downregulated in TNBC cell lines, it codes for a microprotein with tumor-suppressive functions in TNBC (Ye F. et al., 2019). Overexpression of *circFBXW7* suppressed cell proliferation, migration, and reversed tumor growth in TNBC cells (Ye F. et al., 2019). *CircCDYL* promoted autophagy by the miR-1275-ATG7/ULK1 axis to enhance the malignant progression of BC cells, its knockdown slows down tumorigenesis by modulating autophagy (Liang et al., 2020).

Hepatocellular Carcinoma (HCC)

CDR1as is overexpressed in HCC resulting in enhanced proliferation and invasion (Yu et al., 2016). Knockdown of *CDR1as* resulted in increased availability of miR-7 and downregulation of its target genes CCNE1 and PIK3CD, leading to inhibition of cell proliferation and invasion (Yu et al., 2016). Levels of *circMTO1* were decreased in HCC, its overexpression in HCC cells sponges oncogenic miR-9 to promote the expression of tumor-suppressor p21 resulting in reduced tumor cell proliferation, metastasis, and invasion (Han et al., 2017). Expression of *circ-10720* promotes EMT by inducing transcription factor, Twist1 (Meng et al., 2018). It promotes migration, invasion, and EMT by stabilizing mesenchymal marker vimentin by sponging miR-1246, miR-578, and miR-490-5p (Navarro, 2019). Depletion of *circ-10720* inhibited Twist1-induced metastasis (Meng et al., 2018; Navarro, 2019). Elevated *circRNA-100338* induced mTOR signaling *via* the *circRNA-100338*/miR-141-3p/RHEB axis (Huang X. Y. et al., 2020). The depletion of *circ_100338* reduced the activity of mTOR signaling pathway and suppressed HCC tumorigenesis and progression (Huang X. Y. et al., 2020). *Circ_0067934* functions by modulating the miR-1324/FZD5/Wnt/β-catenin axis to enhance migration, invasion, and proliferation of HCC cells (Zhu et al., 2018). Silencing of *circ_0067934* suppressed proliferation, migration, and invasion of HCC cells (Zhu et al., 2018). *CircSMARCA5* is downregulated in HCC tissues and inhibits proliferation, invasion, and metastasis of HCC cells by promoting the expression of the tumor-suppressor TIMP3 by sequestering miR-17-3p and miR-181b-5p (Li Z. et al., 2019). Overexpression of *circSMARCA5* inhibits the proliferation and migration of HCC cells (Li Z. et al., 2019). *CircPTGR1* promoted HCC

progression *via* the miR-449a/MET pathway and its knockdown reduced HCC progression (Chen et al., 2015; Wang G. et al., 2019). *CircRHOT1* facilitated HCC progression by recruiting TIP60, a histone acetyltransferase to the nuclear orphan receptor NR2F6 promoter to enhance its expression (Wang L. et al., 2019). CRISPR/Cas9-based depletion of *circRHOT1* suppressed proliferation, migration, and invasion, and promoted apoptosis in HCC cells (Wang L. et al., 2019). *CircTRIM33-12* modulates TET1-induced DNA demethylation by sponging miR-191 (Zhang P. F. et al., 2019). Overexpression of *circTRIM33-12* inhibited proliferation and invasion of HCC cells (Zhang P. F. et al., 2019). *Circ-BIRC6* facilitates HCC progression by acting as a miR-3918 sponge and thus targeting the miR-3918/Bcl2 axis (Tang et al., 2015). Its knockdown resulted in decreased HCC cell proliferation, migration, and invasion, and enhanced apoptosis (Tang et al., 2015). *Circ_0070269* levels are downregulated in HCC tissues and it facilitates HCC progression by regulating the miR-182/NPTX1 axis (Zhang P. F. et al., 2019). Its overexpression suppresses the proliferation, and invasion of HCC cells (Zhang P. F. et al., 2019). *CircADAMTS13* was downregulated in HCC tissues (Qiu L. et al., 2019). It sequesters oncogenic miR-484, and overexpression of *circADAMTS13* resulted in a significant reduction in HCC cell proliferation (Qiu L. et al., 2019).

Glioblastoma (GBM)

CircNFIX acts as a sponge for miR-34a-5p which targets the Notch signaling pathway in GBM cells (Xu et al., 2018). The knockdown of *circNFIX* inhibited cell proliferation and migration of GBM cells by downregulating *NOTCH1* (Xu et al., 2018). CircRNA *cZNF292* is an oncogenic circRNA that promotes angiogenesis in GBM (Yang P. et al., 2019). Downregulation of *cZNF292* reduced proliferation in GBM cells and suppressed human glioma tube formation by modulating Wnt/β-catenin signaling pathway (Yang P. et al., 2019). *Circ_0037251* enhances GBM progression by sponging miR-1229-3p and upregulating mTOR (Cao et al., 2019). Knockdown of *circ_0037251* inhibited the expression of mTOR leading to increased apoptosis and promoting cell-cycle arrest (Cao et al., 2019). *CircMAPK4* functions as an oncogene to enhance GBM cell survival by sponging miR-125a-3p and regulating the p38/MAPK pathway, its downregulation induces apoptosis of GBM cells (He et al., 2020a). *Circ-U2AF1* enhanced glioma cell proliferation, migration, and invasion by sponging miR-7-5p and increasing the expression of NOVA2 (Li, 2019b). The knockdown of *circ-U2AF1* decreased the migration and invasion abilities of glioma cells by downregulating NOVA2 (Li, 2019a). *Circ_0001946* was downregulated in GBM cells and functions by sponging miR-671-5p (Li, 2019a). Overexpression of *circ_0001946* reduced the migration, invasion, and proliferation of GBM cells by inhibiting the pro-tumorigenic effects of miR-671-5p (Li, 2019a). *CircNT5E* promotes GBM tumorigenesis by sponging miR-422a and its CRISPR/Cas9-mediated deletion suppressed proliferation, migration, and invasion of GBM cells (Wang R. et al., 2018a). *Circ_0029426* facilitates tumorigenesis by sequestering miR-197, its silencing suppressed proliferation, migration, and invasion, and promoted apoptosis of GBM cells (Zhang G. et al., 2019). *Circ-TTBK2* promotes GBM malignancy by modulating the miR-217/HNF1β/Derlin-1 pathway, and its knockdown blocked GBM progression (Zheng et al., 2017). *CircMMP9* elicits its oncogenic function by sequestering miR-124 and upregulating the expression of its targets, cyclin-dependent kinase 4 (CDK4), and aurora kinase A (AURKA) (Wang R. et al., 2018b). Silencing of *circMMP9* inhibited proliferation, migration, and invasion of GBM cells (Wang R. et al., 2018b).

Lung Cancer (LC)

CircRNA_103809 functions as a miR-4302 sponge leading to the ZNF121-mediated increase in MYC expression (Liu W. et al., 2018). Downregulation of *circRNA_103809* resulted in delayed tumor growth and inhibited cell proliferation and invasion in LC cells (Liu W. et al., 2018). *Circ_0020123* sequesters miR-144 and causes upregulation of ZEB1 and EZH2 which are critical for EMT and its knockdown suppresses NSCLC growth and metastasis (Qu et al., 2018). C*ircFADS2* sponges tumor-suppressor miR-498, its silencing reduced invasion and proliferation in LC cells (Zhao F. et al., 2018). *Circ_0000064* levels were elevated in LC tissues and its ablation attenuates cell proliferation and promotes cell apoptosis in LC cells (Luo et al., 2017). *CircRNA_102231* is overexpressed in LUAD tissues and its inhibition resulted in reduced cell proliferation, and invasion (Zong et al., 2018b). *Circ_0033155* is downregulated in NSCLC tissues, and its overexpression resulted in reduced cell proliferation, migration, and colony formation in NSCLC (Gu et al., 2018). *CircRNA_100876* acts as a miR-136 decoy, which targets MMP13 (Yao J. T. et al., 2017). Its silencing suppressed MMP13 expression and increased extracellular matrix formation (Yao J. T. et al., 2017). *CircPTK2* was downregulated in NSCLC cells during TGF-β induced EMT (Wang L. et al., 2018). *CircPTK2* functions as the miR-429/miR-200b3p sponge and reduced the expression of tumor-suppressor T1F1γ, consistent with this its overexpression in NSCLC cells augments T1F1γ expression and reduces TGF-β induced EMT (Wang L. et al., 2018). *CircPVT1* facilitates the increased expression of E2F2 by sponging miR-125b, and its downregulation increased apoptosis *via* E2F2 signaling pathway (Li X. et al., 2018c). Tan et al. identified the oncogenic, *f-circEA-4a* in plasma of NSCLC patients with *EML4-ALK* fusion (Tan et al., 2018). Its silencing reduced cell proliferation, metastasis, and invasion (Tan et al., 2018). The same group identified another oncogenic fusion-circRNA, *f-circEA-2a* produced from *EML4-ALK* fusion bearing an "AA" motif at the junction site. Its overexpression was reported to promote cell migration and invasion in NSCLC cells (Tan et al., 2018). Lower expression levels of *circ-FOXO3* were observed in NSCLC tissues and its overexpression reduced NSCLC development by sponging miR-155 and releasing repression of FOXO3 (Zhang Y. et al., 2018).

Gastric Cancer (GC)

CDR1as modulates PTEN/PI3K/AKT signaling pathway and confers an aggressive oncogenic phenotype to GC cells (Pan et al., 2018). Downregulation of *CDR1as* induced cell death and restricts GC progression (Li X. et al., 2019). *Circ_100269* is downregulated in GC tissues and its overexpression sponges oncogenic miR-630 suppressing GC growth (Zhang Y. et al.,

2017). *Circ_104916* was downregulated in GC tissues and cell lines, its overexpression suppressed cell proliferation, migration, and EMT (Li J. et al., 2017). *CircPDSS1* sponges tumor-suppressing miR-186-5p and upregulate the oncogene NEK2 in GC tissues, and its depletion inhibited cell proliferation (Ouyang et al., 2019). *Circ_0023642* is upregulated in GC and regulates the EMT signaling pathway, and its depletion results in tumor inhibition, reduced cell proliferation, and metastasis due to the downregulation of N-cadherin, Vimentin and Snail (Zhou L. H. et al., 2018). *Circ-ATAD1* promotes GC progression by modulating the miR-140-3p/YY1/PCIF1 signaling axis (Zhang L. et al., 2020). Consistent with its oncogenic function, the depletion of *circATAD1* reduced cell viability and colony formation of GC cells (Zhang L. et al., 2020). Interestingly, *circFN1* was highly expressed in cisplatin-resistant GC tissues and promoted cisplatin-resistance by enhancing cell viability and suppressing apoptosis, by sequestering miR-182-5p (Huang X. X. et al., 2020). The knockdown of *circFN1* promotes cisplatin-sensitivity and apoptosis in GC cells (Huang X. X. et al., 2020). *CircCACTIN* promotes GC progression by sponging miR-331-3p and increasing expression of TGFBR1 (Transforming growth factor-β receptor type 1) (Zhang L. et al., 2019). Knockdown of *circCACTIN* suppressed proliferation, migration, invasion, and EMT of GC cells (Zhang L. et al., 2019). *Circ-CEP85L* is downregulated in GC tissues, it acts as miR-942-5p sponge leading to the upregulation of NFKBIA (NFKB Inhibitor Alpha) (Lu J. et al., 2020). Consistent with this overexpression of *circ-CEP85L* inhibited proliferation and invasion of GC cells (Lu J. et al., 2020).

Bladder Cancer (BCa)

CircRNA-MYLK augments proliferation, migration, tube formation of human umbilical vein epithelial cells (HUVEC) and EMT by sponging miR-29a, and stabilizing its target VEGFA in BCa cells (Zhong et al., 2017). The depletion of *circRNA-MYLK* decreased proliferation, motility, and induced apoptosis in BCa (Zhong et al., 2017). *CircACVR2A* is downregulated in BCa tissues, it sponges miR-626 to upregulate the expression of the tumor-suppressor EYA4 (Dong et al., 2019). Consistent with this overexpression of *circACVR2A* suppressed proliferation, migration, and invasion of BCa cells and metastasis (Dong et al., 2019). In contrast to CRC, *circHIPK3* is downregulated in BCa and serves as a sponge for miR-558 (Li Y. et al., 2017). It prevents angiogenesis by inhibition of heparanase (HPSE), a positive regulator of VEGF expression (Li Y. et al., 2017). Overexpression of *circHIPK3* can be used to reduce aggressiveness and metastasis in BCa cells by targeting the miR-558/heparanase axis (Li Y. et al., 2017). *CircITCH* was downregulated in BCa samples, overexpression of *circITCH* upregulates p21 and PTEN expression by sponging oncogenic miRNAs, miR-17/miR-224, leading to inhibition of BCa cell proliferation, migration, and invasion (Yang C. et al., 2018).

Ovarian Cancer

Circ_0061140 is upregulated in ovarian cancer cell lines and regulates the miR-370/FOXM1 pathway by sequestering miR-370 (Chen Q. et al., 2018). Knockdown of *circ_0061140* suppressed proliferation and migration in GC cells (Chen Q. et al., 2018). *CDR1as* expression is upregulated in OC tissues and it correlated with poor prognosis for TNM stages, lymph node metastasis, and reduced OS (Luo Y. H. et al., 2020). *CDR1as* sponges miR-641 causing up-regulation of ZEB1 and MDM2 expression to promote OC (Luo Y. H. et al., 2020). A large number of circRNAs are misexpressed in primary and metastatic sites of epithelial ovarian carcinoma and their expression exhibits an inverse trend as compared to their linear counterparts in many cancer-related pathways and signaling pathways like NFkB, PI3k/AKT, and TGF-β (Ahmed et al., 2016). Accumulating evidence suggest that circRNA are associated with the initiation and progression of OC (Shabaninejad et al., 2019).

Osteosarcoma (OSC)

CircUBAP2 acts miR-143 sponge and upregulates its target Bcl-2 in OSC (Zhang H. et al., 2017). Depletion of *circUBAP2* suppressed proliferation and induced apoptosis in OSC cells (Zhang H. et al., 2017). *Circ_0009910* sequesters miR-449a which targets IL6R (interleukin 6 receptor), and its knockdown induced cell-cycle arrest, inhibited proliferation and induced apoptosis is OSC cells (Deng et al., 2018). *CircPVT1* was upregulated in the OSC tissues and chemoresistant cell lines, its silencing reversed chemoresistance by decreasing the expression of ABCB1 (ATP Binding Cassette Subfamily B Member 1) (Kun-peng et al., 2018b). *Circ_001564* promotes tumorigenicity by sequestering miR-29c-3p, its depletion suppressed the proliferative activity, induced cell-cycle arrest, and promoted apoptosis (Song and Li, 2018). *Circ_0002052* was downregulated in OSC tissues and suppresses Wnt/β-catenin signaling pathway by promoting APC2 expression *via* sponging miR-1205 (Wu Z. et al., 2018). Overexpression of *circ_0002052* suppresses migration and invasion in OSC cells (Wu Z. et al., 2018). *CircNASP* functions by sponging miR-1253 leading to the upregulation of FOXF1 (Huang et al., 2018). Ablation of *circNASP* by siRNAs inhibits the proliferation, cell-cycle progression, and invasion in OSC cells (Huang et al., 2018).

Head and Neck Squamous Cell Carcinoma (HNSCC)

Esophageal Squamous Cell Carcinoma (ESCC)

Circ_0067934 was upregulated in ESCC tumor tissues and cell lines, also its silencing inhibited proliferation and migration of ESCC cells (Xia et al., 2016). *Circ_0000337* was upregulated in ESCC tissues and sequesters miR-670-5p, its depletion inhibits cell proliferation, migration, and invasion (Song et al., 2019).

Oral Squamous Cell Carcinoma (OSCC)

CircUHRF1 functions as a miR-526b-5p sponge and positively regulates c-Myc, which induces TGF-β1 and ESRP1 (Epithelial Splicing Regulatory Protein 1) expression (Zhao W. et al., 2020). The knockdown of *circUHRF1* reduces migration, invasion, and EMT of OSCC cells (Zhao W. et al., 2020).

CONCLUSION AND FUTURE PERSPECTIVES

CircRNAs which were considered mere splicing artifacts until a few years ago are poised to occupy a center stage in the world of regulatory RNAs. CircRNAs regulate the cellular transcriptome by diverse mechanisms and contribute to a range of cellular functions. They are involved in regulating all the major hallmarks of cancer and can serve as promising biomarkers for cancer diagnosis and prognosis. Unfortunately, so far none of these circRNAs have reached the clinics, and evaluation of a combination of circRNAs as a signature for diagnosis and correlation with clinical features is the likely way forward. CircRNAs also have immense potential for use as therapeutic targets. Novel and effective therapies can be designed by either modulating the endogenous expression circRNAs or by exogenous delivery of artificially engineered circRNAs. At present, the use of circRNAs as therapeutic agents is restricted to the bench and warrants further investigation for clinical use. The circRNA which may be suitable for therapeutic targeting, may also be different for distinct cancer types. However, the aberrant *CDR1as* expression is common to several cancer types, and targeting it for treating many different cancer types has shown promising results *in vitro* and *in vivo*. Development of RNA-based therapeutics for targeting *CDR1as* for clinical use has the potential to emerge as a single-target therapy for multiple cancers and is worth further investigation.

AUTHOR CONTRIBUTIONS

PK and VS conceived and designed the manuscript. AR, SB, VS, and PK wrote the manuscript. All authors read and approved the final version of the manuscript.

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

GLOSSARY

5′UTR, 5′Untranslated region; ABCB1, ATP Binding Cassette Subfamily B Member 1; ADAR1, Adenosine deaminase 1 acting on RNA; ALP, Alkaline phosphatase; AML, Acute myeloid leukemia; APC, Adenomatous polyposis coli; ASOs, Antisense oligonucleotides; AURKA, Aurora kinase A; BAG4, BAG cochaperone 4; BC, Breast cancer; BCa, Bladder Cancer; BCLC, Barcelona clinic liver cancer; CA-724, Carbohydrate antigens-724; CC, Cervical cancer; CCND1, Cyclin D1; CCNE1, Cyclin E1; CDK4, Cyclin dependent kinase 4; CEA, Carcinoembryonic antigen; circRNAs, Circular RNAs; CLL, Chronic lymphocytic leukemia; CML, Chronic lymphocytic leukemia; CNNM3, CBS domain divalent metal cation transport mediator 3; CRC, Colorectal cancer; CSE1L, Chromosome segregation 1 like; CXCL6, Chemokine (C-X-C Motif) ligand 6; DFS, Disease free survival; DHX9, DExH-Box Helicase 9; DLBCL, Diffuse large B-cell lymphoma; dsRBDs, Double stranded RNA-binding domains; E2F5, E2F transcription factor 5; EGFR, Epidermal growth factor receptor; EMT, Epithelial-mesenchymal transition; ER, Estrogen receptor; ESCC, Esophageal squamous cell cancer; ESRP1, Epithelial Splicing Regulatory Protein 1; EYA4, EYA transcriptional coactivator and phosphatase 4; EZH2, Enhancer of zeste homolog 2; FASN, Fatty acid synthase; FBXW7, F-Box and WD repeat domain containing 7; FIGO, Federation of gynecology and obstetrics; FLT3, Fms-like tyrosine kinase 3; FMNL2, Formin like protein 2; FOXO3, Forkhead Box O3; FZD3, Frizzled class receptor 3; GAGE1, G-antigen 1; GBM, Glioblastoma; GC, Gastric cancer; GFRA1, GDNF family receptor alpha 1; HBV, Hepatitis B virus; HCC, Hepatocellular carcinoma; HNRNPL, Heterogeneous nuclear ribonucleoprotein; HNSCC, Head and Neck Squamous cell cancer; HPGD, Hydroxyprostaglandin dehydrogenase; HPSE, Heparanase; HSCC, Hypopharyngeal squamous cell carcinoma; HuR, Hu-antigen R; HUVEC, Human umbilical vein epithelial cells; ICS, Intronic complementary sequences; IGHV, Immunoglobulin heavy-chain variable region; IL6R, interleukin 6 receptor; IRESs, Internal Ribososmal Entry Sites; KIF4A, Kinesin family member 4A; LASP1, LIM and SH3 protein 1,LC, Lung cancer; LFS, Leukemia-free survival; LIF, Leukemia inhibitory factor; LSCC, Laryngeal squamous cell carcinoma; LUAD, Lung adenocarcinoma; m6A, N6-methyladenosine; MACC1, Metastasis associated in colon cancer 1; MG, β -microglobulin; MM, Multiple myeloma; MMP13, Matrix metalloproteins 13; NF1, Neurofibromin 1; NF90, Nuclear factor 90; NF110, Nuclear factor 110; NFKBIA, NFKB Inhibitor Alpha; NPM1, Nucleophosmin; NSCLC, Non small cell lung carcinoma; ORFs, Open reading Frames; OS, Overall survival; OSC, Osteosarcoma; OSCC, Oral squamous cell carcinoma; PCK1, Phosphoenolpyruvate carboxykinase 1; PDK1, 3-phosphoinositide-dependent protein kinase; PFS, Progression-free survival; PIK3CD, Phosphotidylinositol-4,5-Bisphosphate 3-kinase catalytic subunit delta; PLK1, Polo like kinase 1; PRKACB, Protein kinase CAMP-activated catalytic subunit Beta; PTBP1, polyprimidine tract binding protein 1; QKI, Quaking; RBM20, RNA-binding motif protein 20; RBPs, RNA-binding proteins; RFC3, Replication factor C subunit 3; RFS, Recurrance free survival; RT-qPCR, Real-time quantitative polymerase chain reaction; shRNAs, short hairpin RNAs; siRNAs, Small interfering RNAs; SOCS3, Suppressor of cytokine signaling 3; T1F1γ, Transcription intermediatory factor 1-gamma; TET1, Ten-eleven translocation methyclcytosine dioxygenase 1; TERT, Telomerase reverse transcriptase; TGFBR1, Transforming growth factor-β receptor type 1; TIMP3, TIMP Metallopeptidase inhibitor 3; TMZ, temozolomide; TNBC, Triple negative breast cancer; TNKS, Tankyrase; TNM, Tumor; Node and Metastasis; U1snRNP, U1 small nuclear ribonucleoprotein particle; ULK1, Uridine kinase-like protein 1; USP28, Ubiquitin specific peptidase 28; VEGFA, Vascular endothelial growth factor A; YAP, Yes-associated protein 1; ZEB1, Zinc finger E-box binding homeobox 1.

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Identification of circRNAs in the Liver of Whitespotted Bamboo Shark (*Chiloscyllium plagiosum*)

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Whitespotted bamboo shark (*Chiloscyllium plagiosum*), a member of the cartilaginous fish family, has an extremely large liver and demonstrates a strong regeneration ability and immune regulation. Circular RNAs (circRNAs) is an important class of non-coding RNAs. Increasing evidences suggest that circRNAs are a kind of potential regulators. Recently, researchers have isolated and identified different circRNAs from various species, while few reports were on the circRNAs of *C. plagiosum*. In this study, we have identified a total of 4,558 circRNAs in the liver of *C. plagiosum*. This finding suggests that circRNAs are not evenly distributed in the chromosomes and follow the GT-AG rule during cyclization. Alternative back-splicing might exist in shark circRNAs as shown by the authenticity identification of predicted circRNAs. The binding strength of circRNAs (<2,000 bp) and the detected miRNAs in shark liver were simultaneously analyzed to construct an mRNA–miRNA–circRNA network for the Glutathione S-transferase P1 gene, and the circRNA authenticity was simultaneously verified. Our data provide not only novel insights into the rich existence of circRNAs in marine animals, but also a basis for characterizing functions of identified circRNAs in the liver homeostasis of *C. plagiosum*.

Keywords: circRNA, back-splicing, *Chiloscyllium plagiosum*, miRNA, liver homeostasis

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INTRODUCTION

Approximately 530 million years ago, cartilaginous fishes diverged from jawed vertebrates, which are the common ancestors of teleost fish and humans. Similar to teleost fishes, cartilaginous fishes have complex physiological systems that include an adaptive immune system and a pressurized circulatory system. It is the first jawed vertebrate with an adaptive immune system. A member of the cartilaginous fish family, whitespotted bamboo shark (*Chiloscyllium plagiosum*), is mainly distributed in the Indo-West Pacific waters around several East Asian countries, such as Singapore and Indonesia. This marine animal has important research and commercial food value. In contrast to other animals, *C. plagiosum* has a large liver, accounting for approximately 75% of the total weight of its internal organs. The liver is not only an important organ with detoxification function but also has a strong regeneration ability, which endows *C. plagiosum* with many special

characteristics, such as strong immune regulation (Zhou et al., 2011; Wang et al., 2013; Zhang et al., 2013; Masstor et al., 2014).

CircRNAs, a novel type of non-coding RNAs (ncRNAs) (Jeck and Sharpless, 2014; Hsiao et al., 2017), form covalent-closed continuous loops without 5′ to 3′ polarities and poly(A) tails (Liang and Wilusz, 2014; Yaylak et al., 2019). Large amounts of circRNAs have been successfully identified in a variety of organisms, including plants, animals, and humans (Rybak-Wolf et al., 2015; Maass et al., 2017; Ye et al., 2017). According to the circBase statistics[1], approximately 410,000 circRNAs of six species had been identified; however, only one type of marine animal has been detected with circRNA (Pallavicini et al., 2013b). CircRNAs, which are an important part of ncRNA families, are ubiquitously expressed in eukaryotic cells during post-transcriptional processes (Salzman et al., 2012, 2013). CircRNAs form via non-canonical splicing termed back-splicing, while the canonical splicing machinery could control pre-mRNA back-splicing (Kulcheski et al., 2016); as such, the efficiency of circularization may rely on the presence of canonical splice sites (Chen et al., 2016). CircRNAs were thought to have two unique features: high stability due to their resistance to the cellular linear RNA decay machineries, and particular structure because the circular feature may endow circRNAs with unique structure (Liu et al., 2019). Increasing evidence suggests that circRNAs are a kind of potential RNA regulators, and their abnormal expressions are correlated with various human diseases, especially cancer in humans (Meng et al., 2018).

CircRNAs have multiple biological functions: they serve as miRNA sponges or bind to essential proteins as RNA-binding proteins, regulate alternative splicing and gene expression, act as templates for translation, and play other unknown roles (Hansen et al., 2013; Ashwal-Fluss et al., 2014; Li et al., 2015). Emerging evidence suggests that circRNAs can collectively bind and suppress the activation of the kinase PKR, thereby controlling innate immune response (Liu et al., 2019), which is the first line of defense against invading pathogens (Mogensen, 2009). In addition, circRNAs are involved in antiviral immunity; the circRNA–miRNA–mRNA network could regulate host immune function (Chen et al., 2017; He et al., 2017; Zhang et al., 2017), and thus provide efficient protection against viral infections (Wang et al., 2017b). Certain identified circRNAs could activate retinoic acid-inducible gene-I (RIG-I) and confer effective immune protection against viral infections (Chen et al., 2017). Glutathione S-transferases (GSTs) are multifunctional enzymes that are primarily involved in cellular defense against toxins in most living organisms (Hayes et al., 2004). Glutathione S-transferase kappa 1 (GSTκ1) from the big belly seahorse (*Hippocampus abdominalis*) represents an important role in innate immunity and detoxification of harmful xenobiotics (Samaraweera et al., 2019). The GST kappa from *Haliotis discus* responds against immune and stress challenges (Sandamalika et al., 2018). Glutathione S-transferase P1 (GSTP1) is a member of the GST enzyme superfamily and two miRNAs have been verified to target the GSTP1 3′UTR region in the liver of *C. plagiosum* (Ge et al., 2017).

[1]http://www.circrna.org

In this study, circRNAs in the liver of *C. plagiosum* were identified, and their binding strength (<2,000 bp) was determined; the detected miRNAs in shark liver were simultaneously analyzed to construct an mRNA–miRNA–circRNA network for the GSTP1 gene; two of indentified circRNAs that are predicted to interact with each other were cloned on the dual luciferase vector PsicHECK 2. The miRNA was co-transfected into 293T cells, and finally tested by dual luciferase to verify the interaction between circular RNA and miRNA, as well as the authenticity of circRNA to expand current understanding on circRNAs in marine animals (Meng et al., 2018; Zhao et al., 2020).

MATERIALS AND METHODS

Animals

An adult male *C. plagiosum* with length of 40 cm was obtained from the East China Sea. The shark was anesthetized using MS-222, and its liver was collected and stored in liquid nitrogen (Ohta et al., 2000). All procedures were approved by the Zhejiang Sci-tech University Animal Experimental Ethics Committee.

CircRNA High-Throughput Sequencing

In brief, total RNA was pretreated to enrich circRNAs by using a CircRNA Enrichment kit (Cloud-seq, Inc., United States). RNA libraries were constructed using pretreated RNAs with a NEBNext® Ultra II™ Directional RNA Library Prep kit (New England Biolabs, Inc., Massachusetts, United States) in accordance with the manufacturer's instructions. Libraries were controlled for quality and quantified using a BioAnalyzer 2100 system (Agilent Technologies, Inc., United States). Library sequencing was performed on an Illumina Hiseq 4500 instrument with 150 bp paired end reads.

Divergent and Convergent Primer Design

The correction of circRNAs identified from *C. plagiosum* were validated by randomly selecting several circRNAs from a circRNA high-throughput database. As shown in **Figure 1**, a PCR amplification template was made by joining a half sequence of circRNAs from the 3′ end to the 5′ end of the circRNAs. The new amplification template sequence was used to design divergent PCR primers, the initial sequence was used to design convergent PCR primers (Panda and Gorospe, 2018).

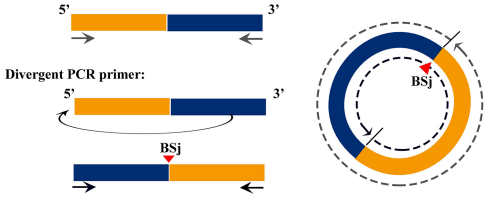

FIGURE 1 | Schematic of the design of divergent and convergent primers, demonstration of the principle of forward and reverse primer design.

TABLE 1 | Sequence of RT-PCR primers.

CircRNA Name	Divergent PCR primers	Convergent PCR primers
328–177	F: TGCAAACCTGGCTTTAAACCAGT	F: TTACTTGTACAGTAAACAGGTATTTAAACAT
	R: ATTGTAAGTGATTACATCATCCACGTG	R: CAACTTGACAGGCAGGTTCTG
294–177	F: TGCGATCGTAGGTTTAAACCAGT	F: TTACTTGTACAGTAAACAGGTATTTAAAAAAT
	R: ACTGTAAGTGATTTCATCATCCATGTG	R: CAATTACACATGCAGGTTCTGGAT
50–170	F: GGTTCTAGAGCAGGAAAAAACATTGT	F: GTGTGCTGGAACACTTGAAGACAC
	R: TGCGCTTTGTGCTGTGCTTC	R: TCCAGCAGACTGTGCAGTCACT
34–434	F: GTTGCTTACCTCGCAACTACAAAAT	F: AACTATCCACCACATTCAGTTTCAC
	R: ATAATTGTTATAAACTAGCTGATTACTACTTTC	R: AGATGCCAAAGCTTTTCCAAG
30–219	F: GAGTCCACAGAACGGTTGGAT	F: AATGAAAAATGCTGGAAACAGAC
	R: ACTGAAGACATTGTTCCGGACAC	R: CTGTGGTTAAAAAGCACTTTGTG
28–237	F: CGGTTGGACGCGATTCCG	F: GTCCGCCACAGATTTCTTCAAG
	R: CTGTGAATGGTGCGGCCAT	R: TAATCACAATGAGAGTGTAGTTGAGGT
22–545	F: GGCTGTCATTGCCTTGATTTTTC	F: TGTAAGCACACCCAGTCTGGC
	R: TTGTGGGCAGAATTGATTTTAG	R: TGGCAGGTGAAGGGGTACTG
21–352	F: CACTGCAGGCATTCCACATC	F: GTTCACAATGTCTGGAAATAAACGT
	R: TGCCTTTAAATGTCGGGGC	R: CTGTGTGTTGGTGCAGGCT
38–1717	F: CAGTTTACTCCTCCGTATTCCT	F: GGTGGAGGATTGGAAGGCT
	R: AAGCCTTCCAATCCTCCAC	R: CGGGTAGGAATACGGAGGAG
6–1096	F: GTATCCTGGCACGATTAAGC	F: TGAACGGACGGAACCTTG
	R: CATCACCATTCGCACTGTTCT	R: CTGCTGGAGGATTGGCCT

F, forward; R, reverse.

Total RNA Isolation and Linear RNA Degradation

The total RNA of the wild whitespotted bamboo shark was extracted from the liver by using a Trizol reagent (Invitrogen, CA, United States) following the manufacturer's procedure. An RNase R digestion reaction was prepared, the mixtures were incubated at 37°C for 30 min, and RNA was immediately isolated.

cDNA Cloning of CircRNAs

Liver cDNA was synthesized using a PrimeScript™ 1st Strand cDNA synthesis kit (Takara, Japan) in accordance with the manufacturer's instructions, added 1 μg total RNA per 10 μL system, and used as templates for PCR amplification. The primers for RT-PCR and expected sizes are shown in **Table 1**. PCR was carried out in accordance with the normal regulation rules, with 2 μL cDNA added to the 50 μL system. The PCR product was separated with 2% agarose gel electrophoresis. The purified PCR product of the junction site and circular sequence of each circRNAs was further validated via Sanger sequencing.

CircRNA–miRNA–mRNA Regulation Network Construction

TargetScan (5.0), and miRanda (3.3a) were used to predict targeting relationships (Hu et al., 2019) between circRNAs with a sequence of less than 2,000 bp and miRNA (Cheng et al., 2017). Based on the results, selected the circRNAs–miRNA [miRNAs that can combine with GSTP1 3′ UTR region (Ge et al., 2017)] pairs with the minimum free energy (mfe) <-20 kcal/mol and TargetScan_score ≥90 to construct a circRNA–miRNA–mRNA regulation network using Cytoskope software. The obtained circRNAs were identified by treating 20 μg of total RNA with 1 μL RNase R (Lucigen, United States), the RNAs treated with RNase R was used for RT-PCR. The primers are shown in **Table 1**.

miRNA-mRNA-circRNA Interaction Study

Through double enzyme digestion, circRNA (38-1717 and 6-1096) predicted to interact with the dual luciferase carrier psiCHECK-2 were connected to form recombinant vectors psiCHECK-2-circ-38-1717 and psiCHECK-2-circ-6-1096, and then the interacting miRNA (ipu-miR-143 targets 38-1717, direct -7a target 6-1096) is sent to Youkang Biological Company to synthesize miRNA mimics, and then the recombinant vector and miRNA mimics are co-transfected into 293T cells.

The plasmid was extracted using Endofee Plasmid Mini Kit II (Omega, United States), 293T cells were obtained from our laboratory. Cells were cultured in DMEM (with L-Glutamine, Gibco, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, United States) and 1% penicillin-streptomycin (Gibco), in an incubator containing 5% CO_2 at 37°C In the cultivation. Twenty four–Forty eight hours prior to transfection, spread 2×10^5 cells/well 293T cells in a 12-well plate and culture overnight to make the cell density about 80% during transfection.

With Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific), the four experimental groups psiCHECK 2 + microRNA mimics-ipu-miR-143 empty plasmid control group, psiCHECK 2-38-1717 + microRNA mimics-ipu-miR-143 experimental group; psiCHECK 2 + microRNA mimics-dre-let-7a empty plasmid control group and psiCHECK 2-6-1096 + microRNA mimics-dre-let-7a experimental group were co-transfected into 293T cells. Add 1.25 μg plasmid and 4 μL

liposomes, 1 μL microRNA mimics to each well. Culture cells for 48–72 h at 37°C, followed by the dual luciferase assay. Incubate the cells at 37°C for 48–72 h, and then use Dual Luciferase Reporter Gene Assay Kit (Yeasen, China) for dual luciferase assay.

RESULTS

Identification of CircRNAs

The total RNAs from the liver of *C. plagiosum* were isolated to investigate circRNAs. The total RNA was treated with a circRNA Enrichment kit to enrich circRNAs and to construct libraries for deep sequencing through the Illumina HighSeq 4000 platform. Sequencing generated 64.12 million nucleotide raw base data. After adaptors were trimmed and low-quality reads were filtered, 64.03 million clean reads were obtained (**Table 2**).

Clean reads were aligned with the *C. plagiosum* genome (ASM401019v1) by using bowtie2 software (Langmead and Salzberg, 2012), and circRNAs were detected and identified via find_circ software (Memczak et al., 2013). The principle of find_circ: basis on the result of Bowtie2 alignment, find_circ extracts 20nt anchor sequences from both ends of the reads that are not aligned to the reference sequence, and aligns each pair of anchor sequences with the reference sequence again. If the 5′end of the anchor sequence is aligned to the reference sequence (the start and stop positions are marked as A3 and A4, respectively), the 3′end of the anchor sequence is aligned to the upstream of this position (the start and stop positions are marked, respectively as A1, A2), and there is a splice site (GT-AG) between A2 and A3 of the reference sequence, then this read is used as a candidate circRNA. Finally, candidate circRNAs with read counts greater than or equal to 2 were used as identified circRNAs. The total number of matched reads was used to normalize the reverse splicing reads (junction reads) of each sample and log2 conversion. According to find_circ analysis, at least 4,558 circRNAs that had one back-splicing junction reads (BSj reads) were obtained. Among them, 2,776 circRNAs (39.1%) had more than one BSj reads, and 967 circRNAs had more than four BSj reads, accounting for 20% of the total reads (**Figure 2**).

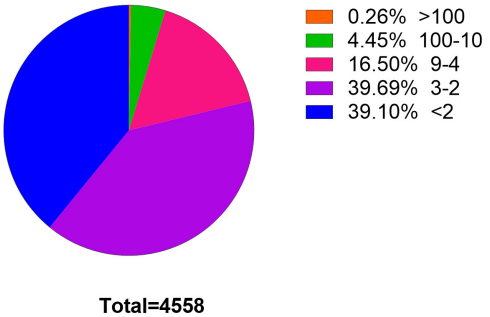

FIGURE 2 | Statistics of circRNAs BSj reads. Among these 4,558 genes, only 0.26% has a reading of more than 100 BSj; 4.45% has a reading of 10–100 BSj reads; 16.5% has a reading of 4–9 BSj reads; while 39.69% for 2–3 BSj reands and 39.10% less than 2 BSj reads.

CircRNA Signature Analysis

We performed a set of counting calculations for total circRNAs and BSj reads >4 circRNAs to determine the characteristics of liver circRNAs in *C. plagiosum*. According to NCBI database, *C. plagiosum* had a total of 51 chromosomes, ranging in size from 4.64 to 156.6 Mb. When referring to the relative expression levels using the ratio of circRNA number to genome size (Mb), the relative expression of *C. plagiosum* circRNAs (∼1.47) is slightly higher than those of humans (∼1.00). Larger number of the chromosome usually have smaller size (**Figure 3A**). Overall, no matter total circRNAs or BSj reads >4 circRNAs, the number of them represent a downward trend with the size of chromosome 1 to 51, and circRNAs detected on chromosome 8 are relatively smaller than those of other chromosomes of similar size. The diversity in the number of matched circRNAs on chromosomes of similar size is pronounced in BSj reads of >4 circRNAs; for example, relatively more BSj reads >4 circRNAs matched on chromosomes 2, 11, 23, and 36 than on chromosomes 8 and 14, which indicates that circRNAs are not evenly distributed on *C. plagiosum* chromosomes.

The length of the circRNAs, total circRNAs, and BSj reads >4 circRNAs exhibited a similar assignment in terms of molecular length (**Figure 3B**). Nearly half of the circRNAs are with a length more than 10,000 bp, which might be due to incomplete genome annotation or the original long circRNAs of sharks.

Identification of CircRNAs

Two pairs of primers for RT-PCR (divergent and convergent primers) were designed to verify the authenticity of identified circRNAs from the *C. plagiosum* liver. Eight highly expressed circRNAs in *C. plagiosum* were selected (**Table 3**) and named "the number of BSj reads—predicted sequence length." The back-splicing junction was detected by RT-PCR. After treated with 1 μL Ribonuclease R per 20 μg of total RNA, the results were compared before and after RNase R degradation of total RNA (**Figure 4A**).

All the sequences of expected sizes of selected circRNAs have been successfully amplified (**Figure 4B**). At the same time, circRNAs, especially 34–434, 22–545, and 21–352, could be enriched through total RNA treated with RNase R, whereas no band was amplified when total RNA and RNase R-treated RNA were used. An RT-PCR product with an expected length could be amplified using convergent PCR primers except 34–434 (**Figure 4C**). 34–434 authenticity was

TABLE 2 | Reads statistics.

Sample	Raw reads	Q30	Clean reads	Clean ratio	Mapped reads	circRNA number
Wt-liver	64,124,640	92.53%	64,033,850	99.86%	42,355,809	4558

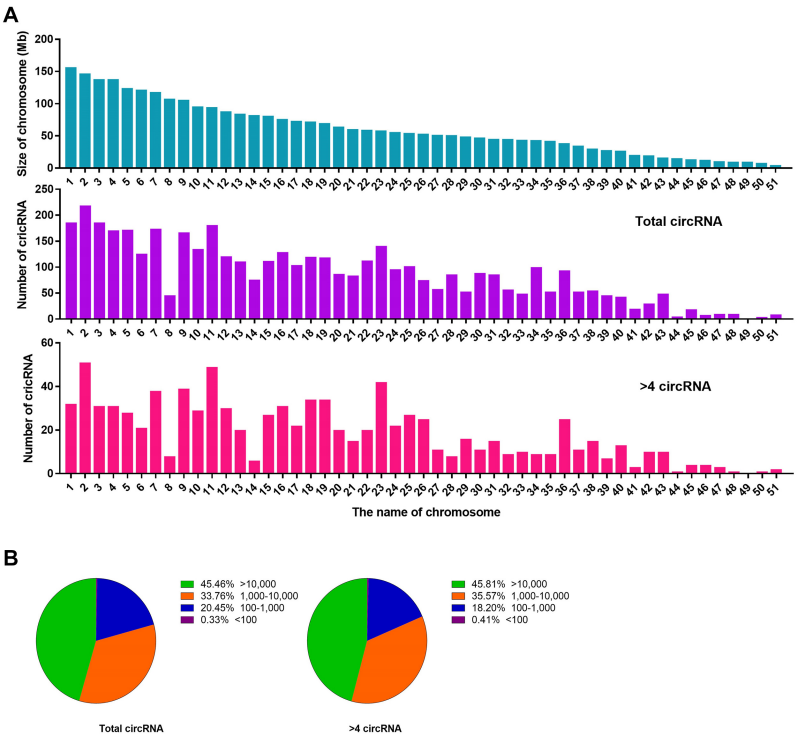

FIGURE 3 | Statistics of circRNAs distribution and length. **(A)** Size of chromosomes in *C. plagiosum* (blue column) and the number of circRNAs that matched on the chromosomes (purple column represents total circRNAs; pink column represents BSj reads >4 circRNAs). **(B)** Statistics of the length of total circRNAs and BSj reads >4 circRNAs; The first pie chart shows the proportion of circular RNA from 100 to 10,000 bp in all circular RNAs; The second pie chart shows the distribution of circular RNA length from 100 to 10,000 bp in all circular RNAs with BSj greater than 4.

TABLE 3 | Information on eight circRNAs with high BSj reads.

CricRNA ID	Number of BSj reads	Predicted sequence length	circRNA name
QPFF01524526.1:668-844 +	328	177	328-177
CM012981.1:19191743-19191919-	294	177	294-177
CM012964.1:995687-995856 +	50	170	50-170
CM012991.1:19134334-19134767-	34	434	34-434
CM012986.1:38607300-38607518 +	30	219	30-219
CM012956.1:24938513-24938785 +	28	273	28-237
CM012988.1:10693863-10694407-	22	545	22-545
CM012971.1:29840270-29840621-	21	352	21-352

verified by amplification using a new convergent PCR primers (**Supplementary Figure 1C**). CircRNAs could be enriched by RNase R treatment (Wang et al., 2017a).

According to the results of Sanger sequencing, three circRNAs, including 30–219, 21–352, and 28–273, showed the same sequences in the database, and the back-splicing junction was simultaneously found in their sequence (**Figure 4D** and **Supplementary Figure 1A**). The sequence identity of 328–177 and 294–177 was approximately 80.2%, and the sequencing results of the two RT-PCR products showed double peaks at specific positions, which suggested that back-splicing occurred on the exon where alternative splicing existed (**Figure 5A**). A similar situation was observed on 50–170 and 22–545 (**Supplementary Figure 1B**).

The sequence 34–434 also had the back-splicing junction, while it was 29 bases ahead of the predicted position. The two sites of back-splicing, which simultaneously followed the GT-AG rule, were found in the database and confirmed via Sanger sequencing (**Figure 5B**). That 34–434 could not be amplified by convergent PCR primers may be due to the primer designed on the 29 bases behind the back-splicing junction.

Binding Prediction of CircRNAs and miRNAs on GSTP1

One crucial function of circRNAs is to act as a miRNA sponge to absorb miRNA and regulate gene expression. According to software prediction, the genes in the miRNAs database

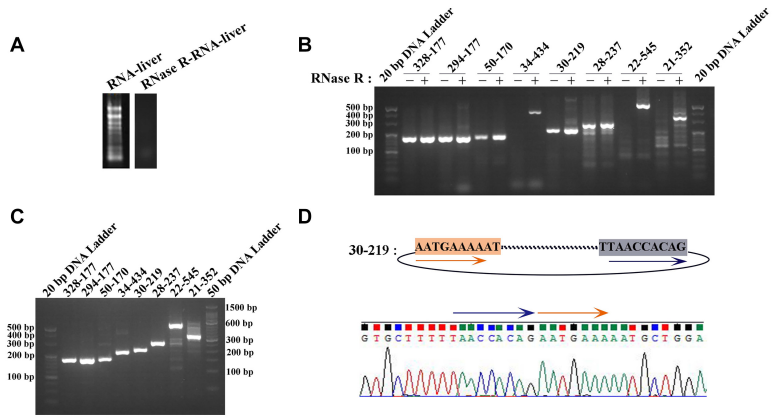

FIGURE 4 | Identification of circRNAs. **(A)** The total RNA was treated by RNase R. **(B)** The amplification result of RT-PCR using divergent PCR primers (" + " represents RNase R-treated RNA as the reverse transcription template, "−" represents the total RNA transcription). **(C)** Amplification result of RT-PCR using convergent PCR primers and RNase R-treated RNA as the reverse transcription template. **(D)** Sanger sequencing of 30-219 confirmed the back-splicing junction in PCR products.

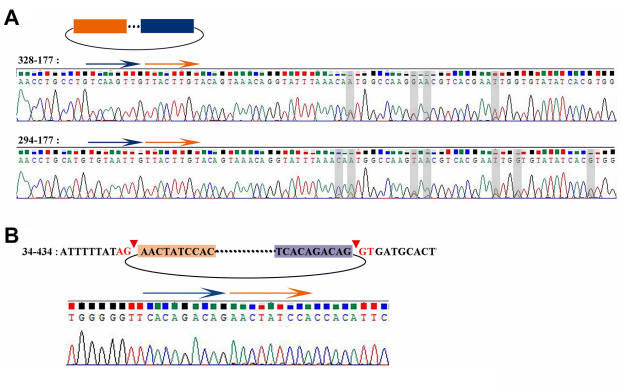

FIGURE 5 | Sanger sequencing result of 328-177, 294-177, and 34-434. **(A)** Sanger sequencing of 328-177 and 294-177 confirmed the back-splicing junction in PCR products. Some overlapping peaks might mean that more than two transcripts formed by alternative splicing. **(B)** Sanger sequencing of 34-434 confirmed the back-splicing junction in PCR products.

could be matched with the circRNA database to obtain the predicted correspondence. Based on a previous research on GSTP1 (Ge et al., 2017), a total of three miRNAs (dre-let-7a, ipu-miR-143, and hsa-miR-143-3p_R + 1_1ss21CA) that bind to the 3′UTR region of GSTP1 were identified in the liver tissue of *C. plagiosum*. Besides, these miRNAs were matched with circRNAs. According to the GSTP1 network of mRNA, miRNAs, and circRNAs (**Figure 6A**), six circRNAs were predicted to combine the three miRNAs. Among them, two circRNAs (named 38-1717 and 6-1096, **Table 4**) were analyzed and predicted to be able to bind miRNAs. The divergent and convergent PCR primers were designed to verify circRNAs, which showed that the two predicted circRNAs were both present in the shark liver tissue (**Figure 6B**). RNA hybrid software analysis indicated that the minimum free energy of the circRNAs and miRNAs was approximately −30.1 kcal/mol (38-1717 and miR-143) and −28.9 kcal/mol (6-1096 and dre-let-7a) (**Figure 6C**), indicating their binding strength and possible ability bind to miRNAs to regulate GSTP1 expression.

Interaction Study of Identified CircRNAs With miRNA

Through the constructed recombinant vector and microRNA mimics, four recombinant miRNA groups: psiCHECK 2 + microRNA mimics-ipu-miR-143 empty plasmid control group, psiCHECK 2-38-1717 + microRNA mimics-ipu-miR-143 experimental group, psiCHECK 2 + microRNA mimics-dre-let-7a empty plasmid control group, psiCHECK 2-6-1096 + microRNA mimics-dre-let-7a were transfected into 293T cells, after 48 h incubation, the luciferase activity was evaluated by Dual Luciferase Reporter Gene Assay Kit (Yeasen, China).

During the analysis of the measured data, and 3 replicates were needed for the experimental group. The first is calculate the ratio (F/R) of Firefly Luciferase/Renilla Luciferase for each hole. Then calculate the average of the three duplicate wells of 38-1717-miRNA-Ctrl and 6-1096-miRNA-Ctrl, called average1. Then use the average1 of the 38-1717-miRNA-Ctrl and 6-1096-miRNA-Ctrl control groups as the standard 1, and use the psiCHECK 2-circ-38-1717-OE group and psiCHECK 2-circ-6-1096-OE F/R three repeated values (Divide by average1 to get three ratios (equivalent to a normalization). Finally, the average value of the three repeated wells of 38-1717-miRNA-Ctrl and 6-1096-miRNA-Ctrl and the three of psiCHECK 2-circ-38-1717-OE group and psiCHECK 2-circ-6-1096-OE. The ratio value is input to Graphpad prism8.0.1 to draw the mutual mapping of circRNA and miRNA (**Figure 7**). The data showed that the experimental group has a large degree of fluorescence value reduction, indicating that circRNA and miRNA interact, suggesting that the prediction is correct, and the two groups of experimental groups and control groups are analyzed by *t*-test (*indicated significant difference).

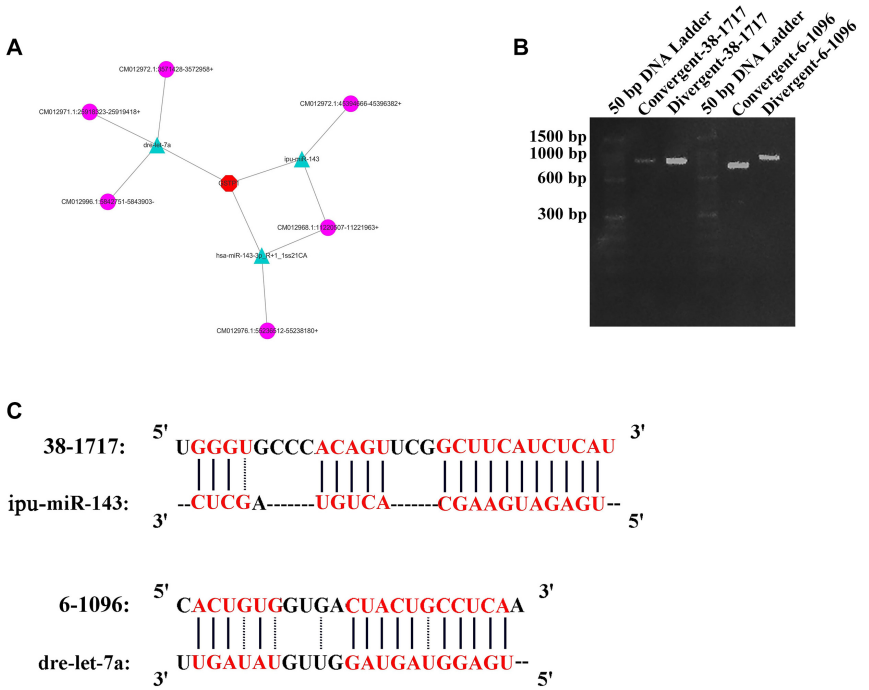

FIGURE 6 | CircRNA analysis for GSTP1. **(A)** Network of mRNA, miRNAs, and circRNAs. **(B)** Amplification result of RT-PCR for 38-1717 and 6-1096. **(C)** Combination of circRNAs and miRNAs.

TABLE 4 | Information on the GSTP1-circRNAs.

circRNA ID	Number of BSj reads	Predicted sequence length	circRNA name	Length of RT-PCR product	
				Convergent	Divergent
CM012972.1:45394666-45396382 +	38	1717	38-1717	867	897
CM012971.1:25918323-25919418 +	6	1096	6-1096	777	907

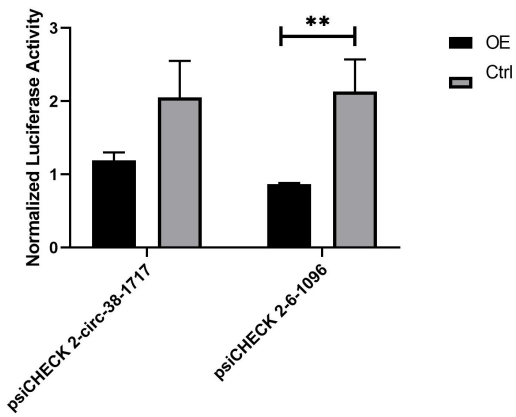

FIGURE 7 | Diagram of the interaction between circRNA and miRNA. They are the psiCHECK 2-circ-38-1717-OE group and psiCHECK 2-circ-6-1096-OE group and its corresponding control group. The *t*-test analysis shows that psiCHECK 2-circ-6-1096-OE and its control group have significant difference. Statistical analysis was performed by Prism 8, $^{**}P \leq 0.01$, indicated significant difference.

DISCUSSION

In recent years, the roles of circRNAs in tumor immunity regulation and immunotherapy have been demonstrated by an increasing number of studies focusing on their function as an miRNA sponge (Xu et al., 2018). CircRNA is particularly less explored in marine animals. *C. plagiosum* is a demersal cartilage fish, with no relative circRNA identified and characterized currently. In this research, 4,558 unique circRNAs were systematically identified, among which more than 45% had a sequence size of more than 10,000 bp. In all 4,558 circRNAs, 967 circRNAs (approximately 20%) contain more than four BSj reads, in which more than 45% are longer than 10,000 bp. This phenomenon is uncommon relative to other species (Pallavicini et al., 2013b; Maass et al., 2017) because none of current data of other fish showed a high percentage of long circRNAs (Pallavicini et al., 2013a). We speculate that this phenomenon might be due to the incomplete genomic information on *C. plagiosum* or the presence of long circRNAs, which could absorb more miRNAs and thus might have regulatory effect on multiple pathways.

Eight randomly selected shark circRNAs were verified, and findings showed that circRNA splicing follows the GT-AG rule. Sequencing results indicated that back-splicing could occur on the exon where alternative splicing existed, which means that alternative splicing occurs not only in mRNA transcription but also in back-splicing. During evolution, sharks might have evolved complex RNA splicing processes to meet their various physiological needs.

GSTs are primarily involved in cellular defense against toxic compounds in most living organisms, and they play an important role in immune responses. Mapping the mRNA–miRNA–circRNA network relationship for GSTP1 may provide information in the further study GSTP1 function in sharks. A complete mRNA–miRNA–circRNA network could not be established because the transcriptome information on *C. plagiosum* is incomplete. Nevertheless, the construction and analysis of the relationship network could be established according to the target gene searching sequence, which would be valuable for subsequent studies on other genes. The relationship between miR-dre-let-7a and circRNA_-6-1096 was confirmed in a dual-luciferase reporter assay. It confirms the authenticity of our prediction and provides a basis for us to verify the interation of CircRNA as a miRNA sponge, and also provides a promising target for various liver diseases in the future.

CONCLUSION

In this study, a total of 4,558 circRNAs were analyzed and identified in the liver tissue of *C. plagiosum*. At least one back-splicing junction reads (BSj reads) was obtained in the identified circRNAs, and further analysis revealed that *C. plagiosum* circRNAs were not evenly distributed on the chromosomes but followed the GT-AG rule during cyclization. Alternative back-splicing existed in shark circRNAs. We found three miRNAs (dre-let-7a, ipu-miR-143, and hsa-miR-143-3p_R + 1_1ss21CA) binding to the 3′UTR region of GSTP1 and generated a potential mRNA–miRNA–circRNA network for GSTP1. The relationship between miR-dre-let-7a and circRNA_-6-1096 was confirmed in a dual-luciferase reporter assay. Confirmed our prediction and confirmed the biological function of circRNA as a sponge of miRNAOur findings may contribute to existing studies on *C. plagiosum* circRNAs in the cartilaginous fish and provide a basis for characterizing the functions of circRNAs in marine animals with a focus on the circRNAs in the liver tissue of *C. plagiosum* (Meng et al., 2018).

DATA AVAILABILITY STATEMENT

The circRNA sequencing data is available in NCBI at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157160.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Zhejiang Sci-Tech University.

AUTHOR CONTRIBUTIONS

WZ: investigation and writing of the original draft. PQ: investigation, writing of the original draft, and editing. XG: writing, review and editing, and use of software. LH: formal analysis and validation. CW: writing and review and editing. GC and JC: validation, project administration, and revision of the manuscript. LW: conceptualization, methodology, and revision of the manuscript. ZL: conceptualization and resources. All authors read and approved the final manuscript.

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CircRNA May Not Be "Circular"

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Circular RNA (circRNA) is a novel regulatory non-coding RNA and participates in diverse physiological and pathological processes. However, the structures and molecular mechanisms of circRNAs remain unclear. In this study, taking advantage of openly databases and bioinformatics analysis, we observed lots of internal complementary base-pairing sequences (ICBPS) existed in plenty of circRNAs, especially in extremely long circRNAs (el-circRNAs, > 5,000 nt). The result indicated that circRNA may not be a simple circular structure. In addition, we put forward the hypothesis of "open-close effect" in the transition for specific circRNA from normal state to morbid state. Taken together, our results not only expand the knowledge of circRNAs, but also highlight the potential molecular mechanism of circRNAs.

Keywords: circular RNA, bio-informatics, biological phenomena, molecular conformation, hypothesis and theory

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INTRODUCTION

CircRNA, a group of endogenous non-coding RNAs, has long been characterized as a single strand covalently closed continuous loop without 5′–3′ polarity and a polyadenylated tail (Hansen et al., 2013). The expression of circRNAs has spatio-temporal specificity, and they may exhibit distinct expression patterns in different diseases or at different stages. It has been recently recommended that cirRNAs have an essential role in regulating genes expression by functioning as a translational regulator, RNA binding protein (RBP) sponge, and microRNA (miRNA) sponge (Memczak et al., 2013; Abbaszadeh-Goudarzi et al., 2020; Mirzaei and Hamblin, 2020). The most thoroughly studied function of circRNA is that it serves as competitive endogenous RNAs (ceRNAs). And studies have demonstrated their activity as miRNA sponges as well as protein sponges (Huang et al., 2020). Though accumulating evidence reveals that circRNAs could exert vital biological functions and serve as novel biomarkers as well as providing promising therapeutic approaches for various human diseases (Borran et al., 2020; Naeli et al., 2020; Razavi et al., 2020), much has not yet to be elucidated about their molecular mechanisms. Chen et al. (Liu C.X. et al., 2019) have recently exhibited the secondary structures of circRNAs and the structures are stable and formed from

Abbreviations: 3′-UTR 3′, untranslated regions; ceRNAs, competing endogenous RNAs; circRNA, circular RNA; circLen, length of circRNAs; CR, complementary ratio; el-circRNAs, extremely long circRNAs; ICBPS, internal complementary base-pairing sequences; IRES, internal ribozyme entry site; m6A, N6-methyladenosine; maxLen, maximum length; medianLen, median length; miRNAs, microRNAs; MREs, miRNA recognition elements; ORF, open reading frame; RBP, RNA-binding protein.

static process. However, the structures of circRNAs, especially their dynamic process are still unclear. In addition, current research of el-circRNAs is scarce. Here, circRNAs with two internal completely complementary regions (≥10 nt) are selected. And the sequences were acquired and regarded as candidate ICBPS. Existence of ICBPS may contribute to the difficulty of exploring el-circRNAs. To better figure out the concepts and theory put forward in the research, an el-circRNA (hsa_circ_0000527) with high complementary ratio (CR) and long maximum length (maxLen) of the ICBPS was taken as an example. Here, we propose a new hypothesis that circRNAs may not be a single strand continuous loop, and they probably have double-strand structure, which may be dynamically reversible and have impact on their mechanism and functions.

RESULTS

Characteristics of ICBPS in circRNA

In our previous study, we detected circRNAs' expression in human plasma using circRNA microarray (GEO accession number: GSE131469) (Wu et al., 2019). Surprisingly, we discovered multiple of internal complementary base-pairing sequences (ICBPS) existed in circRNAs, especially in extremely long circRNAs (el-circRNAs,>5,000 nt). Given to the limited number of circRNAs in the microarray, we used human circRNA database[1] (Liu M. et al., 2019) in the following research. Based on the bioinformatics analysis of this database that containing about 140,790 circRNAs, we got similar results. For most circRNAs, the maximum length (maxLen) of the ICBPS is under 15 or even 10 nt (**Figure 1A**). Next in this study, we emphasized on the analysis of 6,155 circRNAs (4.37% of total circRNAs) with ICBPS ≥ 20 nt, most of which contain more than one pair of ICBPS. The number of circRNAs with different amounts of ICBPS was analyzed statistically, and there are over 2,000 circRNAs containing more than 20 pairs of ICBPS (**Figure 1B**). Through bioinformatics analysis we found that the 6,155 circRNAs contain 58,995 ICBPS in total, and for 90% circRNAs, the median length (medianLen) of the ICBPS is between 20 and 31 nt (**Supplementary Figure 1a**). Also, the number and maxLen of ICBPS were closely correlated with the total length of circRNAs (circLen) (**Figure 1C,D** and **Supplementary Figure 1b**).

Next, we defined the concept of complementary ratio

$$CR,\ CR\ =\ \frac{\left(maxLen\ of\ ICBPS\right)\ \times 2}{circLen}\ \times 100\%.$$

A higher CR would indicate a greater probability of internal base pairing in a circRNA. However, we found that CR of most circRNAs is under 10% or even 5%. And for circRNAs with higher CR, their circLens are all under 200 nt (**Figure 1E**, **Supplementary Table 1**). To better explain and clarified these concepts, here we include a detailed example. Hsa_circ_0000527, an el-circRNA with the circLen of 6,071 nt originating from exon 24 of chromodomain 14 was list as an example. Containing three pairs of ICBPS (≥20 nt), the maxLen of hsa_circ_0000527 is 123 nt, thus its CR is 4.05% (**Figure 1F**, **Supplementary Date 1**).

To characterize the ICBPS of circRNAs, we analyzed the locations and regions of parental genes. We found that for circRNAs that derived from 3′ untranslated regions (3′-UTR) of parental genes, about 60% of them contain ICBPS ≥ 20 nt (**Figure 1G**). Similar statistics analysis was conducted according to different chromosome origins as well as different components originated from the parental genes (**Supplementary Figure 1c** and **Figure 1H**).

Studies have confirmed that, functioning as ceRNA, circRNAs can competitively sponge microRNAs (miRNAs) through miRNA recognition elements (MREs) (Piwecka et al., 2017; Das et al., 2020). Analyzing the 206 circRNAs whose maxLen of ICBPS > 100 nt, we surprisingly observed that about 64% circRNAs have overlap between ICBPS and MREs (**Supplementary Figure 1d**). Meanwhile, circRNAs that containing internal ribozyme entry site (IRES) have the potential to translate proteins, which can be predicted through bioinformatics analysis of its open reading frame (ORF) (Legnini et al., 2017). Based on the analysis of circBank database, there are 6,155 circRNAs contain ICBPS ≥ 20 nt, and about 23/92% of them have overlap with ORF/MRE at the same time (**Figure 1I**).

Possible Structures and Molecular Mechanisms of circRNAs

Synthesizing the above analytical results, we conject that circRNA may not be a simple circular structure. It probably contains double-strand structure internally because of the presence of ICBPS (shown as A and A' in **Figure 2A**). Special situations can exist. For example, there may be one segment of ICBPS that can be complementary paired with multiple ICBPS (shown as B and B'/B" in **Figure 2A**). Or for one continuous sequence, it may have different complementary sequences that set close together or overlap on the same RNA chain (shown as C, D and C', D' in **Figure 2A**). Thus, the "open" or "close" state of the double-strand structures in circRNAs is a sophisticated dynamic process. The formation of this structure makes circRNAs compressed in space, which may help circRNAs bond firmly with RBPs and thus facilitate themselves being exported into the cytoplasm from nucleus. The dynamic process is shown in video 1. However, the formation process of this structure might be reversible. After "escaping" from cell nucleus, circRNAs quickly switch from the "close" state to "open." It is well-known that circRNAs are highly stable, but the degradation mechanism has not been clarified yet. The double-strand structure in circRNA may make them easier to be degraded by relevant enzymes which can probably explain how cells eliminate circRNAs (**Figure 2B**). The process may be regulated by micro-environment or other internal factors such as the length of ICBPS, the binding free energy, the distance between pairing fragments, the secondary structure of RNA, or relevant RNA modification like N6-methyladenosine (m6A), etc. At the same time, it might be an important way to regulate the circRNAs' degradation, translation and adsorption of miRNAs and so on. Last but not least, when the relevant sites of circRNAs are "blocked" due to the occurrence of base pairing, phenomena

[1] http://www.circbank.cn/

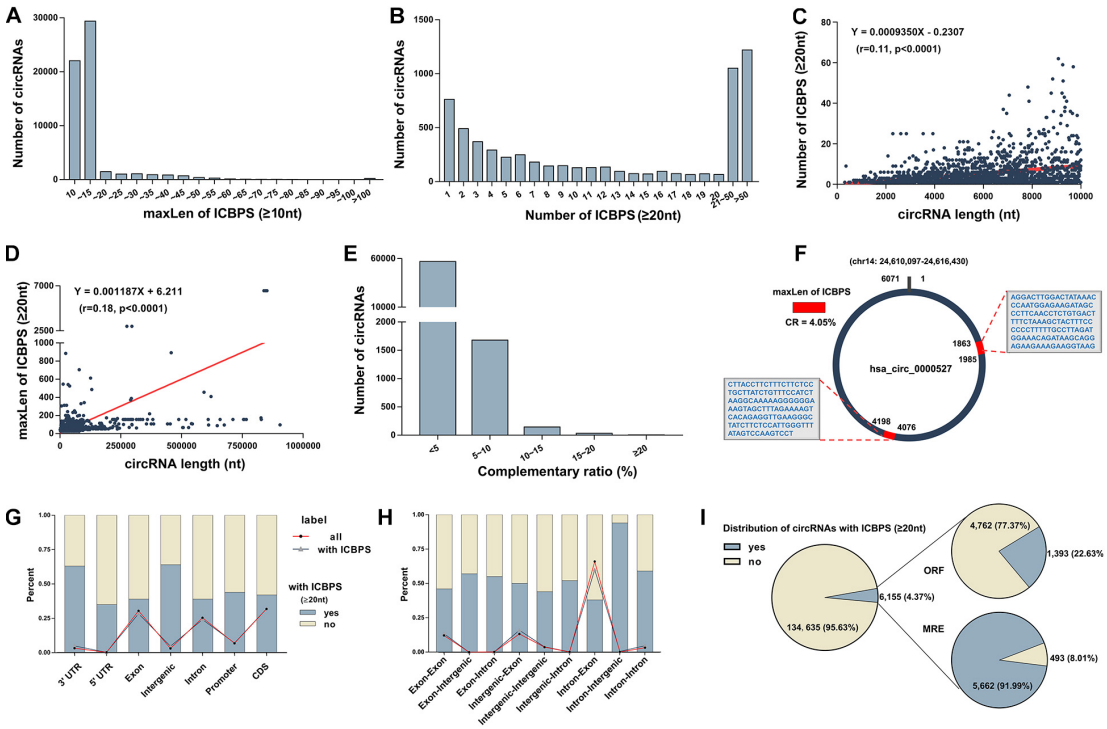

FIGURE 1 | Characteristics of circRNAs **(A)** The number of circRNAs with different maxLen of ICBPS (≥10 nt). **(B)** The number of circRNAs with different amount of ICBPS. **(C)** The correlation analysis between the number of ICBPS (≥20 nt) and the total length of circRNAs (≤10,000 nt). **(D)** The correlation analysis between the maxLen of ICBPS (≥20 nt) and the total length of circRNAs. **(E)** The number of circRNAs with different complementary ratio. **(F)** Hsa_circ_0000527 was taken as an example. **(G)** The percentage of circRNAs (with ICBPS ≥ 20 nt or not) that transcribed from different regions of their parental gene. The polyline graph shows the number distribution of circRNAs in different types. **(H)** The percentage of circRNAs (with ICBPS ≥ 20 nt or not) with different components originated from the parental genes. The polyline graph shows the number distribution of circRNAs in different types. **(I)** The distribution of circRNAs with ICBPS ≥ 20 nt, and the distribution of these circRNAs with ORF/MRE at the same time.

happen that the ability of circRNAs serving as miRNA "sponge" and translating is hindered.

Actually, several researches have questioned about the mechanism of ceRNA (Thomson and Dinger, 2016): some circRNAs were predicted to be able to bind a certain miRNA and regulate the downstream target genes, while this cannot be well proved through related experiments. On the other hand, the abundance changes of some circRNAs cannot effectively regulate the targeted genes. Based on this phenomenon, we put forward the hypothesis of "open-close effect" in the transition for specific circRNA from normal state to morbid state: for those circRNAs with closed ICBPS, even if they are highly expressed, they may not exert corresponding biological functions; for those circRNAs with similar or even lower expression, they may also play important roles through "opening" relevant ICBPS, and vice versa. In addition, RBPs are also known to be sponged by circRNAs and regulate gene expression. And the existence of ICBPS may affect the regulatory process as in miRNAs (**Figure 2C**).

CONCLUSION AND DISCUSSION

The presence of circRNAs is first being discovered in a viroid-infected plant (Sanger et al., 1976). Following evidences approved the critical role of circRNAs in various diseases (Shabaninejad et al., 2019). CircRNAs are ubiquitous, stable, conserved and diverse RNA molecules with a range of activities. They are reported as one of the main players in the regulation of multiple pathways and cellular processes (Yousefi et al., 2020). Some studies also suggested that the expression of circRNAs is dysregulated in virus-infected cells. Virus then uses the cellular mechanism to its advantage (Nahand et al., 2020). Chen et al. (Liu C.X. et al., 2019) first describes the degradation mechanism of circRNAs when cells are infected by virus and exhibited the secondary structures of circRNAs. Structural mapping showed that circRNAs inside cells could form stable secondary structures which contained short imperfect duplexes. Guria et al. (2019) also discussed the possible mechanism of circular RNA biogenesis, its structure and degradation. These studies emphasized the functional importance of the secondary structure within circRNAs. However, the structure and the potential molecular mechanism of circRNAs are still poorly studied.

Here, we provide new ideas and clues for the research of circRNAs. We speculated that circRNA may not be a simple circular structure, which probably contains double-strand structure internally, and the "open" or "close" of the double-strand structures is a sophisticated dynamic process.

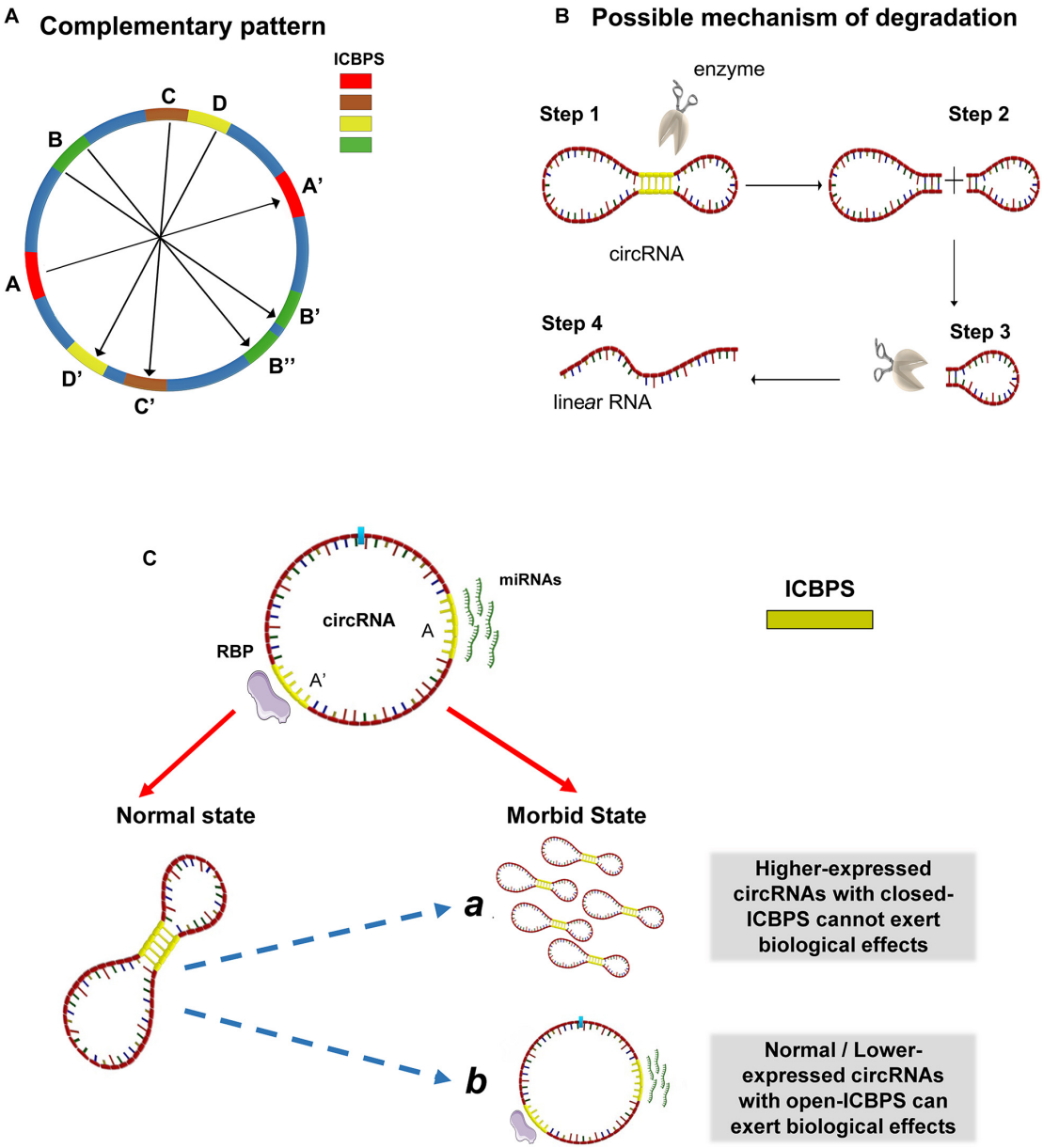

FIGURE 2 | Possible structures and molecular mechanisms of circRNAs. **(A)** The complementary patterns of ICBPS in circRNA. **(B)** Possible mechanism of circRNA degradation due to the presence of double-strand structure. **(C)** The "open-close effect" in the transition for specific circRNA from normal state to morbid state. ICBPS was sequences with two internal completely complementary regions (≥10 nt). The existence of ICBPS affected RBP sponging and miRNA sponging.

The hypothesis indicates that circRNA may play roles in the occurrence and development of disease, not necessarily through its aberrant expression change, but also through the "open-close effect" of related sites on the sequence. If a certain circRNA has both oncogenic and tumor suppressor miRNA binding sites, it may selectively "open" or "close" specific miRNA binding sites, consequently leads to different effects. While the main function of circRNAs is exerted through their activity as miRNA sponges, their second-most important function is exerted *via* circRNA-protein interactions. Interacting with regulatory RBPs through their activity as protein sponges, decoys, scaffolds, and recruiters, circRNAs then affect the fate of their target mRNAs (Conn et al., 2015; Yang et al., 2017). Thus, change in circRNAs' structure due to ICBPS may affect their binding between miRNAs as well as RBPs. In turn, RNA-protein interactions may regulate the synthesis and degradation of circRNAs. The interacted and regulatory relationships induced by ICBPS thereby influence cellular functions and disease processes.

In conclusion, our models may help to provide new ideas and clues for the questions as follows: (1) certain circRNAs cannot be amplified and validated using primers designed according to the design principle; (2) why some circRNAs have no effects on

their predicted target miRNAs and RBPs; (3) how can circRNAs be exported from nucleus into cytoplasma (especially for the el-circRNAs); (4) how can circRNAs be degraded; (5) how does m6A regulate circRNA encoding proteins; and (6) this may help to strengthen the functional research of el-circRNAs. This theory may change the existing circRNA research mode, and more importantly, extend the underlying logic of selecting indicators based on circRNA- sequencing or circRNA array.

MATERIALS AND METHODS

Acquisition of Internal Complementary Base-Pairing Sequences

Through the custom python script, we search all the circRNA sequences in circBank database. CircRNAs with two internal completely complementary regions ($\geq$10 nt) are selected. The sequences were acquired and regarded as candidate ICBPS. We used two pointers to represent the start and end of the query sequence and set pointer1 as position 1 bp and pointer2 as position 10 bp at the beginning. If ICBPS was found, pointers2 will move until find the longest ICBPS. if no ICBPS was found pointer1 move 1 bps. Searching will end when the pointer1 move to 20 bp upstream of the end of the circRNA sequence.

Prediction of miRna Recognition Elements

Through the custom R packages (Pagès et al., 2019), we extracted the ICBPS sequence ($\geq$20 nt). Potential miRNAs that bind to ICBPS were predicted through miRanda (Enright et al., 2003) with relatively strict parameters (free energy $\leq$ 20, alignment score $>$ 150).

Assessment of Coding Potential

Predict The coding potential and open reading frame (ORF) of circRNAs were predicted through CPAT (default parameter) (Wang et al., 2013).

Statistical Analysis

Data analysis was performed using GraphPad Prism7 software (GraphPad Software Inc., La Jolla, CA, United States) and SPSS 20.0 software (SPSS Inc., Chicago, IL, United States). Correlations were analyzed by Pearson's correlation test. P values of $<$0.05 were considered significant.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in circBank database (http://www.circbank.cn) and circBase (http://www.circbase.org/).

AUTHOR CONTRIBUTIONS

HJ, HS, and XD: conceptualization. HS and ZW: Methodology. HS, ZW, and LY: investigation. HS, XD, JZ, and ML: bioinformatical analyses. ZW and HS: writing-original draft. HJ and XD: writing-review and editing. HJ, JL, and LY: supervision and funding acquisition. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Circular RNAs in the Central Nervous System

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Circular RNAs (circRNAs) are endogenous single-stranded RNAs characterized by covalently closed loop structures with neither 5′ to 3′ polarity nor poly(A) tails. They are generated most commonly from back-splicing of protein-coding exons. CircRNAs have a tissue-specific distribution and are evolutionarily conserved, and many circRNAs play important biological functions by combining with microRNAs and proteins to regulate protein functions and their own translation. Numerous studies have shown that circRNAs are enriched in the central nervous system (CNS) and play an important role in the development and maintenance of homeostasis. Correspondingly, they also play an important role in the occurrence and progression of CNS diseases. In this review, we highlight the current state of circRNA biogenesis, properties, function and the crucial roles they play in the CNS.

Keywords: circular RNA, review (article), function, disease, central nervous system

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INTRODUCTION

For many years, although proven to be present, circular RNAs (circRNAs) were overlooked as byproducts of splicing errors (Sanger et al., 1976; Barrett and Salzman, 2016). However, major recent studies have discovered that circRNAs have essential functions and play a novel regulatory role, especially in the nervous system (Memczak et al., 2013), prompting interest from an increasing number of investigators from various fields. Unlike messenger RNAs (mRNAs), circRNAs are mostly derived from different regions of gene loci in eukaryotes through a non-canonical splicing process called "back-splicing." During back-splicing, the downstream 5′ splice site is covalently bonded to an upstream 3′ splice site in a reversed orientation. Due to the lack of 5′–3′ polarity and a polyadenylated tail, circRNAs are much more insusceptible than linear RNAs to degradation by exonuclease RNase R. CircRNAs exert their action mainly by acting as a miRNA sponge and functioning through a competing endogenous RNA (ceRNA) mechanism (Hansen et al., 2013; Memczak et al., 2013), as well as acting as protein sponges or translating proteins (Legnini et al., 2017). Recent genome-wide profiling of circRNAs has shown that numerous circRNAs are widely and dynamically expressed in the nervous system. Furthermore, their expression is dramatically increased in the brain during the aging of multiple organisms (Knupp and Miura, 2018). In addition, circRNAs participate in many processes of neurological diseases. For instance, circ-TTBK2 and circPCMTD1 act as sponges of miR-224-5p to promote glioma progression (Zheng et al., 2017, 2019). As an extension of the brain and a part of the central nervous system (CNS), the retina is another option for investigators to study enigmas of circRNAs. It has been demonstrated that circRNAs are abundant in the retina and play a role in biogenesis and various biological functions (Wang et al., 2018a; Chen et al., 2020c).

BIOGENESIS, PROPERTIES, AND FUNCTIONS OF CIRCRNAS

Biogenesis

Circular RNAs can be generated from different gene loci, such as coding and non-coding exons, introns, both exons and introns, or antisense or intergenic sequences. Due to their different origins, they have different names, such as exonic circRNA (or ecircRNA), intronic circRNA (or ciRNA), and exon–intron circRNA (or ElciRNA) (**Figure 1**; Zhang et al., 2013; Guo et al., 2014; Li et al., 2015). These circRNAs can be generated in different manners. (1) They can be generated from intronic lariat precursors that escape from the debranching step of canonical linear splicing (**Figure 1A**; Zhang et al., 2013). A pre-mRNA can generate linear RNA, lariat introns, Y-structure introns from trans-splicing, and circular exons through exon skipping (Suzuki et al., 2006). In canonical splicing, a lariat intron is generated after the splicing of the linear pre-mRNA and isolated from the ultimate mRNA product. This excised lariat undergoes internal back-splicing. Then, lariats that escape from debranching can lead to the formation of ciRNAs (Zhang et al., 2013; Eger et al., 2018). (2) Lariat formation during exon skipping (**Figure 1B**; Kelly et al., 2015; Petkovic and Muller, 2015). During alternative splicing, an exon-skipping event occurs and creates an exon-containing lariat formation, intronic lariat formation and mRNA with skipped exons (Zaphiropoulos, 1997; Barrett et al., 2015). The exon-containing lariat creates ecircRNAs when undergoing internal back-splicing (Kelly et al., 2015; Petkovic and Muller, 2015). However, not all of the exon-containing lariats will generate circular RNA despite the correlation between exon-skipping and circular RNA formation (Barrett et al., 2015; Kelly et al., 2015). And the smaller skipped exons are less prone to circularize than those in large size (Barrett et al., 2015). (3) Looped by base pairing between inverted repeat elements (such as Alu elements) (Jeck et al., 2013; Ivanov et al., 2015) or RNA-binding proteins (RBPs) (**Figure 1C**; Errichelli et al., 2017). Looping of the intron sequences flanking the downstream splice-donor site and the upstream splice-acceptor site brings these sites into close proximity (Ivanov et al., 2015). RBPs can bind two flanking introns together to promote the formation of circular structures; then, introns will be removed to form junction sites. For instance, muscleblind protein (MBL) provokes circMbl biogenesis in the form of RBPs, resulting in competition between circMbl and its linear transcript (Ashwal-Fluss et al., 2014). Similarly, the immune factors NF90/NF110 also promote circRNA production in the nucleus as RBPs (Li et al., 2017). In addition, some cis-regulatory elements and trans-acting factors participate in back-splicing, such as the spliceosome assembly (Zhang et al., 2016). Although studies have illustrated these mechanisms of circRNA biogenesis, this process has not been fully elucidated.

Properties

As circular structure, circRNAs have some common characteristics. First, circRNAs are prevalent across species and evolutionarily conserved. They can be detected in many species, from plants to animals and from *Caenorhabditis elegans* to humans (Memczak et al., 2013; Sun et al., 2019). With a strict definition, investigators found that 4,522 of 15,849 mouse circRNAs were conserved in humans (Rybak-Wolf et al., 2015). For example, the circTulp4 isoform is derived from homologous exons in humans and mice (Rybak-Wolf et al., 2015). Second, circRNAs have tissue/developmental-stage-specific expression. For instance, circCdr1as is abundantly expressed in the nervous system, whereas it is barely detected in non-neuronal tissues (Memczak et al., 2013; Chen et al., 2020b). In addition, circRNAs are dynamically expressed during development. In the mouse hippocampus, circRNAs derived from protein coding gene loci with synapse-related functions were observed to be upregulated from E18 to P30 [You et al. (2015) profiled four stages: embryonic (E18), early postnatal (P1), the beginning of synapse formation (P10), and late postnatal (P30)]. In contrast, those produced from gene loci without any function were downregulated. Third, circRNAs are much more resistant than linear RNAs. Due to the closed loop structure, circRNAs are stable and can resist degradation from RNase R, which indicates why circRNAs can accumulate in cells for a long time (You et al., 2015). Fourth, circRNAs have incredible diversity. Different circRNAs can consist of one or more exons or even no exons (intronic circRNA), but they principally contain 2–5 exons (Rybak-Wolf et al., 2015). The length of circRNAs ranges from 100 bp to 4 kb (Salzman et al., 2013).

Biological Function

As a novel type of RNA, circRNAs have been proven to play various roles in biological processes. First, individual circRNAs have been posited to function as miRNA or RNA-binding protein sponges (**Figure 2A**). A famous circRNA, ciRS-7, also known as circCdr1as, has more than 70 putative binding sites for miR-7, allowing multiple interactions. Knockdown of circCdr1as decreased the expression of miR-7 target genes, whereas knockout of circCdr1as downregulated miR-7 (Hansen et al., 2013; Memczak et al., 2013; Piwecka et al., 2017). Regarding the combination of RBP, the tumor suppressor gene Foxo3 can produce circFoxo3, and circFoxo3 regulates cell cycle progression by binding to cell division protein kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (p21) (Diallo et al., 2019). Second, circRNA can be translated in cap-independent manners. Under stress conditions, circRNA can use its internal ribosome entry site (IRES) to recruit ribosomes to an internal position of circRNA. For example, circMbl shares the same start codon as the linear mRNA and can be found with increasing IRES activity. The proteins detected by mass spectrometry also provide important evidence (Pamudurti et al., 2017) (**Figure 2B** left). The second mechanism for circRNA translation is the recruitment of eukaryotic initiation factor 3 (eIF3) by methylated adenosine residues in the form of N6-methyladenosines (m6A) in the 5′untranslated region (5′UTR) for translation into small polypeptides (Diallo et al., 2019; Zhou et al., 2021) (**Figure 2B** right). Third, circRNA can participate in transcriptional regulation by interacting with RNA polymerase II (RNA pol II) and other snRNP partners. Experiments have shown that

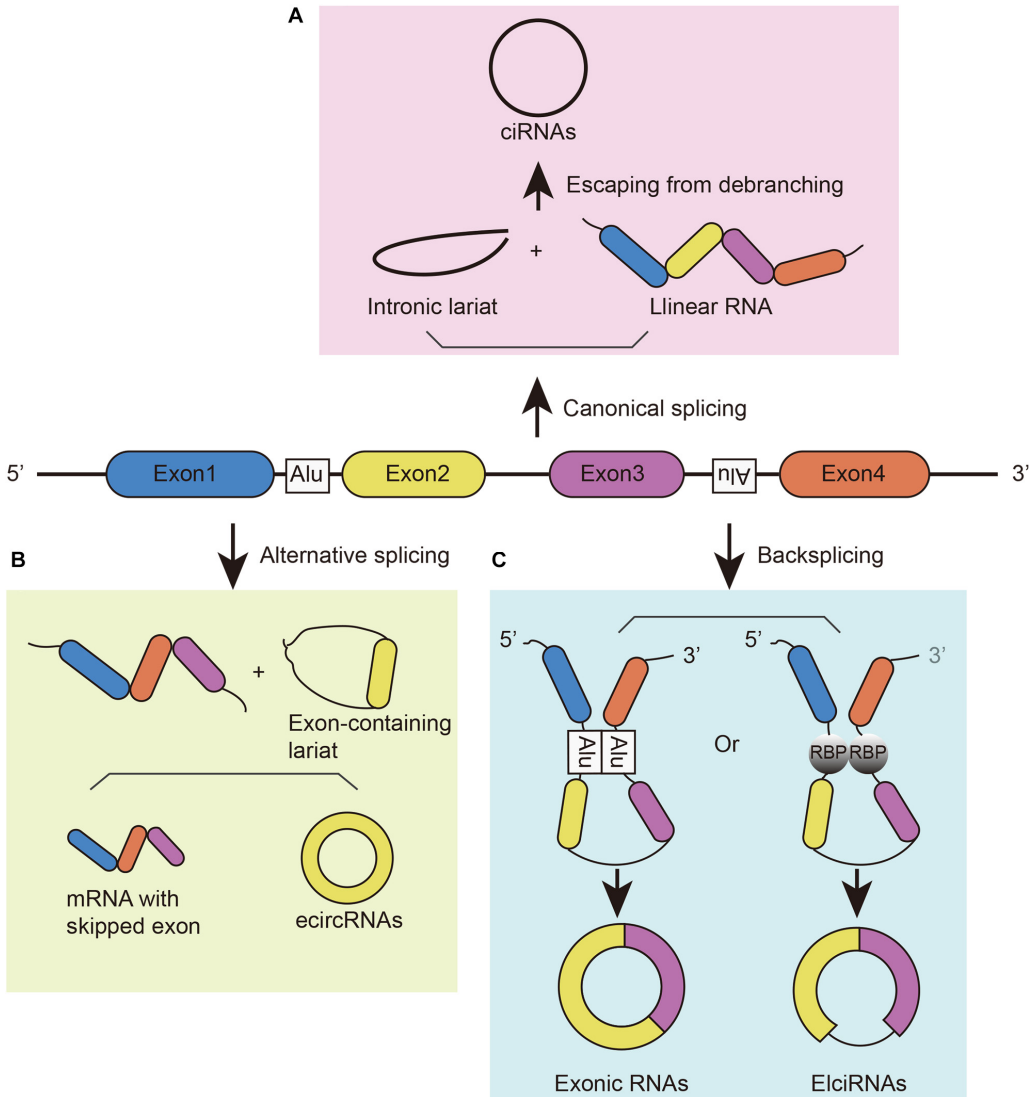

FIGURE 1 | The biogenesis of circRNAs. **(A)** CiRNAs are generated from intronic lariat precursors that escape from the debranching step of canonical linear splicing. **(B)** EcircRNAs can be generated from exon-containing lariats created by an exon-skipping event during linear splicing. **(C)** CircRNAs can be generated from back-splicing mediated by inverted repeat elements and *trans*-acting RNA binding proteins.

circEIF3J and circPAIP2 can regulate the transcription of their parental genes through this mechanism (Li et al., 2015; **Figure 2C**).

DISTRIBUTION, DEVELOPMENTAL-STAGE-SPECIFIC EXPRESSION PROFILE, AND AGE-RELATED ACCUMULATION OF CIRCRNAS IN THE NERVOUS SYSTEM

Distribution

Circular RNAs has been shown to be tissue-specific. We compared the number of circRNAs detected in human tissues in the TSCD database[1] and found that circRNAs are highly enriched in the brain (**Figure 3**; Xia et al., 2017). It was observed that the brain had a dominant role not only in the number of circRNAs but also in the frequency of circRNA hosting genes (approximately 20% of brain protein-coding genes produce circRNAs) (You et al., 2015). Another study reached a similar conclusion by comparing the human frontal cortex, thyroid gland, liver, and muscle (Rybak-Wolf et al., 2015). The enriched circRNAs are not uniformly distributed throughout the nervous system; it has been proven that they vary in different brain areas (Rybak-Wolf et al., 2015). A comparison of the circRNA expression of areas in the human and mouse brain showed that circRNAs were mostly enriched in the forebrain in mice

[1]http://gb.whu.edu.cn/TSCD

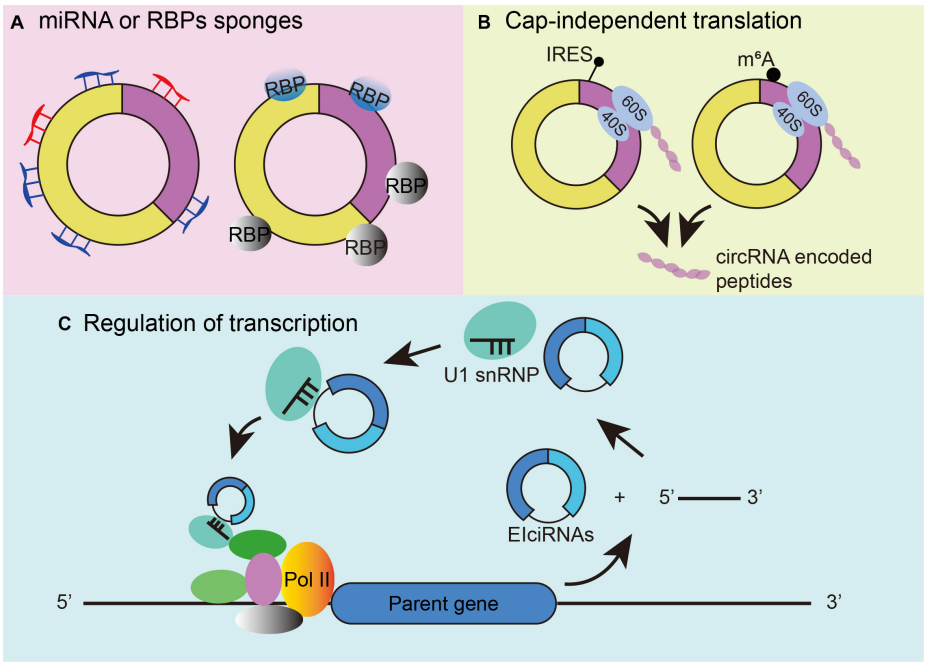

FIGURE 2 | Mechanisms of circRNA functions. **(A)** CircRNAs can function as microRNA and RBP sponges. **(B)** CircRNA cap-independent translation mechanism: IRES-driven circRNA translation (left) and m6A-driven circRNA translation (right). **(C)** Regulation of transcription initiation by EIciRNAs.

and that the prefrontal cortex (PFC) had greater expression than the hippocampus (HC). Investigators assessed genome-wide expression of circRNAs in the HC and PFC of the mouse brain (Chen et al., 2018) and found an opposite result to that of Rybak-Wolf's research; namely, circRNA expression in the HC was greater than that in the PFC. This finding may have occurred because Chen et al. (2018) chose data from the GEO database, while Rybak-Wolf et al. (2015) detected and analyzed these molecules on their own. Another reason may be the sample differences. However, both studies demonstrated the potential function of circRNAs in essential neuronal activities. Afterward, investigators further explored the exact enrichment localization of circRNAs in cells (You et al., 2015). Gene Ontology analysis indicated that circRNAs in the brain are mostly derived from several groups of genes related to synaptic function. Thus, high-resolution *in situ* hybridization (ISH) showed that localization of circRNAs was found in both the cell body and the dendrites of neurons (You et al., 2015). Furthermore, it was found that circRNAs were more abundant in synaptoneurosomes than whole-brain lysate and cytoplasm based on all expression cutoffs when they were normalized to host gene expression (Rybak-Wolf et al., 2015). The localization of circRNAs in the synaptic neuropil suggests that these molecules may play a role in the regulation of gene expression required for synaptic plasticity.

Developmental-Stage-Specific Expression Profile

It has been proven that circRNAs are expressed in a developmental-stage-specific manner. During the maturation of primary neurons, most circRNAs (1,926 circRNAs) were found to be upregulated and only a few were downregulated (797 circRNAs) in the mouse brain (Rybak-Wolf et al., 2015). Investigation of *Drosophila* showed that the expression of circRNAs in neurons was increased throughout life (Westholm et al., 2014). During porcine embryonic brain development (E23, E42, E60, E80, E100, and E115) (Venø et al., 2015), circRNAs were increased from E23 to E60 and reached their peak at E60. Then, expression declined drastically with continuing reduction until E115. These implicit circRNAs may function at specific developmental periods and are important for neuronal function. In addition, investigators found that when compared with mRNA of their host gene, circRNAs were not expressed in the same way. The negative correlation between gene expression and the logarithm of the circular-to-linear ratio (CLR) indicates the independent function of circRNAs (Rybak-Wolf et al., 2015). The expression of circRNAs in the retina was also explored. It was found that many circRNAs were upregulated or downregulated during immature rat retinal development in P3, P7, and P12 (Han et al., 2017). Work from Chen et al. (2020c) in the mouse retina obtained a similar conclusion and indicated that the expression patterns of circRNAs differed from linear transcripts from the same host gene. The developmental stage-specific expression profile of circRNA suggests its important regulatory function in the development and differentiation of the nervous system. The data from the retina are not completely consistent with those from the brain; thus, further research is needed.

Age-Related Accumulation

In addition to the early stages of development, investigators are also interested in the expression of circRNAs in the aging nervous

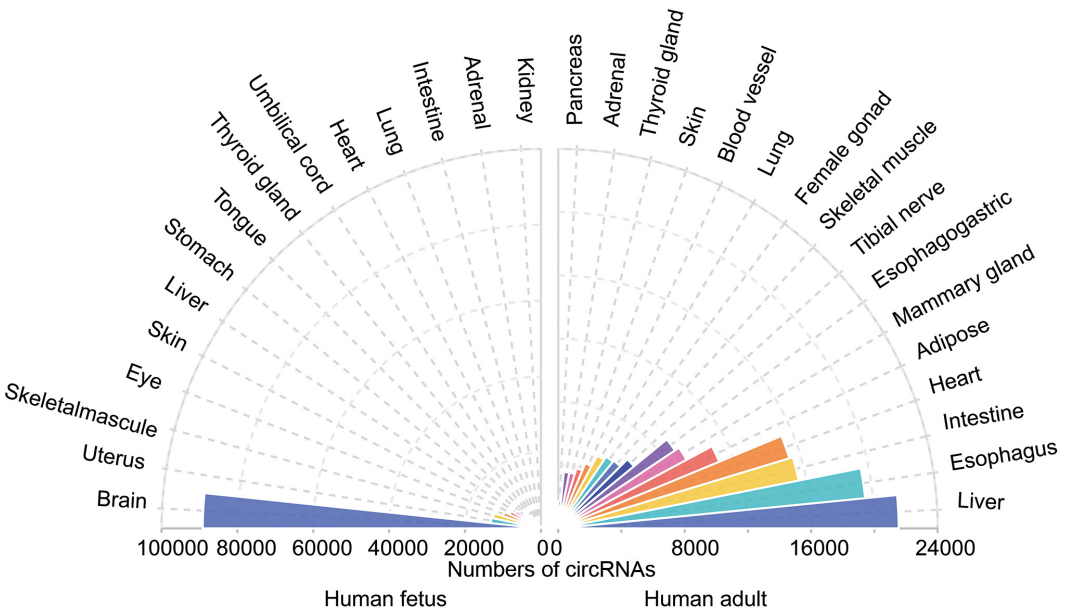

FIGURE 3 | The tissue-specific circRNAs expressed in human tissues. The colorful triangles represent the numbers of circRNAs detected in different human tissues (left, 15 human fetal tissues; right, 15 human adult tissues), respectively. CircRNAs are most abundant in the human fetus brain compared with other tissues.

system. To date, several studies have shown that age-related circRNA accumulation exists in various animals (Westholm et al., 2014; Gruner et al., 2016; Cortes-Lopez et al., 2018; Chen et al., 2019). *Drosophila melanogaster* RNA-Seq libraries reveal this phenomenon for the first time. Westholm et al. found that the expression of 262 circRNAs was significantly upregulated in 20-day heads versus 1-day heads (Westholm et al., 2014). The circRNAs identified in the cortex, HC and heart of 1- and 22-month-old mice were analyzed (Gruner et al., 2016). The study revealed that circRNAs showed significant upregulation in elderly cortex and hippocampal samples but not in the heart. Moreover, investigators performed additional validations using the cortex from an intermediate age of 6 months (Gruner et al., 2016). They found that half of the circRNAs were significantly increased between 1 and 6 months, and all detected circRNAs were significantly increased between 6 and 22 months. This result revealed that the age-dependent accumulation was progressive. In addition, aging-related accumulation of circRNAs was found in *C. elegans* and porcine brain (Cortes-Lopez et al., 2018; Chen et al., 2019). The latest research on rhesus monkeys showed that 11 circRNAs and host mRNAs are involved in regulating brain aging (Xu et al., 2020). One of them, circGRIA1, can regulate age-related synaptic plasticity by negatively regulating its host gene in the nucleus. Interestingly, the accumulation of circGRIA1 is also male-biased, which warrants further study.

The above results revealed that circRNAs are highly expressed in the nervous system. The uneven distribution of circRNAs in different brain regions indicates that they have potential functions in spatial learning and memory. The age-related accumulation of circRNAs suggests that they may contribute to neuronal aging and age-related diseases such as Alzheimer's disease (AD) (Lukiw and Circular, 2013) and age-related macular degeneration (AMD) (Jin et al., 2019; Chen et al., 2020a). Current studies show some evidences in these diseases but still requires further research.

CIRCRNAS IN NEUROLOGICAL DISEASES

With such abundant expression in the nervous system, circRNAs play important roles in neurological diseases, such as AD, Parkinson's disease (PD), and immune-mediated demyelinating diseases (Ghosal et al., 2013; Lukiw and Circular, 2013; Zhao et al., 2016; He et al., 2019). Briefly, we summarized some circRNAs involved in neurological diseases (**Table 1**) and three representative ones were chosen for detailed depiction (**Figure 4**).

AD

The miR-7 circRNA system was shown to be dysregulated in the hippocampal CA1 region of sporadic AD patients by Northern blot hybridization techniques and the circularity-sensitive circRNA probe RNase R (Lukiw and Circular, 2013). It has been demonstrated that ubiquitin protein ligase A (UBE2A) is a target downstream gene of miR-7. UBE2A is the core effector of the ubiquitin 26S proteasome system, which acts by proteolysis to remove amyloid peptides. The expression of miR-7 is significantly increased in the brains of sporadic AD patients, which may be related to the downregulation of circCdr1as expression (Zhao et al., 2016). In addition, circCdr1as can promote the degradation of APP and BACE1 via the proteasome and lysosome (Shi et al., 2017; **Figure 4A**). Furthermore, the atlas of cortical circular RNA expression in AD and normal patients' brains showed that circRNA expression

TABLE 1 | CircRNAs in neurological diseases.

Disease/Model	Host		CircRNAs	Mechanism	Reference
AD		Human	circCDR1as	As miR-7 sponge	Zhao et al., 2016; Shi et al., 2017
		Human	circHOMER1	As miR-651 sponge	Dube et al., 2019
		Human	circCORO1C	As miR-105 sponge	Dube et al., 2019
AD	HN cell	Human	circHDAC9	Alleviated Aβ42-induced HN cell neurotoxicity via miR-142-5p	Zhang Y. et al., 2020
	cellular AD model	Rat	circ 0000950	circ 0000950 enhanced neuron apoptosis and inflammatory response in AD through acting as a miR-103 sponge	Yang et al., 2019
PD		Human	circCDR1as	As miR-7 sponge	Ghosal et al., 2013
		Mouse	circDLGAP4	miR-134-5p/CREB pathway	Feng et al., 2020
Immune-mediated demyelinating disease		Human	hsa circ 0087862 hsa circ 0012077	Biomarker	He et al., 2019
CNS injury	HT22 cells with oxygen-glucose deprivation/reoxygenation (OGD/R)	Mouse	mmu-circRNA-015947	mmu-miR-188-3p, mmu-miR-329-5p, mmu-miR-3057-3p, mmu-miR-5098, mmu-miR-683 sponge	Lin et al., 2016
	Microglia-induced hippocampal neuronal apoptosis	Rat	circPTK2	MiR-29b-SOCS-1-JAK2/STAT3-IL-1β pathway	Wang et al., 2019
	Traumatic injury	Rat	circ-Spidr	PI3K-Akt signaling pathway	Mao et al., 2019a
	Nerve crush model	Rat	circ-Ankib1	miR-423-5p, miR-485-5p, and miR-666-3p	Mao et al., 2019b
Retinal disease	RB	Human	hsa circ 0001649	AKT/mTOR signaling pathway	Xing et al., 2018
	AMD/RPE cell line	Human	circNR3C1	circNR3C1-miR-3 82-5p-PTEN network	Chen et al., 2020a
	Rat model of glaucoma	Rat	circ-ZRANB1	circ-ZRANB1/miR-217/RUNX2 network	Wang et al., 2018b
	Rat model of glaucoma	Rat	cZNF609	As miR-615 sponge	Wang et al., 2018a
Glioma	Glioma cell lines	Human	circ-ZNF264	As miR-4493 sponge	Zhang et al., 2019
		Human	circPCMTD1	As miR-224-5p sponge	Zheng et al., 2019
	Human tissue sample and cell line	Human	circ-TTBK2	As miR-217 sponge	Zheng et al., 2017
	Human brain sample	Human	circ-FBXW7	Encode protein	Yang et al., 2018
	Human tissue sample and cell line	Human	circPOSTN	CircPOSTN/miR-361-5p/TPX2 axis	Long et al., 2020

AD, Alzheimer's disease; PD, Parkinson's disease; CNS, central nervous system; RB, retinoblastoma; AMD, Age-related macular degeneration.

levels are significantly correlated with both neuropathological and clinical measures of AD severity (Dube et al., 2019). In this study, circHOMER1 was most significantly correlated with AD and may be involved in AD as a sponge of miR-651, which regulates the AD-related genes *PSEN1* and *PSEN2* (Agarwal et al., 2015). Recent *in vitro* studies found additional circRNAs involved in AD. In Aβ42-treated HN cells, circHDAC9 overexpression can promote cell viability and repress cell apoptosis and inflammation via sponging miR-142-5p (Zhang N. et al., 2020). In a rat cellular AD model, circ_0000950 was found to promote neuronal apoptosis and the inflammatory response in AD via sponging miR-103 (Yang et al., 2019). The mechanism of circRNAs in AD remains to be studied in the future, which will provide a new direction for the treatment of AD.

PD

MiR-7 also plays a role in PD by regulating alpha-synuclein (Ghosal et al., 2013). Alpha-synuclein is overexpressed with the development of PD and plays an essential role in PD. Overexpression of miR-7 can reduce the level of alpha-synuclein (Doxakis, 2010). Considering the interaction between mir-7 and circCdr1as, circCdr1as may participate in the PD pathological process by acting as a sponge. In addition, investigators found that circDLGAP4 had neuroprotective effects by modulating the miR-134-5p/CREB pathway in a PD mouse model (Feng et al., 2020), although the mechanism underlying circRNAs in PD is still unclear.

Immune-Mediated Demyelinating Disease

CircRNA expression was dysregulated in cerebrospinal fluid from patients with immune-mediated demyelinating disease compared with that of healthy controls (2,364 were upregulated and 2,730 were downregulated) (He et al., 2019). The enrichment analysis of GO and KEGG showed that these circRNAs are most likely to participate

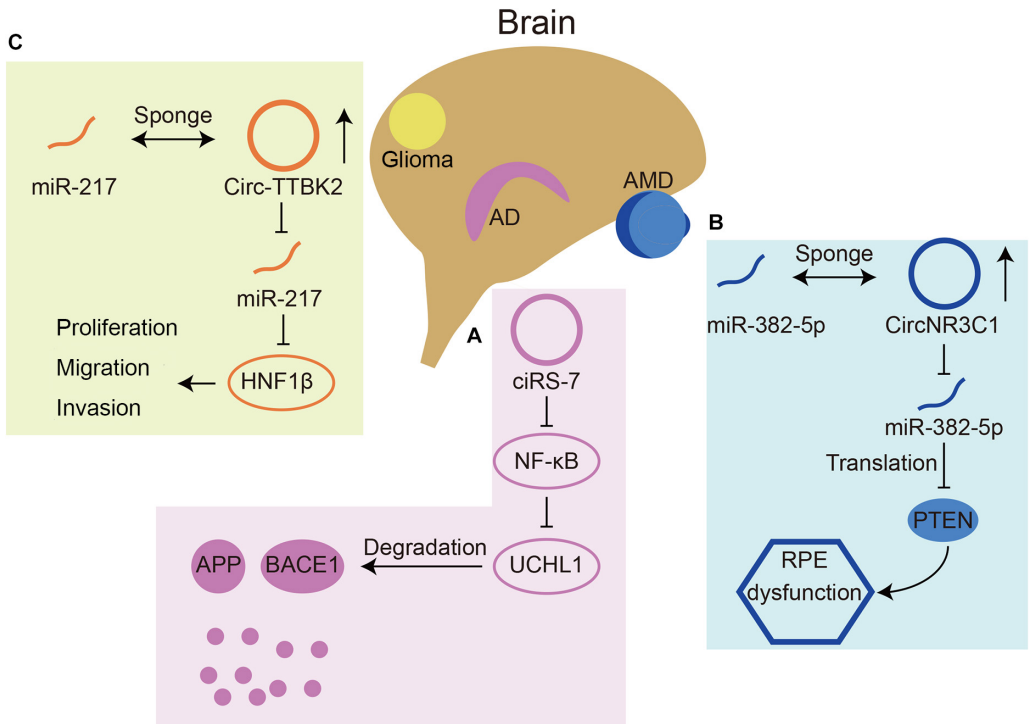

FIGURE 4 | Three circRNAs that play roles in neurological diseases. **(A)** CiRS-7(also named circCDR1as) can inhibit translation of NF-κB, resulting in upregulated expression of UCHL1. UCHL1 can promote the degradation of APP and BACE1 in AD. **(B)** CircNR3C1 acts as a miR-382-5p sponge, which can inhibit translation of an AMD related gene PTEN. **(C)** Circ-TTBK2 acts as a miR-217 sponge, which can inhibit HNF1β. HNF1β can promote glioma malignancy.

in the process of macromolecule metabolism, membrane-bound organelles and protein binding. CircRNAs can also influence the immune response of viral infections through binding with immune factors, for example, NF90 and NF110 (Li et al., 2017).

CNS Injury

CircRNAs participate in various types of neuronal injury. To investigate the mechanisms of circRNAs in cerebral ischemia-reperfusion-injury (IRI)-induced neuron injury, Lin et al. (2016) tested circRNA expression in HT22 cells with oxygen–glucose deprivation/reoxygenation (OGD/R) and found that three circRNAs were upregulated and 12 were downregulated. It was further shown that circPTK2 could inhibit miR-29b expression. Downregulated miR-29b expression can upregulate the JAK2/STAT3 signaling pathway and finally lead to neuronal apoptosis induced by OGD-activated microglia (Wang et al., 2019). Irreversible axonal damage is the main cause of neurological dysfunction in neurodegenerative diseases or after traumatic injury. Mao et al. (2019a) found for the first time that circRNAs could be involved in axon regeneration of injured neurons. These results showed that circ-Spidr enhances axon regrowth *in vitro* and *in vivo*. In addition, the investigators found another circRNA that had the opposite effect. Circ-Ankib1 inhibits axon regeneration by inhibiting Schwann cell proliferation in the sciatic nerve after crush injury (Mao et al., 2019b). These studies indicated a therapeutic possibility of circRNAs for CNS injury.

Retinal Disease

As the retina is part of the CNS, many retinal diseases have also been shown to be related to circRNAs. The expression of circ-ZRANB1 was significantly upregulated in the aqueous humor of a rat model of glaucoma. Circ-ZRANB1 is mainly derived from Müller cells, which can bind to miR-271 to regulate the expression of RUNX2. Finally, the circ-ZRANB1/miR-217/RUNX2 network influenced retinal neurodegeneration caused by Müller cells (Wang et al., 2018b). Circ-ZNF609 has a similar function in retinal neurodegeneration induced by glaucoma by binding with miR-615 (Wang et al., 2018a). In AMD patients, circNR3C1 expression was found to be downregulated in the blood serum (Chen et al., 2020a). Investigators further assessed the possible mechanism of this circRNA through RPE cell lines and found that circNR3C1 protected RPE functions via the circNR3C1-miR-382-5p-PTEN network (**Figure 4B**). Retinoblastoma (Rb) is an important cause of blindness in early childhood (Liu et al., 2020). CircRNAs have also been shown to be dysregulated in RB and some RB cell lines. It was found that hsa_circ_0001649 was significantly downregulated and could regulate apoptosis and cell proliferation by the AKT/mTOR signaling pathway (Xing et al., 2018). There are currently few studies that have assessed the mechanism of circRNAs in retinal diseases.

Glioma

In addition, some studies have shown the function of circRNAs in nervous system tumors. Circ-ZNF264, circPCMTD1, and circ-TTBK2 (**Figure 4C**) promote cell proliferation, migration and invasion in glioma cell lines by regulating their miRNAs (Zheng et al., 2017, 2019; Zhang et al., 2019). Circ-FBXW7 has a tumor suppressor effect in glioma cells by encoding a novel protein and is positively correlated with the overall survival rate (Yang et al., 2018). A recent study found that, apart from regulating cell growth and apoptosis, circ POSTN participated in aerobic glycolysis in glioma via the miR-361-5p/TPX2 axis (Long et al., 2020). Additionally, a recent review reported that most circRNAs function as miRNA sponges in glioma (Sun et al., 2020).

CircRNAs also play a role in psychiatric diseases such as bipolar disorder and major depressive disorder (Luykx et al., 2019; Zhang Y. et al., 2020).

Overall, the aforementioned studies indicate that circRNAs play a key role in many neurological diseases. However, the underlying mechanisms of circRNAs in many diseases have not been fully elucidated. Further study is required to elucidate how circRNAs exert their effects biologically.

PERSPECTIVES

CircRNAs are novel RNAs that are abundantly expressed in numerous organisms. Numerous studies have shown that circRNAs are enriched in the nervous system. Many of them are derived from genes related to synaptic function. Synapses are critical in information transmission and regulation of neuronal activities. CircRNAs are usually small, and many circRNAs can be detected in exosomes. They may be transported by synaptic vesicles to adjacent cells to transmit information. However, more evidence is required. A recent study by Xu et al. (2020) showed that circRNAs in the nucleus can regulate synaptic plasticity. This finding has encouraged more investigators to identify more mechanisms of circRNAs in the nervous system.

The rapid development of biochemical methods, such as BaseScope ISH and high-throughput circRNA sequencing analysis, provides investigators with powerful tools to investigate the exact subcellular location and interaction with other molecules. If we can determine the location of circRNAs in exact types of neurons, we may be able to distinguish more types of circRNAs and further elucidate the functions of the emerging RNAs. These studies will provide powerful guidance for clinical diagnosis and treatment.

The mechanism by which age-related circRNAs accumulate in the brain is still unclear, probably because of their circular structure. This characteristic makes them resistant to RNase R and difficult to degrade, ultimately leading to their age-related accumulation. However, recent studies have found sex-biased accumulation, indicating that this phenomenon may be caused by multiple factors.

AUTHOR CONTRIBUTIONS

M-LL and WW collected the data and drafted the manuscript. Z-BJ revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Genetics

REVIEW
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Investigating the Underlying Mechanisms of Circular RNAs and Their Application in Clinical Research of Cervical Cancer

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Circular RNAs (circRNAs) are non-coding RNA molecules, and these are differentially expressed in various diseases, including cancer, suggesting that circRNAs can regulate certain diseases. CircRNAs can act as miRNAs sponges, RNA-binding protein (RBP) sponges, and translation regulators, and they can become an important part of the regulation of gene expression. Furthermore, because of their biomedical features in body fluids, such as high abundance, conservation, and stability, circRNAs are seen as potential biomarkers for various cancers. Cervical cancer (CC) is one of the main causes of cancer-related death in women, and there have been a large number of studies that analyze circRNAs as a new object to be evaluated in CC. Therefore, this review, by understanding the role of circRNAs in CC, may create innovative strategies in the future clinical diagnosis, treatment, and prognosis of CC and promote the development of personalized and highly accurate cancer therapy.

Keywords: cervical cancer, circRNAs, precision medicine, molecular marker, miRNAs sponges

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INTRODUCTION

Circular RNAs (circRNAs) are a special class of non-coding RNA molecules that do not have a 5′ terminus cap and a 3′ terminus poly (A) tail. Unlike traditional linear RNA, circRNAs exhibit closed ring structures, are not affected by exonuclease RNaseR, and are more stable in expression. CircRNAs are mainly generated from gene exons, but there are other types, such as those from introns and those that are inter genic, antisense, and sense overlapping (**Figure 1**). Owing to *trans*-shear, circRNA is abundant in the cytoplasm of eukaryotic cells, and its localization in the cytoplasm is a sufficient and necessary condition to study the mechanism of miRNA sponge. Small numbers of intron-derived circRNAs are noted in nucleic acids and have certain tissue, timing, and disease specificities, and they are thus suitable for molecular markers (Zhang Z. et al., 2018). CircRNAs have become a research hotspot because they are closely related to the progress of disease.

CircRNA MECHANISMS OF ACTION

There has been much research on circRNAs. They can be used as competing endogenous RNA (ceRNA) to regulate the physiological activities of cells; some circRNAs located in the nucleus can regulate the transcription of parental genes by binding to RNA

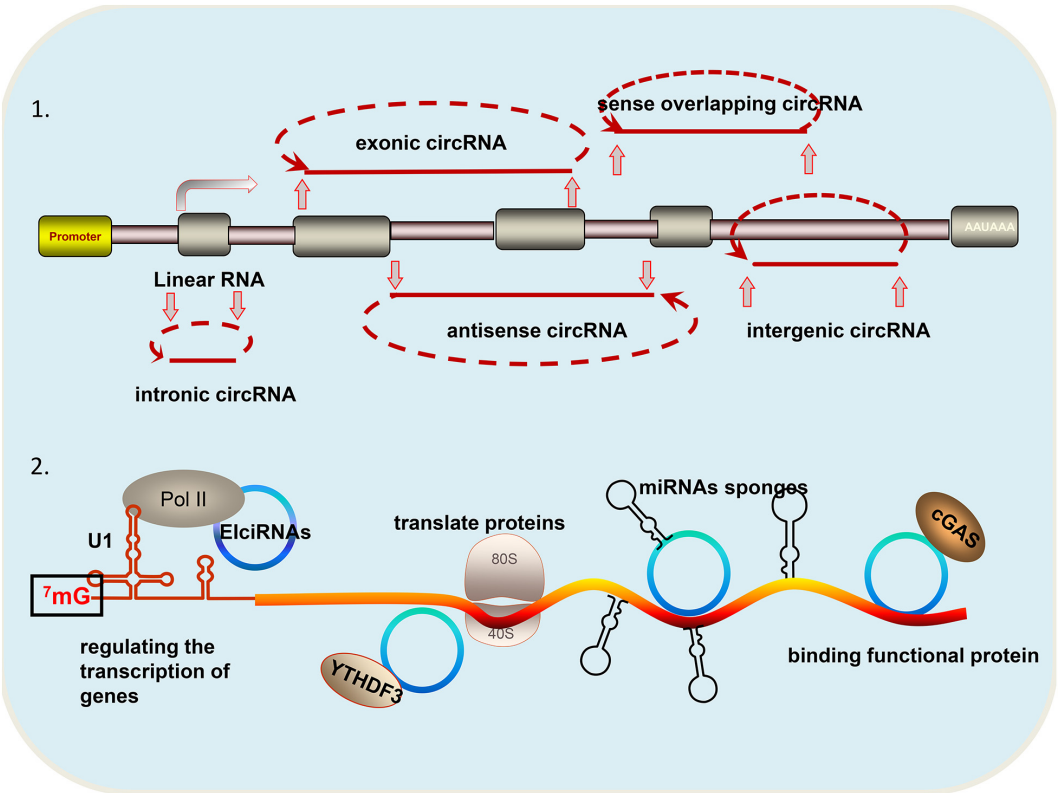

FIGURE 1 | Different types and functions of circRNAs: (1) According to the source of circRNA, it can be divided into the following categories: circular intronic RNAs (ciRNA) derived from gene introns; exonic circRNA (ecircRNAs) derived from gene exons; intergenic circRNAs derived from breakpoints between known genes; antisense circRNA derived from the antisense strand of a known gene; and sense overlapping circRNA: derived from the same gene locus as the linear transcript. (2) CircRNAs have the following four functions: regulating the transcription of genes; translating proteins; acting as miRNA sponges; and binding functional proteins.

polymerase II; they can also bind to proteins, affecting the cell cycle; and they can even encode proteins like mRNA. Especially in recent years in the field of cancer research, this has opened up a fresh direction for circRNAs (Zhou et al., 2018). Several cancer studies have found dysregulation of circRNA expression in *in vitro* and *in vivo* trials, in clinical cancer sample tissues, and even in patients' body fluid samples, which are associated with clinical features of cancers (Huang et al., 2017), such as reproduction and gynecological diseases (Liu et al., 2019), cancer of the digestive system (Sheng et al., 2018), glioblastoma (Zhang et al., 2017; Yang et al., 2018), drug resistance (Shao et al., 2018), and so on. The unique role of CircRNAs in carcinogenesis may provide potential targets for cancer therapy and may also inhibit or regulate the malignant behavior of cancer cells through novel transcriptional therapies (Zhao and Shen, 2017).

CircRNA Acting as miRNA Sponge

Nikolaus Rajewsky first discovered antisense circRNA with cerebellar degeneration-related protein 1 transcript (CDR1as), which can be combined with miR-7 in neural tissue to prove that circRNA can act as a sponge (called ciRS-7) (Memczak et al., 2013). The data suggest that circRNA may act as a post-transcriptional regulator to competitively inhibit other RNAs from binding to miRNA and RBPs. It can often have a role in regulating local free concentrations of RBP, RNA, or their binding sites. While circRNA can completely resist the instability of miRNA target genes, it significantly inhibits miR-7 function, leading to elevated miR-7 target levels. Other studies have shown that sex-determining region Y (Sry), a kind of testis-specific circRNA,

Abbreviations: circRNA, circular RNA; RBPs, RNA binding proteins; CC, cervical cancer; ceRNA, competing endogenous RNA; CDR1as, cerebellar degeneration-related protein 1 transcript; Sry, sex-determining region Y; HCC, human hepatocellular carcinoma; HESCs, human embryonic stem cells; RIP, RNA binding protein immunoprecipitation; HSEs, hematopoietic stem cells; LT-HSC, long-term hematopoietic stem cells; IFN, interferon; FISH, fluorescence *in situ* hybridization; EIciRNAs, exon-intron circRNA; snRNP, small nuclear ribonucleoproteins; IRES, internal ribosome entry site; m6a, modified n6- methyladenosine; PPI, protein-protein interaction; GEO, Gene Expression Omnibus; CGA, Cancer Genome Atlas; ANT, adjacent non-tumor tissue; CSCC, cervical squamous cell carcinoma; DE, differentially express; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; HPV, human papillomavirus; PI3K, phosphatidylinositol 3-kinase; TGF-β, transforming growth factor-beta; MAPK, mitogen-activated protein kinase; EMT, epithelial-mesenchymal transition; HOXA1, homeobox a1; RNA-FISH, RNA fluorescence *in situ* hybridization; QREs, QKI response elements; EGFR, epidermal growth factor receptor; ATG, autophagy-related genes; MDM4, murine double minute 4; FOXK2, Forkhead Box K2; SSBP1, Single-Stranded DNA Binding Protein 1; OS, overall survival; HR, hazard ratio; MACC1, metastasis-associated in colon cancer 1; CNNM3, cyclin-M3; ROC, receiver operating characteristic; AUC, area under curve; RFS, recurrence-free survival; SCC Ag, squamous cell carcinoma antigen; SCT, Spatholobi Caulis tannin.

is an miR-138 sponge, indicating that some circRNAs contain response elements of miRNA that can act as ceRNA, thereby relieving miRNA inhibition of target genes and upregulating their expression (Hansen et al., 2013). High expression of circRNA_104075 in human hepatocellular carcinoma (HCC) cell lines adsorbs miR-582-3p of targeted inhibition of YAP expression through the sponge mechanism and promotes HCC occurrence and progression. It also reveals that circ_104075 has up to 96% sensitivity and 98.3% specificity in diagnostic HCC and has potential as a biomarker for diagnosis (Zhang X. et al., 2018).

CircRNA Regulating Gene Expression

To further understand the function of circRNA, CDR1as was further studied. The authors selected four regions of the high expression of brain CDR1as in CDR1as mice and CDR1as-knockout (KO) mice for mRNA sequencing. Many of the genes identified as miR-7 targets showed significant changes. Consistent with changes in gene expression, mutant mice did exhibit abnormal neuronal activity in a series of *in vivo* behavioral experiments, suggesting circRNA plays a critical role in mouse behavior (Piwecka et al., 2017). Exon2, an abundant characterizing circRNA, derived from HIPK3, is called circHIPK3, and that silencing, rather than HIPK3 mRNA silencing, considerably inhibits human cell proliferation. Through a luciferase screening assay, circHIPK3 was noted to directly combine with miR-124 and then suppress miR-124 activity, suggesting that circRNA generated by precursor mRNA can regulate cell behavior (Zheng et al., 2016).

Functions of two circRNAs (circBIRC6 and circCORO1C) are related to pluripotent states, demonstrating that circRNAs also have the function of regulating human pluripotency, identifying subsets of circRNA enriched in human embryonic stem cells (hESCs). They also promote a pluripotent state by inhibiting their mediated reduction of NANOG and OCT4 expression and then inhibiting hESC differentiation through sponge mechanisms that bind directly to miR-34a and miR-145 (Yu et al., 2017). Unlike this, circCSNK1G3 and miR-181b/d in prostate cancer have synergies. Overexpression of miR-181b/d in PC-3 cell lines significantly decreased the abundance of tumor suppressor CBX7 in prostate cancer, upregulated cell cycle genes such as CDK1, CDC25A, increased the proliferation of PC-3 cells and, more importantly, attenuated circCSNK1G3 knockdown induced cell proliferation arrest. Taken together, circCSNK1G3 promotes the proliferation of prostate cancer cells through interactions with miR-181b/d, and this new regulation differs from the sponge mechanism (Chen S. J. et al., 2019).

CircRNA Binding Functional Protein

Circular RNA can not only bind to the miRNA to play the role of a sponge, but it can also bind to functional proteins. One study explored circANRIL binding proteins through RNA pull down, and it was reverse confirmed by RNA binding protein immunoprecipitation (RIP) that circRNA was involved in the maturation process by binding to PES1 proteins (Holdt et al., 2016). Sub-populations of hematopoietic stem cells (HSEs) were significantly altered only after interfering with circRNA-Cia-cGAS transcribed by D430042O09Rik. The long-term hematopoietic stem cells (LT-HSC) decreased significantly, and the expression of type I interferon (IFN) increased in cia-cGAS KO mice. Enzymatic studies have demonstrated that cia-cGAS in the nucleus inhibits its enzyme activity by binding DNA-sensitive cGAMP synthetase (cGAS) and then preventing the resting LT-HSC from being cGAS depleted. Additionally, cia-cGAS has a stronger affinity to cGAS compared with linear cGAS and thereby inhibits the generation of type I IFN in LT-HSCs, which was mediated by cGAS. These experiments found a novel circRNA—cia-cGAS that binds cGAS and inhibits its activity, regulates the LT-HSC resting state, and reduces energy depletion and apoptosis caused by type I IFN (Xia et al., 2018).

CircRNA Regulating the Transcription of Genes

Circular RNAs can regulate transcription through specific RNA-RNA interactions and then regulate source gene expression. Through CLIP-seq, researchers found that RNA Polymerase II incorporated some circRNAs, formed by introns, between exons and retained exons named exon-intron circRNA (EIciRNAs), such as circEIF3J and circPAIP. By fluorescence *in situ* hybridization (FISH) localization, the researchers concluded that it was located in the nucleus and could regulate the expression of the genes from which it originated. Experiments then showed that EIciRNAs co-conjugated with Pol II, U1, and small nuclear ribonucleoproteins (snRNP) at 300 bps upstream of the transcription start site of the gene from which it originated and also that the binding with U1 and snRNP was necessary (Li et al., 2015).

CircRNA Translating Proteins

Originally, we thought that circRNAs belonging to non-coding RNA could not play the function of coding proteins; Yun Y. et al., proved that circRNA can translate proteins, but most circRNAs are non-coding RNAs. CircRNA does not have a free 5′ and 3′ terminus; if it can be translated, it must thus be done in a way that does not depend on the 5′ cap structure, such as through the internal ribosome entry site (IRES) or modified N6-methyladenosine (m6A) modification. The study detected no IRES on the circRNA, but it can also translate proteins. A series of experiments confirmed that the recognition proteins YTHDF3 recognized the m6A modification occurring on the circRNA and recruited translation initiation factors such as eIF3A and eIF4G2 (Yang et al., 2017). The circRNAs are widely m6A modified and present cell-specific features. The m6A modification enzyme of circRNA is consistent with the mRNA, while the m6A modification sites are different (Zhou et al., 2017). Moreover, circRNA-encoded proteins are functional, such as FBXW7-185aa, a new 21 kDa protein, which was encoded by circ-FBXW7. Upregulation of FBXW7-185aa inhibits cell proliferation but accelerates the cell cycle, whereas knockdown of FBXW7-185aa promotes the malignant behavior of cells *in vivo*

and *in vitro*. Furthermore, circ-FBXW7 expression is correlated with the prognosis of glioblastoma patients (Yang et al., 2018).

BUILDING circRNA RELATED INFORMATION NETWORKS IN CERVICAL CANCER

Building Networks Through Bioinformatics Data

Through the use of the Molecular Complex Detection building protein–protein interaction (PPI) network to analyze the comprehensive bioinformatics of cervical cancer, seven central genes were identified (RRM2, CEP55, CHEK1, KIF23, RACGAP1, ATAD2, and KIF11). A circRNA-miRNA-mRNA network was constructed that included five circRNAs (hsa_circRNA_101958, hsa_circRNA_400068, hsa_circRNA_103519, hsa_circRNA_104315, and hsa_circRNA_000596), two mRNAs (hsa-miR-106b and hsa-miR-15b), and seven mRNA corresponding to the seven central genes, and 22 circRNA-miRNA-mRNA control axes were determined in the sub-net (Yi et al., 2019). Similarly, another study also constructed ceRNA networks for CC through Gene Expression Omnibus (GEO) database and the Cancer Genome Atlas (CGA) database, used the STRING database to analyze protein interactions, and used the MCODE plugin to identify hub genes (Gong et al., 2019). These results can provide some insight for future research on the molecular mechanism of CC, but since the research is based on computer data, further experiments are necessary in order to verify it.

Building Networks Through High-Throughput RNA Sequencing (RNA-Seq)

Tumor tissues and adjacent non-tumor tissue (ANT) tissues were studied in patients with cervical squamous cell carcinoma (CSCC) by RNA-seq. It identified 19 lncRNAs, 99 circRNAs, and 28 miRNAs; 304 mRNAs were differentially expressed (DE); and it constructed ceRNA networks of coded and non-coding RNA to predict interactions between lncRNA, circRNA, miRNA, and mRNA. The findings revealed for the first time that circRNA may be involved in CC and that ceRNA of DE may develop further as biomarkers (Wang H. et al., 2017).

Furthermore, through RNA-seq, the DE of circRNA between radiated CC cells and non-radiated CC cells was studied, and cytoscape-mapped circRNA–miRNA–target gene interaction networks were described. Studies revealed that, through different mechanisms, radiotherapy can kill HeLa cells and promote cell migration and invasion. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis indicated that the MAPK signal transduction pathway is the most abundant pathway, and high expression of target genes may be related to angiogenesis and cell metastasis. The exploration of circRNA associated with radiation resistance in HeLa cells of radiotherapy may be used to provide appropriate treatment strategies and improve prognosis in resistant cancers (Yu et al., 2018).

Network Constructions via Microarray

Bioinformatic prediction and the widespread application of RNA-seq technology have identified a large number of circRNAs. However, at present, to fully characterize these circRNAs we require samples of interest, which we currently lack. Microarray detection may show effective detection efficiency. One study provides abundant resources from 41 microarray datasets, providing strong evidence for the expression of circRNA in CC (Li et al., 2018). Using high-throughput microarray technology, the expression of circRNAs was also studied by transfecting E7 siRNA in HPV16-positive CaSki cells. Eight of them are strongly expressed, the expression of hsa_circ_0026527, hsa_circ_0056353, hsa_circ_0035918, hsa_circ_0037213, hsa_circ_0038475, and hsa_circ_0048867 was downregulated, and the expression of hsa_circ_0052602 and hsa_circ_0051620 was upregulated. Further analysis clarified through the GO and KEGG databases showed that several circRNAs can be used as ceRNAs to regulate the occurrence and development of CC, and DE of circRNAs may be related to the proline metabolism, glutathione metabolism, and the mTOR signaling pathway (Zheng et al., 2018). The miRNA target of circRNA was predicted by TargetScan Human and miR Base, but the point of whether it was directly directed to sponge circRNAs or targeted to regulate its expression to cause tumorigenesis was not made.

MECHANISM OF circRNA IN CERVICAL CANCER

Cervical cancer is the most common gynecologic tumor and can provoke a large number of deaths every year. For the most part, a high-risk subtype of human papillomavirus (HPV) is in charge of the disease, which is largely preventable (Cohen et al., 2019). CircRNAs are the focus of current research, and several studies have speculated on the role of circRNAs and inferred their potential mechanisms in CC (Chaichian et al., 2019). A series of studies have proven the effects of circRNA on CC cell lines in *in vitro* and *in vivo* experiments (**Table 1**); further studies have indicated that circRNA is involved in the progression of cervical tumors through a variety of mechanisms (**Figure 2**), with miRNA sponges as the main mechanism, and they have even shown that circE7 produces E7 proteins by m6A modification (Zhao et al., 2019). We reviewed some studies conducted so far to explore the functions of circRNAs in the occurrence and development of CC and the relationship between circRNAs and metastasis, invasion, recurrence, and chemical resistance of cervical cancer.

Participation in Related Signaling Pathways

Controlling PI3K-Akt Signaling Pathways

One study found that circ-0033550 was upregulated in CC, and its related gene was AKT1, and the circ-0033550 was thus renamed circ-AKT1. AKT1, as a serine/threonine kinase, has highly conserved properties and is a core node of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. AKT1 is involved in multiple biological manners, including metabolism,

TABLE 1 | The effects of circRNA on CC cell lines.

circRNA	miRNA	CC cell lines and Xenograft	Function	Type of detection methods	References
circ-0033550 (circRNA-AKT1)	miR-942-5p	HeLa, CaSki, SiHa, C33A, 293T, and H8 cell lines; Male BALB/c nude mice	Promotes cell proliferation and invasion; Increased the volume and weight of xenografts	CCK-8, transwell, colony formation, FISH, Immunofluorescence (IF) staining, dual-luciferase reporter, 5-Ethynyl-2′-deoxyuridine (EdU), RNA pull-down, and RIP assays	Ou et al., 2020b
circAGFG1	miR-370-3p	HeLa, C-33A, SiHa, HCC94, and End1/E6E7 (normal cervical cell line)	CircAGFG1 downregulation restrained cell viability, proliferation and migration, and promoted cell apoptosis	loss-of function, bioinformatics analysis, mechanism experiments, and rescue assays	Wu and Zhou, 2019
circular RNA hsa_circ_0000515	miR-326	Hela and SiHa cell lines; Clean grade female BALB/c nude mice	Hsa_circ_0000515 silencing attenuated cell proliferation and invasion; promoted apoptosis and autophagy; and reduced tumor volume and weight	EdU, Monodansylcadaverine (MDC) staining, flow cytometric, transwell, FISH, RIP, dual-luciferase reporter, and TdT-mediated dUTP-biotin nick end-labeling (TUNEL) staining assays	Tang et al., 2019
circular RNA hsa_circ_0023404	miR-136	SiHa, C-33a, CaSki, C4-1 and Hela, and human cervical epithelial cells (CerEpiC)	Hsa_circ_0023404 knockdown significantly suppressed cell proliferation and colony formation	CCK-8, transwell, colony formation, cell cycle distribution, and luciferase reporter assays	Zhang Z. et al., 2018
circCLK3	miR-320a	SiHa, HeLa, CaSki, C-33A, MS751 cell lines; BALB/c athymic nude mice	Promotes cell proliferation, migration, and invasion. Promoted growth and metastasis of tumor *in vivo*	CCK-8, cell colony, wound healing, transwell migration and invasion, RNA pull-down, RIP, luciferase reporter, and rescue assays	Hong et al., 2019
circular RNA HIPK3	miR-338-3p	SiHa and C-4I cell lines	Circ-HIPK3 silencing inhibited cell proliferation, migration and invasion, while induced apoptosis	CCK-8, cell formation, cell apoptosis, transwell migration and invasion, point mutation, RNA pull-down, luciferase reporter, and rescue assays	Qian et al., 2020
circ_0067934	miR-545	SiHa, CaSki, Hela, and C4-1 and immortalized cervical epithelium NC104 cell line; BALB/c nude mice	Promotes proliferation, colony formation, migration, invasion, and epithelial-mesenchymal transition of CC cells. Promoted tumor growth *in vivo*	CCK-8, colony proliferation, transwell, and luciferase reporter assays	Hu et al., 2019
circEIF4G2	miR-218	HeLa, CasKi, C33A, and SiHa cells	CircEIF4G2 knockdown significantly inhibited cell migration and invasion	CCK-8, colony formation, wound healing, transwell migration and invasion, RIP, and dual-luciferase reporter assays	Mao et al., 2019
circSLC26A4 (hsa_circ_0132980)	miR-1287-5p	CaSki, SiHa cell lines; Male BALB/c nude mice	CircSLC26A4 silencing inhibited cell proliferation, invasion, and effectively repressed tumor growth *in vivo*	CCK-8, colony proliferation, transwell, RIP, dual-luciferase reporter, and RNA-FISH assays	Ji et al., 2020
circSMARCA5	miR-620	HeLa, CaSki, SiHa, C33 cell lines, and 293T cells	CircSMARCA5 dramatically inhibited the proliferation and colony-forming abilities of CC cells	Luciferase activity, MTT, and cell invasion assays	Dai et al., 2018
circ-ATP8A2	miR-433	HeLa and SW756 cell lines	Circ-ATP8A2 knockdown inhibited cell proliferation, migratory and invasive capacities and increased apoptotic cells	CCK-8, acridine orange/ethidium bromide (AO/EB), flow cytometric, transwell, and dual-luciferase reporter assays	Ding and Zhang, 2019
circMTO1	miR-6893	HeLa, CaSki, C-33A, C-4 II, SiHa and immortalized epithelial cells of human ectocervix Ect1/E6E7 cell lines; NOD-SCID immunodeficient mice	CircMTO1 knockdown suppressed cell migration, invasion, chemoresistance and markedly impaired tumor growth ability *in vivo*	Wound healing, transwell invasion, MTT, TUNEL, and luciferase reporter assays	Chen M. et al., 2019
circRNA hsa_circ_0023404	miR-5047	HeLa, SiHa cell lines and 293T cell	Knockdown in HeLa and SiHa cells reduced the number of invaded cells;promotes metastasis of Human Dermal Lymphatic Endothelial Cells (HDLEC)	Transwell invasion, lymphatic vessel, MTT, flow cytometry analysis, and luciferase reporter assays	Guo et al., 2019

(Continued)

TABLE 1 | Continued

circRNA	miRNA	CC cell lines and Xenograft	Function	Type of detection methods	References
circ_0005576	miR-153	HeLa, SiHa, Caski, C-33A and immortalized cervical epithelium cell lines (HcerEpiC)	Promotes cell proliferation, migration, and invasion	CCK8, colony formation, transwell, RNA pull-down, luciferase reporter, and RIP assays	Ma et al., 2019
hsa_circ_0018289	miR-497	HeLa, CaSki, SiHa, HT-3,C33A and human epidermal cell (HaCaT); Male BALB/c nude mice	Hsa_circ_0018289 knockdown inhibited cell proliferation, migration and invasion. Decreased the tumor volumes and weights	CCK8, transwell, and luciferase reporter assays	Gao et al., 2017
circRNA-000284	miR-506	HeLa, CaSki, SiHa, C-33A, and SW756 cell lines	Promotes cell proliferation and invasion	CCK8, transwell, dual-luciferase reporter, and RNA-FISH assays	Ma et al., 2018
circular RNA hsa_circ_0000263	miR-150-5p	HeLa, CaSki, SiHa, C-33A, and SW756 cell lines	Hsa_circ_0000263 downregulation inhibited cell proliferation and migration; promoted cell apoptosis	CCK-8, cell proliferation, transwell, flow cytometric, RIP, and dual-luciferase reporter assays	Cai et al., 2019
hsa_circ_0007534	miR-498	HeLa, SiHa, and CaSki, and human cervical epithelial immortalized cell line H8	Hsa_circ_0007534 inhibition impeded cell proliferation and invasion	CCK-8, colony proliferation, transwell, RNA pull-down, and dual-luciferase reporter assays	Rong et al., 2019
circAMOTL1	miR-485-5p	HeLa, CaSki cell lines; BALB/c nude mice (C-33A cells)	Induced cell proliferation and migration. Promoted cervical cancer development *in vivo*	CCK-8, wound healing, transwell, EdU incorporation, RNA pull-down, FISH, and luciferase reporter assays	Ou et al., 2020a
circ-ITCH	miR-93-5p	SiHa, Caski, HeLa, and C33A cell lines; SPF grade BALB/c nude mice	Inhibited cell proliferation, migration and invasion. Significantly inhibited tumor growth *in vivo*	CCK-8, colony proliferation, transwell, luciferase reporter, and tumor xenograft assays	Li et al., 2020
circ-MYBL2	miR-361-3p	HeLa, CaSki cell lines	Circ-MYBL2 inhibition suppressed cell proliferation and invasion	CCK-8, colony formation, transwell invasion, RNA pull-down, and luciferase reporter assays	Wang J. et al., 2019
hsa_circ_0075341	miR-149-5p	SiHa and CaSki cell lines	Promotes cell proliferation and invasion	CCK-8, colony formation, transwell invasion, and dual-luciferase reporter assays	Shao et al., 2020
hsa_circ_0001038	miR-337-3p	HeLa, SiHa, C-33A, SW756, and normal cells (HcerEpiC)	Hsa_circ_0001038 knockdown inhibited cell proliferation, migration and invasion	CCK-8, flow cytometric, transwell, AO/EB staining, loss/gain-of function, and dual-luciferase reporter assays	Wang Y. et al., 2020
circRNA8924	miR-518d-5p/519-5p	SiHa and HeLa cell lines and 293T cells	CircRNA8924 knockdown significantly inhibited cell proliferation, migration and invasion	CCK-8, flow cytometric, transwell, loss/gain-of function, and luciferase reporter assays	Liu et al., 2018
circ_0000388	miR-337-3p	HeLa and SiHa cell lines	Notably enhances cell migration and invasion	CCK-8, TUNEL, wound healing, transwell, RT-PCR, Western blot, RIP, and luciferase reporter assays	Meng et al., 2020
circ_103973	miR-335	HeLa, CaSki, C33A, and SiHa cell lines	Circ_103973 knockdown promoted cell apoptosis and inhibited cell proliferation	Flow cytometric, MTT, colony formation, dual-luciferase reporter, and RNA pull-down assays	Zhu et al., 2020
circRNA_0000285	–	HeLa, SiHa, C4-1, and C-33A and normal cervical epithelium cell line (NC104); NOD/SCID mice	CircRNA_0000285 knockdown inhibited cell proliferation and invasion; inhibited tumor formation and metastasis	CCK-8, cell cycle, and transwell assays	Chen R. X. et al., 2019
Hsa_circRNA_101996	miR-8075	SiHa, C33A, CaSki, and Hela cell lines; BALB/c nude mice	Promotes cell proliferation, migration, and invasion. Led to the decrease of the tumor size and weight	CCK-8, cell invasion, colony formation, and luciferase reporter assays	Song et al., 2019

cell survival, and migration. The loss/gain-of-function assays showed that circ-AKT1 regulated AKT1 and then promoted cell proliferation and invasion in CC *in vitro*, and it was verified that the tumor growth was promoted in CC by *vivo* assays. Mechanically, circ-AKT1 raised AKT1 through sponging miR-942-5p. Moreover, transforming growth factor-beta (TGF-β) induces circ-AKT1 and AKT1. All in all, circRNA-AKT1/miR-942-5p upregulated AKT1 and then facilitated CC tumorigenesis. The circ-AKT1/miR-942-5p/AKT1 axis may provide novel molecular targets for therapeutic improvement in

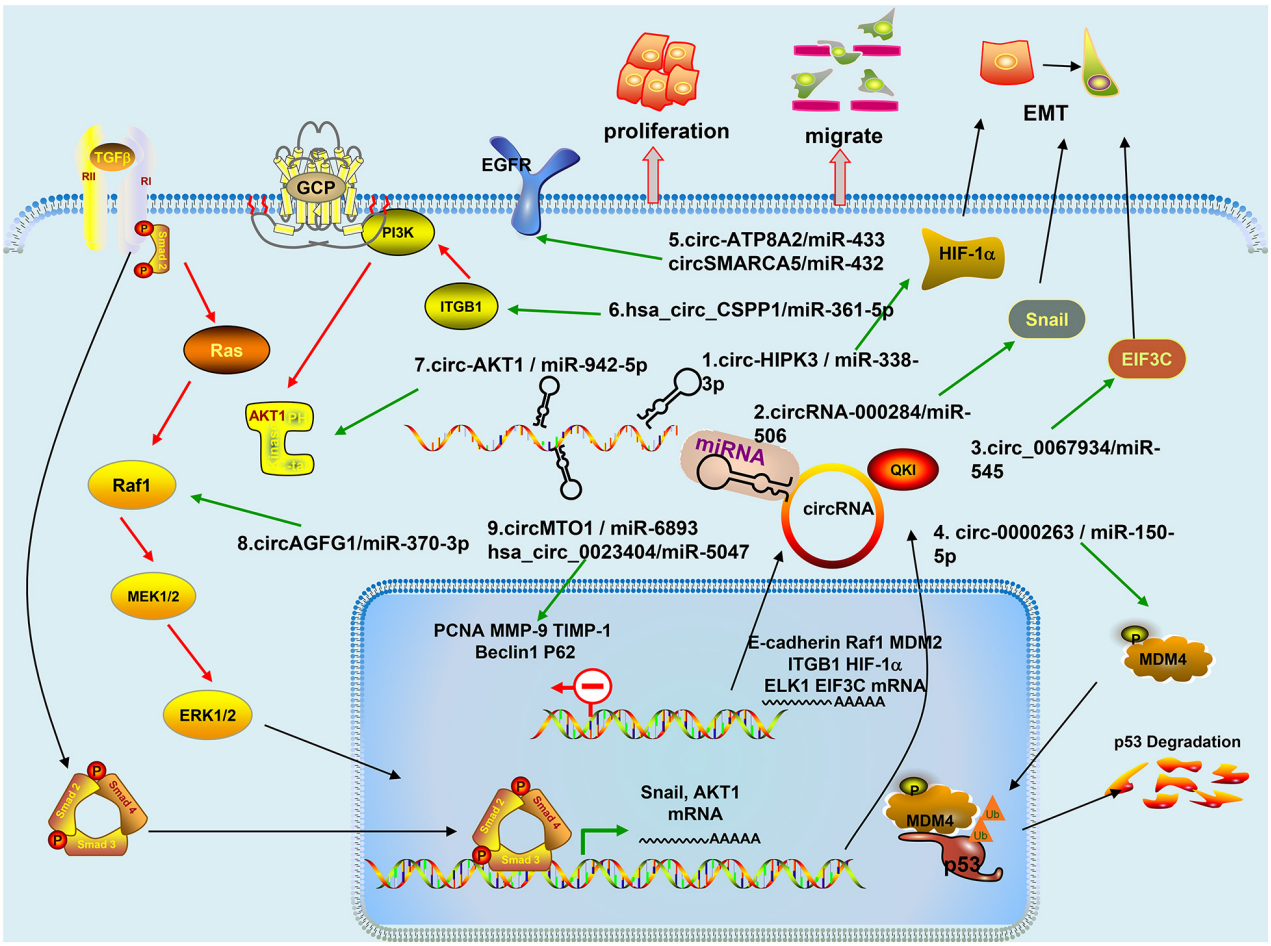

FIGURE 2 | Mechanism of circRNAs in Cervical Cancer: (1) Circ-HIPK3 acts as a competing endogenous RNA of miR-338-3p, via regulating HIF-1α mediated EMT; (2) CircRNA-000284 suppresses the expression of Snail-2 via sponging miR-506; (3) Circ_0067934 modulates EIF3C by sponging miR-545; (4) Circ-0000263, as a competitive endogenous RNA, regulates MDM4 expression by sponging miR-150-5p and may play an important role affecting the expression of p53 gene; (5) Circ-ATP8A2 sponges miR-433 to release its suppression on EGFR expression. CircSMARCA5 modulates miR-432 via the ERK signaling pathway; (6) Hsa_circ_CSPP1 regulates miR-361-5p/ITGB1 in the PI3K-Akt signaling pathway; (7) CircRNA-AKT1 sequesters miR-942-5p to upregulate AKT1; (8) CircAGFG1 enhances the activity of RAF/MEK/ERK pathway by sponging miR-370-3p and further regulating RAF1; and (9) CircMTO1 directly interacts with miR-6893 and their inhibitor enhanced Beclin 1 expression and downregulated p62 level. Hsa_circ_0023404 knockdown can also promote p62 protein levels and attenuate Beclin 1 level.

CC (Ou et al., 2020b). In other studies, hsa_circ_CSPP1 can regulate the proliferation and migration of CC cells through the miR-361-5p/ITGB1 axis, which is a part of the PI3K/AKT pathway. Hsa_circ_CSPP1 acts as a sponge of miR-361-5p, and, meanwhile, miR-361-5p, by downregulating ITGB1, inhibits cell activity and mobility and induces cell apoptosis, thereby inhibiting cervical cancer. Thus, inhibiting hsa_circ_CSPP1 and then inhibiting the expression of downstream genes such as PI3K, AKT, and ITGB1 prevents tumor growth (Yang and Xie, 2020).

Involvement in MAPK Signaling Pathways

CircAGFG1 was upregulated in CC cell lines *in vitro* and, the decrease in circAGFG1 levels was proven to inhibit the proliferation and migration ability of CC cells. CircAGFG1 depleted miR-370-3p, which regulates RAF1 expression, and then activated RAF/MEK/ERK pathways through bioinformatics analyses and further experiments. Thus, circAGFG1 can influence the proliferation and migration of CC cells through miR-370-3p/RAF1 (Wu and Zhou, 2019). The overexpression of hsa_circ_0000515 in cervical cancer samples was identified by microarray data analysis (GSE102686), and it was predicted that hsa_circ_0000515 directly regulated miR-326 using a biological database and RT-qPCR. Further experimental results emphasized that hsa_circ_0000515 can be used as an miR-326 ceRNA to increase ELK1 expression. Furthermore, enhancing ELKI expression leads to increase proliferation and invasion but inhibits apoptosis and autophagy of CC cells. Experiments *in vivo* further confirmed that hsa_circ_0000515 silencing inhibited tumor growth. ELK1 is the integration point of other pathways associated with mitogen-activated protein kinase (MAPK) signal pathways, and we may thus infer that potential MAPK pathways may be highly likely

involved in hsa_circ_0000515/miR-326/ELK1 axis (Tang et al., 2019). Some findings also provide a hypothesis that the mechanism of hsa_circ_0101996 involved in the progression of CC may be achieved by activating MAPK signaling (Wang Y.-M. et al., 2017).

Involvement in Hippo Signaling Pathways

YAP1 is the transcription effector of the Hippo signaling pathway and mediates the activity of stem cell and tissue expansion through interactions with TEAD transcription factors (Schlegelmilch et al., 2011). One study has shown that hsa_circ_0023404 regulated TFCP2 via sponging miR-136, and TFCP2 is an activator of YAP pathways. Experiments proved that hsa_circ_0023404 activated the Hippo pathway by stimulating miR-136, promoting the expression of TFCP2 in CC, which leads to the development and progression of CC. The study uncovered a novel therapeutic ring in the process of cervical cancer, which is hsa_circ_0023404/miR-136/TFCP2/YAP axis (Zhang J. et al., 2018).

Regulation of Relating Genes Expression Through Sponge Mechanisms

Mediating Epithelial-Mesenchymal Transition (EMT)

The downregulation of E-cad is generally considered to be a result of EMT and is recognized as a key link promoting progression and metastasis of cancer. In SiHa and CaSki cells, the low expression level of circ-0745 decreased cell proliferation, invasion, and migration *in vitro* and also reduced the tumor size *in vivo*. The knockdown of hsa_circ_0000745 can reduce the expression of E-cad, and the upregulation of circ-0745 can inhibit E-cad expression, thereby facilitating the transfer of cells and leading to the development of cancer (Jiao et al., 2020). Similarly, the circCLK3/miR-320a/FoxM1 axis also impacted the progression of CC through EMT. Overexpression of miR-320a inhibited the activity of circCLK3 to increase the expression of E-cadherin. MiR-320a targets FoxM1 increased E-cadherin expression but downregulated the expression of N-cadherin and Vimentin (Hong et al., 2019). In another study, TGF-β targets circ-AKT1/miR-942-5p/AKT1 axis and then promotes EMT of CC cells (Ou et al., 2020b). The expression of circ-HIPK3 in cervical cancer tissues was markedly upregulated compared to ANT. Through sponging miR-338-3p, circ-HIPK3 upregulated hypoxia-inducible factor-1α (HIF-1α). Overexpression of HIF-1α or miR-338-3p silencing can rescue suppression of CC malignant features caused by the knockdown of circ-HIPK3. Therefore, circ-HIPK3 as ceRNA of miR-338-3p regulates EMT mediated by HIF-1α and further promotes CC cell growth and metastasis. Targeting the circ-HIPK3/miR-338-3p/HIF-1α axis will be a new type of regulating strategy for CC (Qian et al., 2020). Circ_0067934 also facilitates cell proliferation, migration, and invasion, which was caused by EMT in CC *via* a series of assays. An RNA pull-down assay found that circ_0067934 precipitated miR-545 through using biotin-labeled circ_0067934 probes in SiHa and HeLa cells, which indicated direct interaction between circ_0067934 and miR-545. Further study showed that miR-545 inhibits cell proliferation and invasion, and the effect of circ_0067934 knockdown can be rescued by the recovery of EIF3C. This proves that circ_0067934 promotes CC development through miR-545/EIF3C axis (Hu et al., 2019). By culturing exo-circ_PVT1/exo-vector with C33A, the researchers found that circ_PVT1 induced the downregulation of E-cadherin and the upregulation of Vimentin, N-cadherin, and SNAIL by targeting miR-1286 (Wang H. et al., 2020). This suggests circ_PVT1 can induce EMT in CC cells through the exosome pathway, which is a new mechanism for the progression of cervical cancer.

Targeted HOXA Cluster Gene

CircEIF4G2 in cervical cancer tissues was strongly higher than that in matched ANT and was evidently related to tumor size, lymph node metastasis, and poor prognosis but not related to the stage of tumor lymph node metastasis or the patient's age. Moreover, circEIF4G2 can sponge miR-218 to enhance the expression level of the target gene homeobox a1 (HOXA1). Transfection of cells with miR-218 inhibitors attenuated the suppression of malignant cell behavior due to circEIF4G2 knockdown. Silencing HOXA1 can reverse the effect of miR-218 inhibitors on Hela cells. These results show that circEIF4G2/miR-218/HOXA1 pathways promote tumor formation and metastasis (Mao et al., 2019). CircSLC26A4 and miR-1287-5p have a negative correlation function and are both located in the cytoplasm of CC cells, which is proven by RNA fluorescence *in situ* hybridization (RNA-FISH). Additionally, miR-1287-5p can combine with HOXA7 through their complementary binding domains. Therefore, circSLC26A4 may act as miRNA sponges to mediate CC tumor phenotypes (Ji et al., 2020).

Targeting Epidermal Growth Factor Receptor (EGFR)

CircSMARCA5 overexpression inhibits CC tumor phenotypes. The results of the study suggest that circSMARCA5 can target miR-432 that interacts with EGFR by binding to the 3′-non-translational region. Thus, circSMARCA5 may regulate miR-432 and then, through the ERK signaling pathways, act as a vital role in the progression of CC (Huang et al., 2020). Another finding found that circSMARCA5 can also bind to miR-620 and inhibit the proliferation and invasion of CC cells, and it is involved in the development of CC through regulating the circSMARCA5/miR-620 axis (Dai et al., 2018). Hence, targeting circSMARCA5 can be considered as an emerging therapeutic option for cervical cancer. Circ-ATP8A2 was detected in CC specimens and cells and interacted with miR-433. Immunoblotting analysis showed that the expression of EGFR decreased considerably after silencing circ-ATP8A2, while co-transfection with the miR-433 inhibitor or EGFR vector efficiently increased the expression level of EGFR. Circ-ATP8A2 released the inhibition of EGFR expression caused by miR-433 at the posttranscriptional level, i.e., circ-ATP8A2 can promote CC cell progression through the miR-433/EGFR axis (Ding and Zhang, 2019).

Regulation of Autophagy-Related Genes (ATG)

CircMTO1 can interact with miR-6893 and is significantly upregulated in CC cells in an *in vitro* experiment. Meanwhile, a xenograft tumor assay proved that miR-6893 inhibitors can save the tumorigenic effects of CC cells caused by circMTO1 knock-down. S100A1 was identified as the target for circMTO1

and miR-6893 induction tumorigenesis of CC. Western blot analysis showed that both circMTO1 and miR-6893 inhibitors can enhance Beclin 1 expression and downregulate the levels of P62 protein, thereby regulating the proliferation and apoptosis of CC cells, but autophagy inhibitor 3-MA can reverse these effects (Chen M. et al., 2019). Same as circMTO1, western blot results found that the combination of hsa_circ_0023404 decreased the levels of P62 and Beclin 1 protein (Guo et al., 2019). Studies have also shown that hsa_circ_0023404 enhanced chemical resistance to cisplatin in CC cells by regulating autophagy signaling. Hsa_circ_0023404 can be bound to miR-5047 directly. Knockout hsa_circ_0023404 reduced the number of invasive cells and markedly attenuated lymphatic formation. VEGFA can partially restore CC cells metastasis regulated by hsa_circ_0023404 or miR-5047, suggesting that VEGFA is an essential downstream effector of hsa_circ_0023404/miR-5047-mediated CC cell migration. Hsa_circ_0000515 silencing also inhibits cancer progression by regulating autophagy genes. For example, the expression of PCNA, MMP-9, TIMP-1, and P62 decreased, which is associated with higher expression of caspase 3, caspase 9, Beclin 1, LC3, and LC3-II/LC3-I rates (Tang et al., 2019).

Regulation of Other Genes Through Sponge Mechanisms

Using microarray screen CC specific circRNA, such as circ_0005576 (Ma et al., 2019),hsa_circ_0018289 (Gao et al., 2017) and circRNA-000284 (Ma et al., 2018). Target genes KIF20A, KIF20A of circ_0005576 were screened by GEPIA and Starbase database, which was extremely elevated in CC tissues. CC tissues also have sequences complementary to miR-153-3p. Downregulation of circ_0005576 in CC cell lines decreases the levels of KIF20A protein, and using the miR-153-3p inhibitor can partially retrieve the downregulation of KIF20A caused by circ_0005576 knockdown. Study disclosed that circ_0005576/miR-153-3p/KIF20A axis can encourage the development of CC, which can provide a novel understanding of the tumorigenesis of CC (Ma et al., 2019). Through bioinformatics prediction procedures, luciferase reporter assays, and RIP assays, hsa_circ_0018289 and miR-497 may interact via sponge mechanisms (Gao et al., 2017). CircRNA-000284 regulates cell proliferation and invasion via binding to miR-506 and subsequently targeting Snail-2 genes that are upregulated by circRNA-000284 (Ma et al., 2018).

Hsa_circ_0000263 (Cai et al., 2019), hsa_circ_0007534 (Rong et al., 2019), and circAMOTL1 (Ou et al., 2020a) was dramatically upregulated in CC by RT-qPCR analysis. They all promoted CC cell colony formation *in vitro*. Downregulation of circ-0000263 and circAMOTL1 could reduce tumor size *in vivo* experiments. There are direct interactions between hsa_circ_0000263 and miR-150-5p, between hsa_circ_0007534 and miR-498, and between circAMOTL1 and miR-485-5p through the sponge mechanism. Rescue experiments suggest that downregulation of hsa_circ_0007534 inhibits cell proliferation and migration by interacting miR-498/BMI-1 axis (Rong et al., 2019). The MiR-485-5p/AMOTL1 axis participates in cervical cancer progression, which was mediated by circ_AMOTL1 (Ou et al., 2020a). It was proven that hsa_circ_0000263 sponging miR-150-5p could adjust downstream factor murine double minute 4 (MDM4) expression and ultimately regulate the expression of p53 genes, revealing the important function of hsa_circ_0000263/miR-150-5p/MDM4/p53 pathways in CC (Cai et al., 2019). Circ-ITCH also performed tumor suppressor activity and affected the expression level of Forkhead Box K2 (FOXK2) by sponging miR-93-5p (Li et al., 2020).

Encoding Cancer Proteins

Researchers have invented a pipeline that could find back-splice junctions from viral genomes to screen the circRNA presenting in HPV. They detected HPV16 circE7 from cells transformed by HPV16, preferentially located in the cytoplasm, which is related to polyribosomes. HPV16 and HPV35 circE7s are both predicted to have miRNA binding sites between HPV16 and HPV35 circE7 species, but none of them are conserved, so circE7 cannot be an miRMA sponge. RIP experiments confirmed that circE7 was m6A modified and was able to produce E7 protein in the form of heat shock regulation. Inhibiting circE7 mutations formed by DRACH motif mutations or splicing site mutations can prevent the translation of E7 proteins. Although the quantification of northern imprinting indicates that circE7 accounts for about 1–3% of the total E7 transcript and has lower transcript abundance compared to linear HPV transcript, and circE7 has a crucial impact on the function of CaSki cells transformed by HPV16. Targeting the circE7 back-splicing connection rather than targeting siRNA of linear homotypes prevents the production of E7 oncoproteins *in vitro* and decreases cellular proliferation in tumor xenografts. At the same time, circE7 only existed in the cell lines with free HPV and are found in TCGA RNA-Seq data of HPV-positive cancers (Zhao et al., 2019). These studies demonstrate that virus-derived, protein-coding circRNA have biological functions and are linked to the transformation properties of certain HPV. Results of circE7 formation may generate new understandings about how HPV can infect, lurk, and cause tumors to develop, and further studies that detect circE7 reverse joint connections may also have diagnostic significance.

Interacting With RNA-Binding Proteins (RBPs)

QKI, a kind of RBP, can activate biogenesis and cyclization in tumorigenesis of circRNA. It was indicated that QKI could be bound to specific QKI response elements (QREs) in the flanking introns of circSLC26A4 using RIP analysis. In order to prove this, several fusion vectors with QRE mutants were constructed, and circSLC26A4 expression was detected by qRT-PCR, proving that QKI can effectively activate circSLC26A4 by interacting with two QRE in flanking introns, and they can then target miR-1287-5p/HOXA7 to impact the progression of cervical cancer (Ji et al., 2020). Another RBP, called Single-Stranded DNA Binding Protein 1 (SSBP1), interacts with hsa_circ_0072088 (circZFR). They can form an SSBP1-circZFR complex that activates the CDK2/cyclin-E1 complex to induce p-Rb phosphorylation, thereby releasing the E2F1 transcription factor. Therefore, this

promotes the progression of the CC cell cycle and induces the proliferation of CC cells (Zhou et al., 2021). In another study, circCDKN2B-AS1 promoted the aerobic glycolysis of CC cells by recruiting IM3 protein, which is also a kind of RBP, to regulate the stability of the rate-limiting enzyme Hexokinase 2 (HK2) mRNA in the aerobic glycolysis pathway. Moreover, the bond between the cyclic KN2B-AS1 and IM3 proteins can be blocked by IIP, which is a synthetic inhibitory peptide, opening up a new way for the treatment of cervical cancer (Zhang et al., 2020).

APPLICATION OF circRNA IN CLINICAL RESEARCH OF CERVICAL CANCER

Impact on Clinical Severity

RT-qPCR confirmed in CC cell lines and tumor tissues the expression of circ-MYBL2 (Wang J. et al., 2019), hsa_circ_0075341 (Shao et al., 2020),hsa_circ_0001038 (Wang Y. et al., 2020), circRNA8924 (Liu et al., 2018), circ_0005576 (Ma et al., 2019), circCLK3 (Hong et al., 2019), and circ_0018289 (He et al., 2020) was significantly upregulated. Their elevation is closely related to clinical severity, including tumor size, FIGO staging, lymph node metastasis, and myometrial invasion, and this was also associated with poor prognosis in CC patients. The potential circRNAs that could be used as biomarkers in CC are shown in **Table 2**.

Mechanically, circ-MYBL2 binds to miR-361-3p in CC cells, and the inhibition of miR-361-3p reverses the effect of si-circ-MYBL2 on the progression of CC cells (Wang J. et al., 2019). Hsa_circ_0075341 negatively regulates miR-149-5p in CC progression. AURKA has been demonstrated to combine with miR-149-5p and is positively regulated by hsa_circ_0075341 in CC. Low miR-149-5p expression was associated with the poor overall survival (OS) rate (hazard ratio (HR) = 0.96, $P < 0.05$) of CC patients. Expression of AURKA was upregulated and also related to poor OS ($P < 0.05$) and lymph node metastasis in cervical cancer (Shao et al., 2020). The patients with higher expression of hsa_circ_0001038 can reduce OS more than those with a lower expression ($P = 0.030$). Hsa_circ_0001038 also negatively correlated with miR-337-3p expression in CC tissues. The expression of hsa_circ_0001038 in clinical tissues was also positively correlated with the expression level of metastasis-associated in colon cancer 1 (MACC1) and cyclin-M3 (CNNM3). Hsa_circ_0001038 allows miR-337-3p to release the inhibition of CNNM3 and MACC1 and can be associated with metastasis, thus promoting the growth and invasion of CC cells (Wang Y. et al., 2020). CircRNA8924 and the miR-518d-5p/519-5p family regulates CBX8 expression levels through competitive bonding, and miR-518d-5p/519-5p expression levels are thus negatively correlated with circRNA8924. Upregulation of CBX8 expression enhances malignant phenotype of CC cells (Liu et al., 2018).

Besides, upregulation of circ_0000388 expression in CC clinical samples is associated with poor pathological indicators. Circ_0000388 overexpression significantly hindered miR-337-3p expression and enhanced TCF12 expression to play a carcinogenic role in CC tissue and cells (Meng et al., 2020). High expression of circ_103973 is also related to poor prognosis in CC patients, and circ_103973 is an miR-335 sponge that directly targets PPP6C in cells (Zhu et al., 2020). The expression level of circRNA_0000285 was strongly higher than the corresponding normal tissues in CC samples. The growth and migration ability of CC cells was significantly inhibited after knockout of circRNA_0000285 *in vitro*, and the expression of FUS was also downregulated. Moreover, in nude mice, knockdown circRNA_0000285 can obviously inhibit the production and metastasis of the tumor (Chen R. X. et al., 2019). At the same time, the expression of circRNA_0000285 was raised in patients with radioresistant nasopharyngeal carcinoma compared with patients with radiosensitive nasopharyngeal carcinoma (Shuai et al., 2018). However, whether it relates to the radiosensitivity of CC needs further exploration. CircRNA_101996 obtained using circRNA microarrays is positively correlated with CC staging and negatively correlated with OS rate ($P = 0.032$), promoting CC progression through hsa_circRNA_101996/miR-8075/TPX2 networks (Song et al., 2019).

As a Diagnostic Biomarker

The expression levels of five circRNAs (hsa_circ_0101996, hsa_circ_0104649, hsa_circ_0104443, and hsa_circ_0101119) are markedly increased in the peripheral blood of 87 CC patients and 55 healthy controls. It is speculated that the expression of circRNA of whole blood in CC patients may have specific changes. We used the Receiver Operating Characteristic (ROC) curve to plot the expression level of circRNA in CC tissue and its paired ANT to evaluate the diagnostic value of circRNA. The area under the ROC curve (AUC) of hsa_circ_0101996 and hsa_circ_0101119 in peripheral whole blood was 0.906 and 0.887, respectively. The combined AUC increased to 0.964 (Wang Y.-M. et al., 2017). Low expression of serum circFoxO3a was significantly associated with positive lymph node metastasis ($P = 0.008$) and deeper stromal invasion depth ($P = 0.005$). CC patients with lower expression of serum circFoxO3a negatively correlated with OS rate and recurrence-free survival (RFS) (Tang et al., 2020). Hsa_circ_0101996, hsa_circ_0101119, and circFoxO3a in peripheral whole blood of CC patients can be combined as diagnostic biomarkers in routine clinical diagnosis. ROC analysis showed that the AUC of circ_0018289 was 0.907 (He et al., 2020), the AUC of hsa_circ_0107593 was 0.869 (Liao et al., 2020), and the AUC of circZFR was 0.88, which could significantly separate tumor tissue from ANT. Moreover, the clinical pathological features of 40 patients with CC revealed a positive correlation between circZFR expression and lymphatic metastasis, Ki67 values, and squamous cell carcinoma antigen (SCC Ag) value (Zhou et al., 2021). So circ_0018289, hsa_circ_0107593, and circZFR can also serve as potential disease monitoring biomarkers of CC.

As a Drug Target

Some preliminary studies have demonstrated that Spatholobi Caulis tannin (SCT), as a traditional Chinese medicine, has anti-cancer properties. A three-dimensional microfluidic chip demonstrates that SCT can reduce the survival rate of Hela cells and regulate the cell cycle, and it has been shown to affect pathogenic proteins by molecular docking

TABLE 2 | The potential circRNAs that could be used as biomarkers in clinical application.

circRNA	miRNA	Target gene	Clinical application	Type of detection methods	References
Hsa_circ_0101996 and hsa_circ_0101119	–	–	The expression of Hsa_circ_0101996 and hsa_circ_0101119 were significantly upregulated in peripheral whole blood from CC patients	RT-qPCR and ROC analysis	Wang Y.-M. et al., 2017
circCLK3	miR-320a	FoxM1	Significantly correlated with poor tumor differentiation, advanced FIGO stages, and large depth of stromal invasion but was negatively related with OS and disease-free survival of CC patients	qRT-PCR and Kaplan–Meier Plotter analysis	Hong et al., 2019
circ_0005576	miR-153	KIF20A	Upregulated circ_0005576 was positively associated with advanced FIGO stage, lymph node metastasis, but was negatively related with OS of CC patients	qRT-PCR, Kaplan–Meier Plotter analysis, and IHC	Ma et al., 2019
circ-MYBL2	miR-361-3p	–	The expression of circ-MYBL2 was significantly upregulated and positively associated with advanced FIGO stage, larger tumor size, lymph node metastasis, and poor prognosis in CC patients	qRT-PCR and Western blotting	Wang J. et al., 2019
hsa_circ_0075341	miR-149-5p	AURKA	The expression of hsa_circ_0075341 was significantly upregulated and associated with larger tumor size, advanced FIGO stage, and lymph-node metastasis in CC patients	qRT-PCR, IHC, and Kaplan-Meier curves	Shao et al., 2020
hsa_circ_0001038	miR-337-3p	CNNM3, MACC1	Hsa_circ_0001038 highly expressed in CC tissues and was closely related to lymph node invasion and myometrial invasion	qRT-PCR, Western blotting, Kaplan-Meier curves, Fisher's exact test, and Pearson correlation analysis	Wang Y. et al., 2020
circRNA8924	miR-518d-5p/519-5p	CBX8	The level of circRNA8924 expression was significantly correlated with tumor size, FIGO staging and myometrial invasion, but it has no correlation with patient's age or tumor differentiation or lymph node metastasis	qRT-PCR and Western blotting	Liu et al., 2018
circ_0000388	miR-337-3p	TCF-12	The circ_0000388 expression was notably correlated with the FIGO stage, lymph node metastasis, and depth of invasion	qRT-PCR and Western blotting	Meng et al., 2020
circ_103973	miR-335	PPP6C	Higher levels of circ_103973 were correlated to a worse prognosis of CC patients	qRT-PCR	Zhu et al., 2020
circRNA_0000285	–	FUS	CircRNA_0000285 expression was significantly higher in CC tissue samples	qRT-PCR	Chen R. X. et al., 2019
Hsa_circRNA_101996	miR-8075	TPX2	The expression level of hsa_circRNA_101996 in CC tissues was positively correlated with TNM stage, tumor size, and lymph node metastasis	qRT-PCR and Kaplan-Meier analysis	Song et al., 2019
circular RNA_0018289	–	–	Circ_0018289 expression was positively correlated with pathological grade, tumor size, lymph node metastasis, and FIGO stage	RT-qPCR, ROC analysis, and Cox's regression analysis	He et al., 2020
circular FoxO3a	–	–	Low serum circFoxO3a levels to be a poor prognostic factor for both OS and RFS, independent of positive lymph node metastasis	RT-qPCR, Kaplan-Meier analysis, and multivariate Cox regression analysis	Tang et al., 2020
circZFR	–	SSBP1/CDK2/cyclin E1	CircZFR was positively associated with lymphatic metastasis and was not associated with age, tumor stage, invasion depth, or vascular invasion	qRT-PCR and ROC analysis	Zhou et al., 2021

technology (Wang et al., 2018). Differential genes of cervical cancer were screened by the TCGA database and GEO and KEGG analysis. Using molecular docking studies and other bioinformatic analyses predicted SCT target proteins to further identify circRNAs associated with SCT target genes. The expression levels of circFANCB, circE2F3, circATR, and circBLM gradually increased following the decrease of drug concentration in HeLa cells. RegRNA analysis provides the following projections: circFANCB combined with hsa-miR-4692 and circE2F3 combine with hsa-miR-5006-3p, hsa-miR-3960, hsa-miR-4739, hsa-miR-4459, hsa-miR-211-5p, and hsa-miR-4632. SCT regulates circRNAs associated with CC to promote apoptosis and inhibit the proliferation of cells. Therefore, SCT can be used as a booster for the treatment of CC (Wang N. et al., 2019).

CONCLUSION

At present, the era of "precision medicine" has come; "precision medicine" is used to provide cancer patients with more individualized diagnosis, treatment, and follow-up, and improving efficacy and quality of life and reduce the treatment-related toxicity response has thus become possible. CircRNA is one of the hot topics in the study of transcriptomes. Because of its rich, stable, and specific expression, circRNA has a broad clinical application prospect and has the potential to be a molecular marker for tumor diagnosis and prognosis as well as a therapeutic target. Nevertheless, the current research on the mechanism of circRNA action in cervical cancer is not deep enough, mostly limited to the sponge mechanism between circRNA and miRNA, and its regulation needs further exploration and in-depth research.

AUTHOR CONTRIBUTIONS

JL: writing manuscripts and collecting literature. HZ: collecting literature and modifying manuscripts. LF: proposing amendments. TX: designing the content of the article and proposing amendments. All authors contributed to the article and approved the submitted version.

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Cancer-Associated circRNA–miRNA–mRNA Regulatory Networks: A Meta-Analysis

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Recent advances in sequencing technologies and the discovery of non-coding RNAs (ncRNAs) have provided new insights in the molecular pathogenesis of cancers. Several studies have implicated the role of ncRNAs, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and recently discovered circular RNAs (circRNAs) in tumorigenesis and metastasis. Unlike linear RNAs, circRNAs are highly stable and closed-loop RNA molecules. It has been established that circRNAs regulate gene expression by controlling the functions of miRNAs and RNA-binding protein (RBP) or by translating into proteins. The circRNA–miRNA–mRNA regulatory axis is associated with human diseases, such as cancers, Alzheimer's disease, and diabetes. In this study, we explored the interaction among circRNAs, miRNAs, and their target genes in various cancers using state-of-the-art bioinformatics tools. We identified differentially expressed circRNAs, miRNAs, and mRNAs on multiple cancers from publicly available data. Furthermore, we identified many crucial drivers and tumor suppressor genes in the circRNA–miRNA–mRNA regulatory axis in various cancers. Together, this study data provide a deeper understanding of the circRNA–miRNA–mRNA regulatory mechanisms in cancers.

Keywords: circRNA, microRNA, cancer, TCGA, driver genes, tumor suppressor genes

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INTRODUCTION

The cellular processes governing gene expression regulation are controlled at the molecular level. The aberrations in these regulations are linked to severe consequences, including cancers. Unfortunately, the biological processes pivotal to cancer growth and metastasis remain undefined despite the years of dedicated research. As a result, there is a dearth of molecular targets for diagnostics, prognostics, and therapeutics for many cancers. Improved insights into the molecular mechanisms of cancer development and progression will help develop the strategies for early diagnosis, prognosis, and treatment.

Recent developments in the high-throughput sequencing technologies led to the discovery of novel therapeutic biomolecules, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and poorly characterized circular RNAs (circRNAs) (Guria et al., 2019). The miRNAs and lncRNAs are well known for their critical role in cancer development and metastasis (Huang et al., 2013; Slack and Chinnaiyan, 2019). In recent years, an increasing number of researchers are focusing their efforts on explaining the biological functions of circRNAs. The circRNAs, also known as competitive endogenous RNAs (ceRNAs), are a large family of covalently closed single-stranded stable RNA molecules with a regulatory potential (Salzman et al., 2012; Ledford, 2013).

They are generated from exons and/or introns with a certain degree of evolutionary conservation and show tissue-specific expression patterns (Chen, 2016). Although the biological functions of the majority of circRNAs are not known, the accumulating pieces of evidence established that circRNAs regulate gene expression by sponging miRNAs and binding with RNA-binding proteins (RBPs), and by direct translation into proteins (Panda, 2018; Huang et al., 2020).

Circular RNAs are widespread; however, their expression is tissue-specific. A growing body of research indicates that circRNAs are involved in various types of pathophysiology, including aging, diabetes, glycolysis (Mirzaei and Hamblin, 2020), myogenesis (Das et al., 2020), virus infections (Nahand et al., 2020), and cancer (Haque and Harries, 2017; Cai et al., 2019; Shabaninejad et al., 2019; Shang et al., 2019; Borran et al., 2020; Li et al., 2020; Razavi et al., 2021). Since circRNAs are stable and tissue-specific, many studies have explored their diagnostic and prognostic potential in cancers. The emerging evidence indicates that circRNA can be an excellent biomarker for the development of new diagnostic and prognostic strategies. For instance, CDR1as and circ-FOXO3 are involved in regulating the development of breast cancer by acting as miRNA sponges (Lu, 2017; Yang et al., 2019). Furthermore, circ-ITCH inhibits the Wnt/β-catenin pathway in esophageal squamous cell carcinoma by sponging miRNAs (Li et al., 2015). Several studies suggested that the circRNA–miRNA–mRNA axis plays a crucial role in regulating various cellular events critical for cancer progression. However, the molecular mechanisms of circRNA–miRNA–mRNA regulatory axis in the carcinogenesis and progression of cancer are not well studied.

Understanding the circRNA–miRNA interaction can give important clues about the molecular mechanism of the pathogenesis in a given cancer. In the current study, the expression profiles of circRNAs, miRNAs, and mRNAs in different cancers have been collected from Gene Expression Omnibus (GEO) database, The Cancer Genome Atlas (TCGA), and research publications. The circRNA–miRNA–mRNA regulatory networks consisting of the differentially expressed (DE) circRNAs and their downstream miRNAs and target mRNAs have been constructed for seven cancer types. The circRNAs that may play active roles in regulating the driver genes and tumor suppressor genes in those cancers are also identified. The analysis of target mRNAs for the functional pathways using the protein–protein interaction network (PPIN) and gene ontology (GO) enrichment analysis revealed the potential mechanism of circRNAs in the initiation and progression of various cancers. Together, this research provides new insights into the regulation of carcinogenesis by the circRNA–miRNA–mRNA regulatory axis.

RESULTS

Identification of DE circRNAs in Different Cancers

The data mining to identify DE circRNAs (DECIs) in different cancers resulted in more than 1,300 articles (i.e., research and review articles). Only research articles for the last 8 years were considered to find circRNAs associated with cancer (**Figure 1** and **Supplementary Table 1**). The supplementary data from the reports were analyzed for DECIs, especially for those having a significant expression (p value < 0.05). The results from different circRNA databases were also included. The data were compiled such that all the circRNAs had at least a circBase ID and genomic coordinates. Other relevant information such as the gene symbol, type of circRNA (exonic/intronic), regulation (up- or downregulated), and strand were also included wherever available. These DECIs were classified into different types such as exonic, intronic, intergenic, intragenic, sense overlapping, and antisense circRNAs (**Supplementary Table 2**).

Identification of circRNA–miRNA and miRNA–mRNA Interactions and Construction of the ceRNA Network

The expression of the mRNAs is tightly controlled at the posttranscriptional level by RBPs and miRNAs. The miRNAs can regulate the expression of mRNAs by promoting their 3′ degradation, whereas the circRNAs can exert their control by acting as a miRNA sponge and thereby controlling mRNA regulation indirectly. Understanding the circRNA–miRNA and miRNA–mRNA interactions can give important clues about the molecular mechanism of the pathogenesis in a given cancer. The *in silico* prediction algorithms were utilized to produce the circRNA–miRNA and miRNA–mRNA interaction map.

After finding the DECIs, the next step was to find the circRNA–miRNA interaction. The miRNAs that have two or more binding sites on circRNAs were considered only to create the circRNA–miRNA interaction. The analysis of these miRNAs in mirTarBase resulted in the identification of many mRNAs as targets, resulting in the miRNA–mRNA interaction. To identify significantly DE miRNAs (DEMIs) and mRNAs, we analyzed transcriptomics data in TCGA for seven different cancers. We included only DEMI and DE mRNA (DEM) in chosen cancers for this study. Hence, the number of circRNA–miRNA and miRNA–mRNA interactions were reduced (**Supplementary Table 3**). Furthermore, these interactions were merged to form a circRNA–miRNA–mRNA triad (**Figure 2**). The identified triad with a positive correlation between the circRNA and mRNA expression was only considered further. A network of triads was generated to understand their interrelation and possible role in the pathogenesis of cancer. Besides, we also scrutinized the presence of tumor driver and suppressor genes in DEMs. The circRNAs, hsa_circ_0036186| PKM2, are known to regulate 14-3-3-ζ expression by functioning as a ceRNA in the development and progression of head and neck squamous cell carcinoma (HNSCC) (Li B. et al., 2018). It is important to note that SFRP4, a driver gene, upregulated in HNSCC, is regulated by five different circRNAs, namely, hsa_circ_0008309| CUL3, hsa_circ_0001387| WHSC1, hsa_circ_0036186| PKM2, hsa_circ_0002667| MGAT2, and hsa_circ_0001821| circPVT1, in the identified triad. SFRP4, which drives the process of carcinogenesis in HNSCC, has not been reported anywhere about its interaction with circWHSC1. In this study, it is seen to be

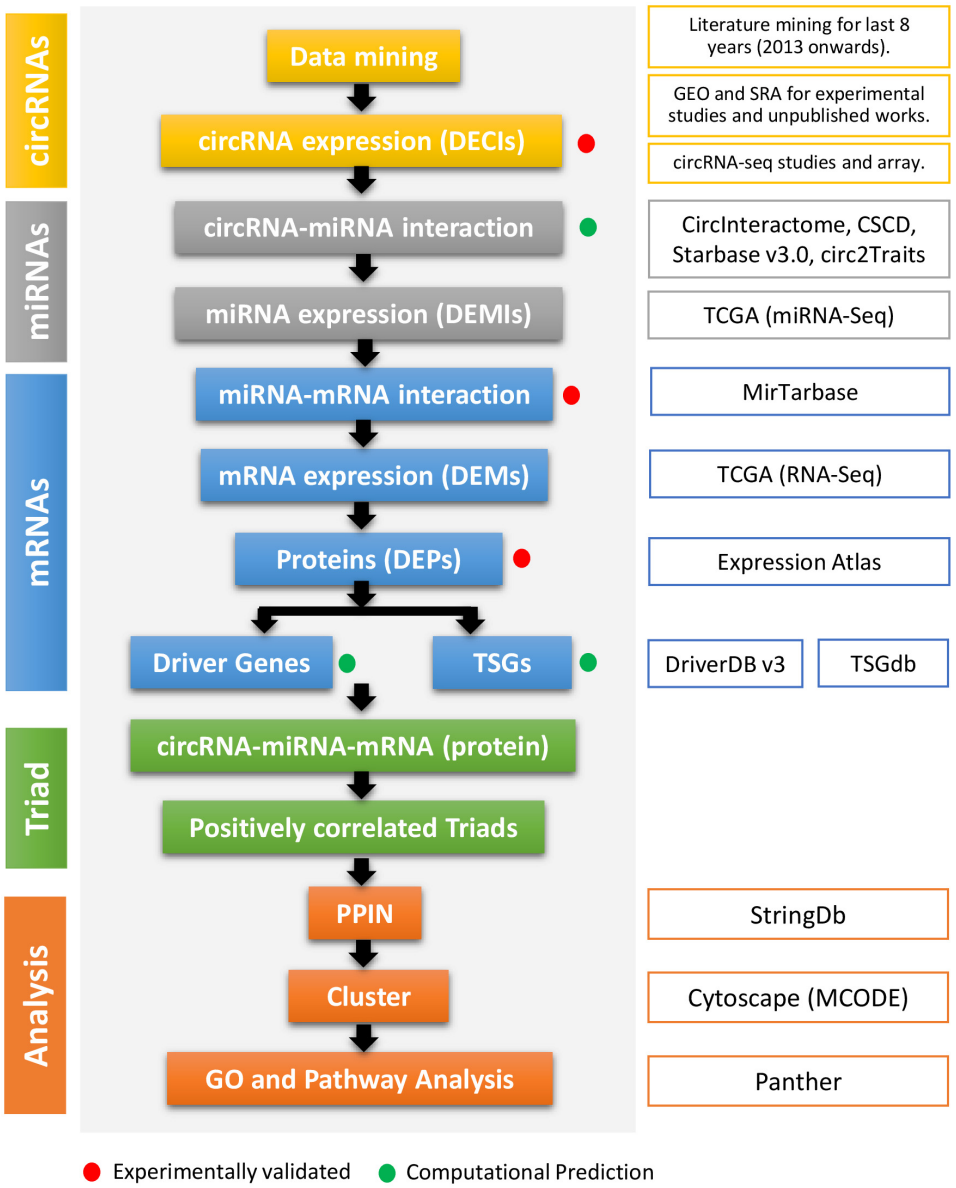

FIGURE 1 | The flowchart of the method used in this study. The first column represents the types of regulatory elements (color-coded). The second column mentions the method cascade. The third column gives the detail about every step, especially the source of the study. The red and green dots represent if the step and output are experimentally validated or computationally predicted.

regulated by the circRNA hsa_circ_0001387| WHSC1 through miR-942. Similarly, in lung cancer, the circRNAs, namely, hsa_circ_0051620| SLC1A5 and hsa_circ_0066954| POLQ, are upregulated and are shown to interact with and regulate driver genes, namely, ADAM17, CDH2, RUNX2, and ZBTB18, through miR-338-3p. The miRNA-338-3p, however, is known to suppress tumor proliferation (Ni et al., 2013). Although circRNA function is not yet understood completely, the circRNA–miRNA–mRNA network analysis suggests that these circRNAs may act as a miRNA sponge and regulate the driver genes, thereby modulating carcinogenesis. The driver and tumor suppressor genes regulated by the miRNA and circRNA were marked in the network as driver and tumor suppressor triads, respectively (**Supplementary Table 4**). We now have a DE triad of circRNA–miRNA–mRNA for seven different cancers (**Supplementary Table 5**). A circRNA–miRNA–mRNA triad network was made for each cancer (**Supplementary Material**).

Construction of PPIN and Extraction of Clusters

The DEMs from the circRNA–miRNA–mRNA triads were used to create the PPIN in the form of a network graph for each cancer (**Figure 3**). The analysis of a large PPIN can give information

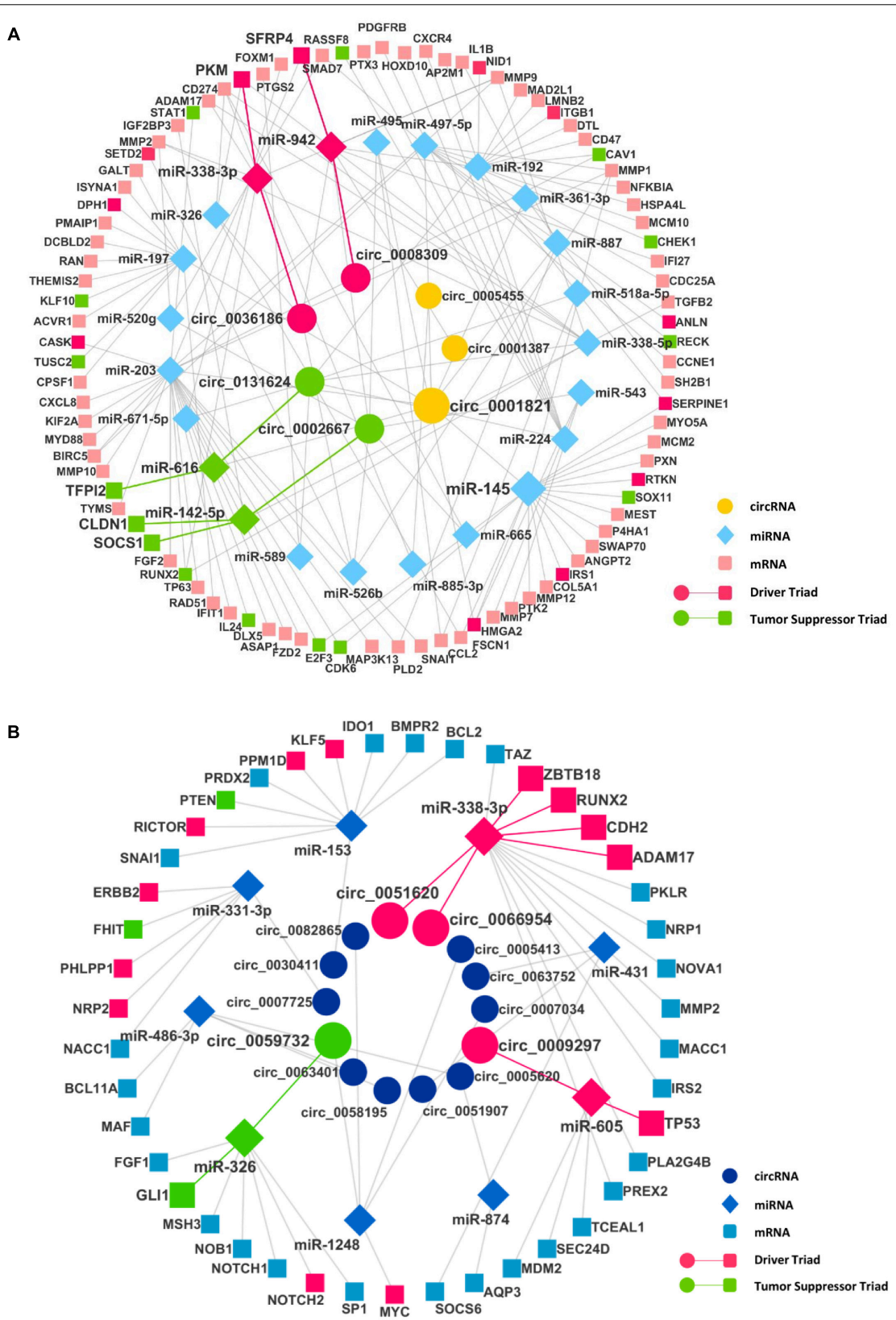

FIGURE 2 | The circRNA–miRNA–mRNA regulatory networks in **(A)** head and neck squamous cell carcinoma (HNSCC) and **(B)** lung cancer. Nodes (inner to outer) represent differentially expressed circRNAs, miRNAs, and mRNAs, respectively. The circular nodes represent circRNA, diamonds represent miRNAs, and square nodes represent the mRNAs. The red and the green highlighted circRNA–miRNA–mRNA interaction represent a driver and tumor suppressor triads, respectively.

about small subnetworks, also known as "clusters." In this study, the advantage of the clusters in a PPIN is to get information about specific processes represented by them. The modulation of any protein expression in the PPIN by the circRNA–miRNA–mRNA networks may affect the function of the cluster and the pathway. This analysis can also give information about the hub genes that may be important for the stability and functioning of a given PPIN. In this study, the MCODE algorithm was used to extract clusters from the generated PPIN (**Figure 4**). The PPIN for different cancers resulted in different number of clusters (**Supplementary Table 6**).

GO and Pathway Analysis

The GO analysis for the biological process, molecular function, and cellular component can give important information about the processes and pathways in which a group of genes may be involved. Such information is vital for the generation of hypotheses and the design of further studies. The enriched GO terms and pathways for different clusters were analyzed to see the pathways and processes in which they are involved (**Supplementary Table 7**). Every cluster has its significance in terms of functions; hence, the need to classify each cluster differently in terms of their processes helps us relate these essential processes to the circRNAs indirectly through the triad. The R package ggplot2 was used to plot the graphs, where GO was combined in dot plots and pathways as bar plots (**Supplementary Material**). The most common processes among cancers were extracellular matrix organization (GO:0030198), cellular process (GO:0009987), metabolic process (GO:0008152), catalytic activity (GO:0003824), metallopeptidase activity (GO:0008237), hydrolase activity (GO:0016787), extracellular region (GO:0005576), and nucleus (GO:0005634), and the pathways were Alzheimer's disease–presenilin pathway (P00004), p53 pathway (P00059), and angiogenesis (P00005). The topmost common gene ontologies and pathways from every largest cluster for each cancer are taken and plotted (**Figure 5**).

Impact of DEMIs and DEMs on Patient Survival

The role of many coding RNAs and non-coding RNAs (ncRNAs) has been studied in various cancers to determine their impact on the survival of the patients. Besides mRNA, there are many ncRNAs, such as miRNAs (onco–micro RNAs), whose expression has been seen to affect the survival of the patients. Several DEMs and DEMIs from the triads were common, with the top DE genes and miRNAs playing a significant role in the overall survival of patients (**Supplementary Material**). The circRNAs interacting with those DEMIs and DEMs can be predicted to have similar functions. For example, the circRNAs in a triad hsa_circ_0131624| TUBB2A–hsa-miR-338-5p—PKM in HNSCC, hsa_circ_0080517| CLDN4–hsa-miR-145—SERPINE1 in gastric cancer, and hsa_circ_0000228| ZEB1–hsa-miR-526b—MMP1 and hsa_circ_0009022| PPP4R1–hsa-miR-526b—MMP1 in liver cancer, where both the miRNAs and mRNAs from the triad play a significant role in survival, might be important in the prognosis of these cancers. Hence, we plotted the survival curves for HNSCC, gastric cancer, liver cancer, lung cancer, and breast cancer using DEMs and DEMIs in triads (**Figure 6**). We did not find any significant DEMs and DEMIs in our triads for pancreatic cancer and thyroid cancer, affecting the overall survival (**Supplementary Table 8**).

MATERIALS AND METHODS

Data Mining

PubMed was used to collect the circRNAs associated with 15 different cancers (**Supplementary Table 1**). The search was performed using the keywords "name of cancer" and "circular RNA," for example, "HNSCC" or "head and neck squamous," "cancer" or "tumor," and "tumor" or "carcinoma," and "circRNA" or "ciRNA" or "Circular RNA" were used for HNSCC. The results were further filtered using the following inclusion criteria: (i) research articles for the last 7 years (2013 onward), (ii) studies done exclusively on humans, (iii) circRNAs with the expression as $|\text{Log 2 Fold Change} \geq 1|$ and p value (<0.05). The review articles, redundant papers (update of a previous work done by the same authors), and circRNAs having ArrayStar Id without genomic coordinates were excluded.

Moreover, the NCBI GEO was explored for the unpublished experimental work, including the microarray and total RNAseq datasets for the role of circRNAs and their expression in the selected cancers. The studies containing circRNA expression data for at least three replicates (tumor and non-tumor) were chosen for further analysis. Additionally, other circRNA databases such as CircR2Disease (Fan et al., 2018), CircFunBase (Meng et al., 2019), CircInteractome (Dudekula et al., 2016), Circ2Traits (Ghosal et al., 2013), StarBase (Li et al., 2014), CircNet, CircBase (Liu et al., 2016), CIRCpedia (Dong et al., 2018), CSCD (Xia et al., 2018), TSCD (Xia et al., 2017), circRNADb (Chen et al., 2016), and ExoRbase (Li S. et al., 2018) were also searched. We considered only those circRNAs where the cancer type and expression data were available.

Identification of circRNA–miRNA and miRNA–mRNA Interactions

To understand the circRNA–miRNA and miRNA–mRNA interactions, various bioinformatics tools, such as Cancer-Specific CircRNA (CSCD), CircInteractome, Circ2Traits, and StarBase, for each cancer were used. Additionally, to avoid bias by one algorithm, the circRNA–miRNA interaction identified by at least two algorithms was considered for further studies. The next part was to find the targets of the miRNAs identified in the previous step. For that, mirTarBase (Chou et al., 2018), a database that contains >360,000 experimentally validated miRNA–mRNA interactions (MTIs), was used. The target genes validated by at least more than one experimental method were selected for further analysis.

Mining of DECIs, DEMIs, and DEMs

We identified DECIs, DEMIs, and DEMs using TCGA data (Cancer Genome Atlas Research Network [CGARN], Weinstein et al., 2013). The RNA-seq data for mRNAs and miRNAs for selected cancers were downloaded from TCGA and the

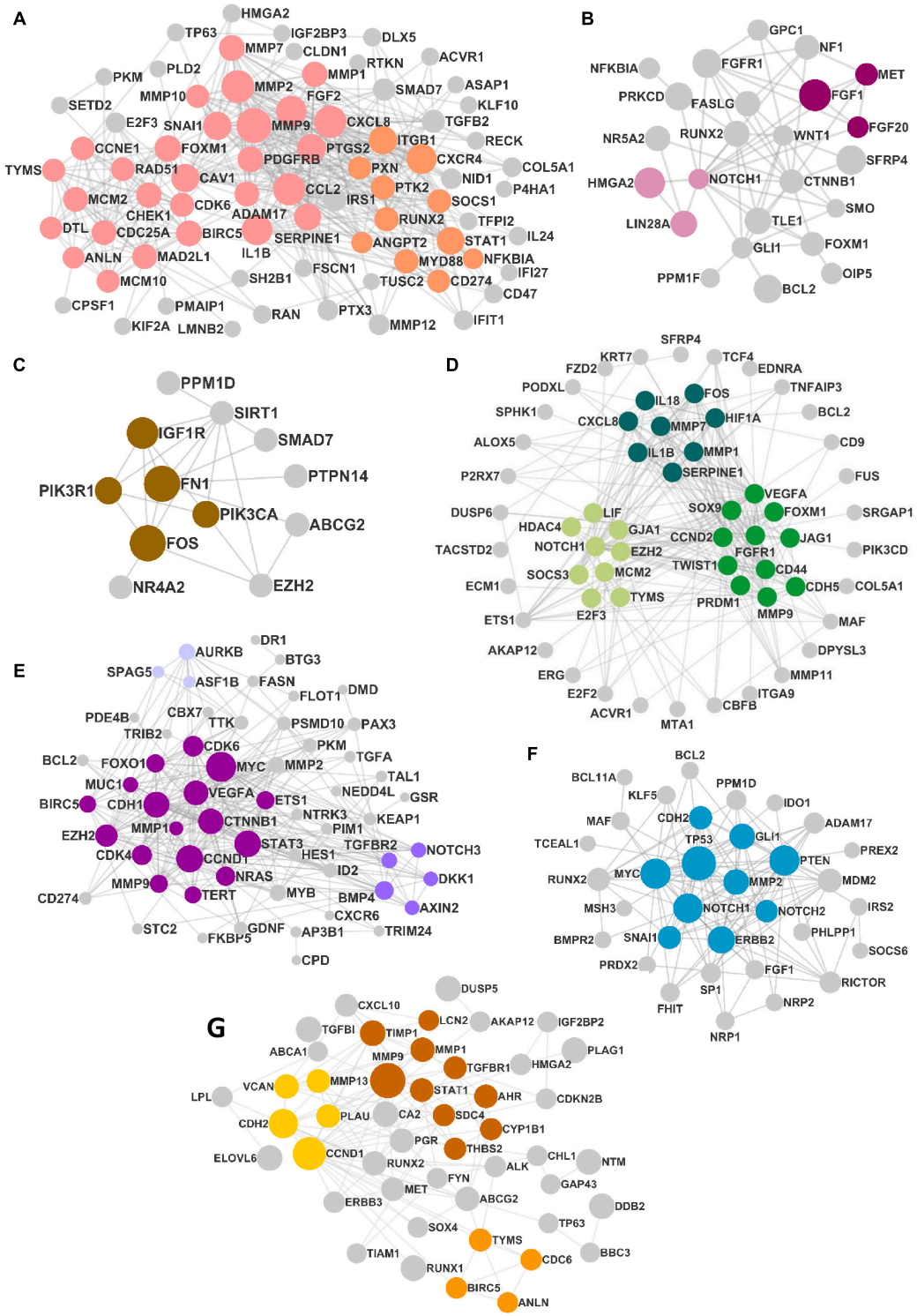

FIGURE 3 | The protein–protein interaction network (PPIN) in **(A)** head and neck squamous cell carcinoma (HNSCC), **(B)** breast cancer, **(C)** pancreatic cancer, **(D)** gastric cancer, **(E)** liver cancer, **(F)** lung cancer, and **(G)** thyroid cancer. The size of the nodes is distributed according to the number of in and out degrees.

differential expression analysis was performed using the DESEQ2 package (Love et al., 2014) in Bioconductor. A miRNA/mRNA was considered DE if $|\log2(\text{fold change})| \geq 1$ and p value ≤ 0.05. Moreover, we also wanted to see if these DEMs are translated into proteins. We used Expression Atlas (Papatheodorou et al., 2018) to check if the DEMs are

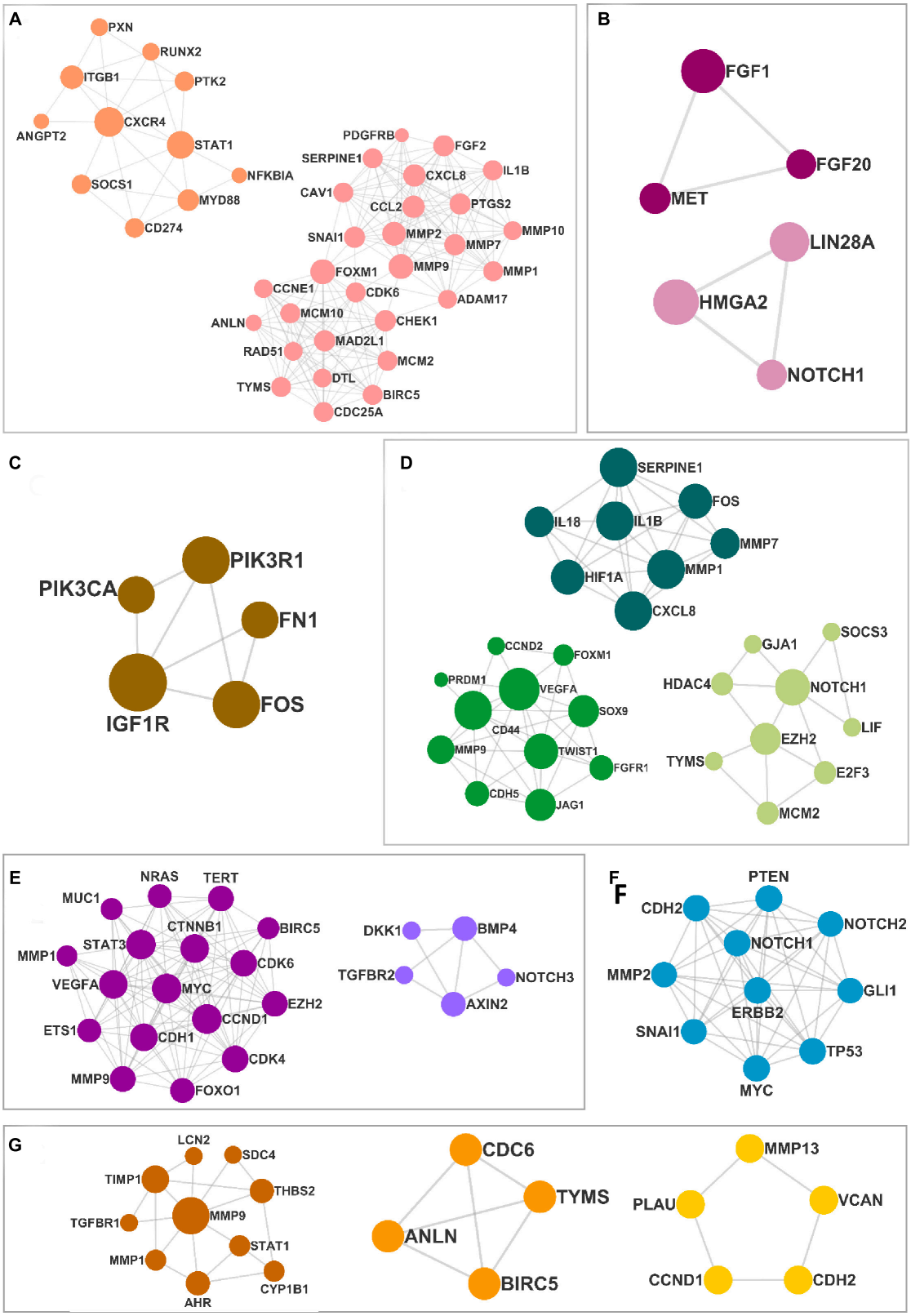

FIGURE 4 | The clusters extracted from the protein–protein interaction network (PPIN) for **(A)** head and neck squamous cell carcinoma (HNSCC), **(B)** breast cancer, **(C)** pancreatic cancer, **(D)** gastric cancer, **(E)** liver cancer, **(F)** lung cancer, and **(G)** thyroid cancer.

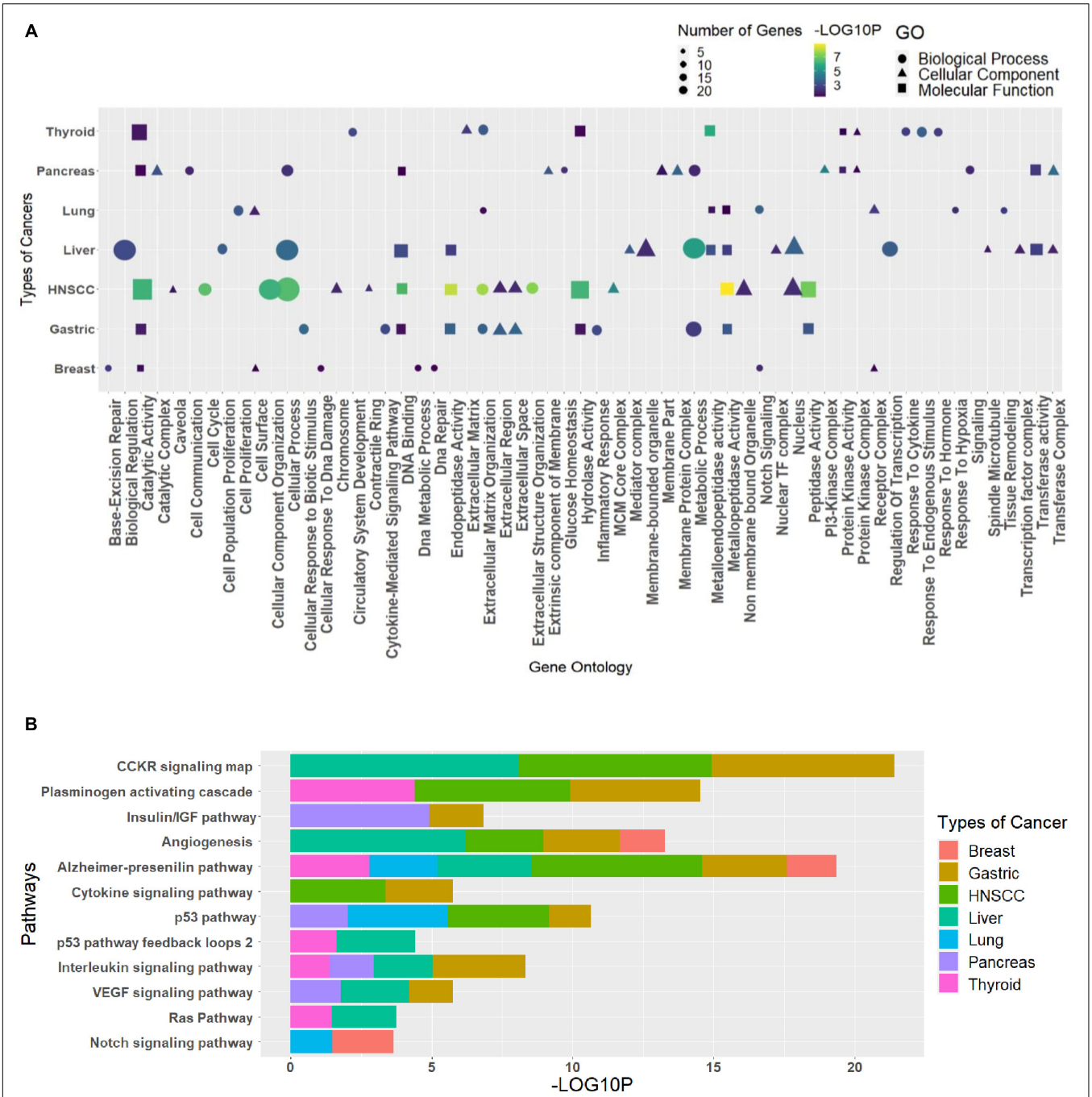

FIGURE 5 | The topmost common **(A)** gene ontologies and **(B)** pathways, taken from the largest cluster of each cancer. The graph has been plotted based on the significant *p* values ($p \leq 0.05$), taken as the negative logarithm of the *p* value ($-\log_{10}p$).

expressed in a given tissue for a particular cancer. We also considered mRNAs only if they are expressed as proteins in a particular cancer.

Identification of Cancer Driver Genes

Although many mRNAs are DE during cancer development and progression, only a few are responsible for driving the disease process. These cancer driver genes give cells a growth advantage, especially when they are mutated or otherwise dysregulated. In this study, we used the DriverDBv3 (Jin et al., 2020) that uses 12 algorithms, such as ActiveDriver (Reimand and Bader, 2013), CoMET (Martin et al., 2015), Dendrix (Vandin et al., 2012), DriverML (Han et al., 2019), DriverNet (Bashashati et al., 2012), e-Driver (Porta-Pardo and Godzik, 2014), iPAC (Ryslik et al., 2013), MEMo (Ciriello et al., 2013), MSEA (Jia et al., 2014), MUTEX (Babur et al., 2015), NetBox (Cerami et al., 2010),

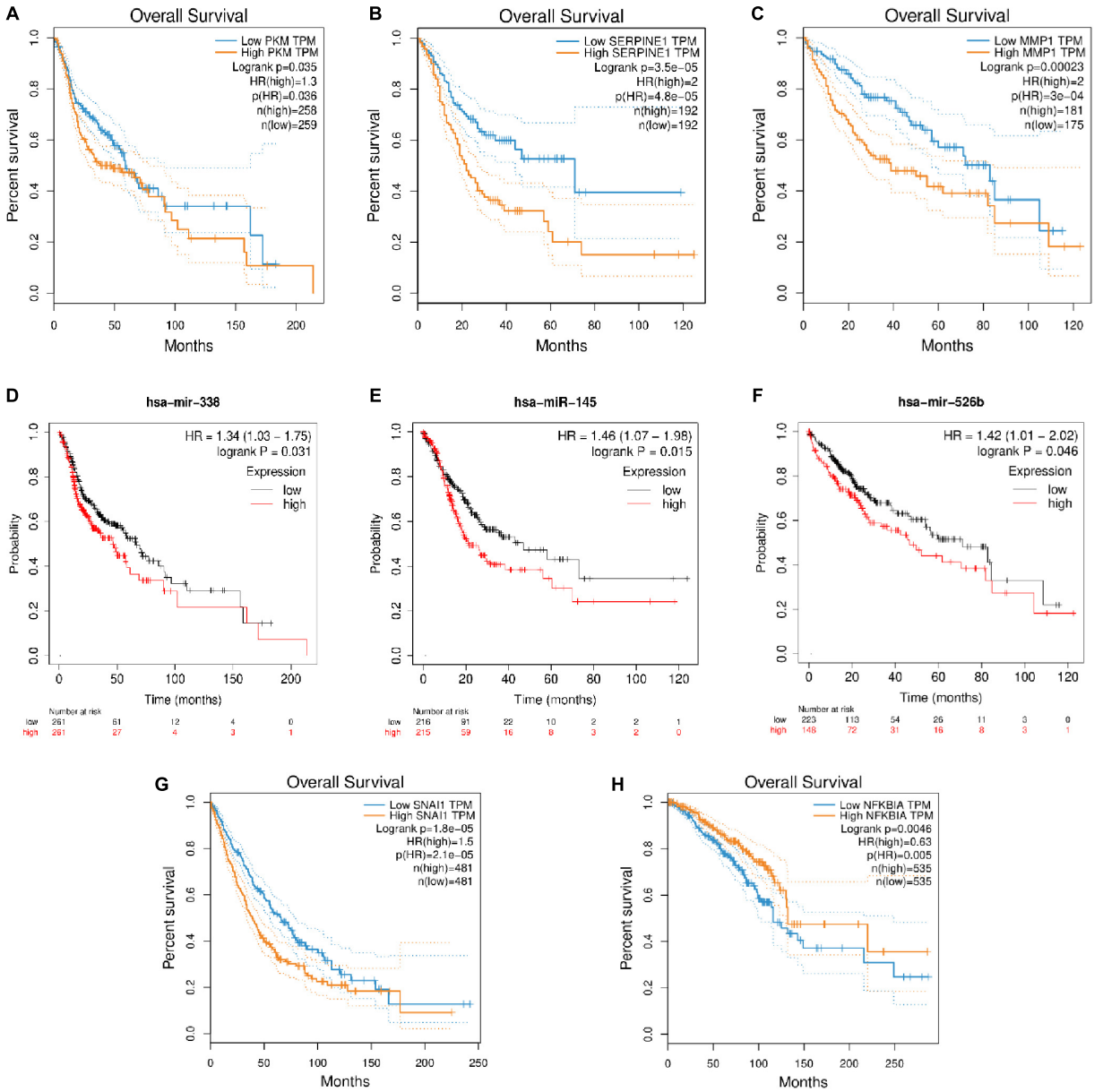

FIGURE 6 | The survival plots for **(A)** PKM and its corresponding miRNA, **(D)** has-miR-338-5p in HNSCC, **(B)** SERPINE1 and its corresponding miRNA, **(E)** hsa-miR-145 in gastric cancer, **(C)** MMP1 and its corresponding miRNA, **(F)** hsa-miR-526b in liver cancer, **(G)** SNAI1 in lung cancer and **(H)** NFKBIA in breast cancer.

and OncodriveCLUST (Tamborero et al., 2013), to predict the cancer driver genes.

Identification of Tumor Suppressor Genes

Apart from the driver genes, another category is called anti-oncogene or tumor suppressor genes that help in cell growth regulation. These genes act like breaks in cell growth and multiplication. Mutations in tumor suppressor genes may also lead to cancer. Therefore, their study can identify essential mRNAs and, in turn, essential circRNAs regulating their activity. We used TSGene 2.0 (Zhao et al., 2016) to determine the tumor suppressor mRNAs. It contains 1,217 human TSGs (1,018 protein-coding and 199 non-coding genes) curated from more than 9,000 articles. Additionally, TSGene 2.0 provides thousands of expression and mutation patterns derived from TCGA.

Generation of circRNA–miRNA–mRNA Triad

The circRNA–miRNA and miRNA–mRNA interactions were combined to form a circRNA–miRNA–mRNA regulatory triad. The next step was to identify high-priority triads based on the

observed expression levels of coding and non-coding RNAs. As stated earlier, the circRNA may regulate the expression of mRNA by sponging the intermediate miRNAs. Thus, a positive correlation between the expression levels of circRNA and mRNA can be expected. It may indicate a strong effect of circRNA on the expression of given mRNA. A triad, in which circRNA is up and its corresponding mRNA(s) are also significantly up or circRNA is down and its corresponding mRNA(s) are also significantly down, is considered to be in a positive regulation. Therefore, we retained the triads if there exists a positive correlation between circRNA and mRNA expression levels.

Construction and Analysis of PPIN

In the current study, the proteins related to the mRNAs from positive triads were used to create a PPIN for each cancer using the String database (Szklarczyk et al., 2016), where the minimum required interaction score was set at 0.4 (medium confidence). The PPIN was imported into Cytoscape v3.7.2 (Shannon et al., 2003) for further analysis. The highest interconnected component, also known as the "giant component," was extracted. The clusters were then identified using MCODE (Bader and Hogu, 2003) algorithm that identifies densely connected nodes in a network. The layout was designed using the "combined score" for each protein combined with gene fusion, phylogenetic cooccurrence, homology, coexpression, experimental validation scores, and the node attribute.

GO and Pathway Analysis

The gene ontologies and pathways were analyzed for the identified clusters using PANTHERv14.0 (Mi et al., 2019). The statistical overrepresentation test was used to find the enriched GO terms and pathways by matching the gene list with the human genome, applying Fisher's exact test with Bonferroni correction. The GO and pathway analysis for the clusters relates the genes with specific processes and pathways. This information can be used to relate the circRNAs in the regulation of those processes and pathways. The R package, ggplot2 (Wickham, 2011), was used to plot the GO as dot plots and pathways as bar graphs based on the p values.

Impact of DEMIs and DEMs on Patient Survival

We used GEPIA (Gene Expression Profiling Interactive Analysis) (Yang et al., 2020) for DEMs, and OncomiR (Wong et al., 2018) and UALCAN (Chandrashekar et al., 2017) for DEMIs, which considers the RNAseq and miRNAseq data from TCGA to see if the DEMs and DEMIs in our triad have a significant impact on survival. The Kaplan–Meier method (Nagy et al., 2018) was used to plot the overall survival curve for the DEMIs. The survival plots for both DEMs and DEMIs were considered significant, only if the log rank p value ≤ 0.05.

DISCUSSION

The advent of NGS technologies coupled with user-friendly tools has spurred research in deciphering the genome and its regulation. These technologies have arguably impacted the research in circRNA and have resulted in identifying many circRNAs with the myriads of functions. Since 2013, there has been a lot of attention given to circRNA research due to their novel functions such as miRNA sponging, RBP regulation, and translational capabilities. The circRNAs have sparked considerable interest as potential biomarkers due to their tissue-specific expression and high stability.

The circRNA–miRNA–mRNA axis plays a vital role in cancer initiation and progression (Jamal et al., 2019). For instance, the members of the circ-ZEB1 family are reported to play a role in the suppression of lung cancer progression *via* the sponging of miR-200. The circMOT1 sponges miR-9 to allow the expression of the tumor suppressor gene p21. The emerging oncogenic function of circRNAs is of particular interest, as it might make them candidates for new biomarkers and therapeutic targets in cancer. This study was made to find circRNAs in different cancers and group them into triads to see their role in controlling the downstream regulatory elements and their process.

This study has assimilated information from multiple sources such as literature search and online databases, including CircR2Disease, CircFunBase, CircInteractome, Circ2Traits, and StarBase TCGA, and GEO to establish a "Triad Regulatory Network." The circRNA enhances gene expression by acting as miRNA sponges. This network was made based upon the established mechanism of interaction among circRNA, miRNA, and mRNA. If circRNA is overexpressed, it competitively binds to the miRNA and inhibits their activity, hence rescuing the mRNA degradation or *vice versa*. Therefore, we chose those triads with a change in the same direction for circRNAs and mRNA expression. We also established the driver and tumor suppressor triads based on the driver and tumor suppressor genes in our triad, followed by the GO and pathway analysis for the clusters. The hub genes (clusters), which were explicitly driver and tumor suppressor genes extracted from PPIN, were further analyzed for their therapeutic role(s).

In HNSCC, the driver gene PKM plays a vital role in carcinogenesis through cell proliferation. It is targeted by miR-338-3p, which in turn is targeted by hsa_circ_0036186| PKM2. As predicted in this study, this particular driver triad gains more importance as the survival study on the driver gene (PKM) and the miRNA that it interacts with (hsa-miR-338-3p) shows that both the gene and miRNA cause a decrease in the survival of the patients with HNSCC. One of the circRNAs, hsa_circ_0001387| WHSC1, predicted from this study and shown in our triad in HNSCC is upregulated and known to be circulating in the peripheral blood. This can act as a diagnostic marker for HNSCC.

In the lung cancer, the driver triad hsa_circ_0051620| SLC1A5–hsa-miR-338-3p—CDH2 and hsa_circ_0066954| POLQ–hsa-miR-338-3p—CDH2 shows the downregulation of the driver gene CDH2, which increases the survival of the patients. Since the circRNAs hsa_circ_0051620| SLC1A5 and hsa_circ_0066954| POLQ interact with CDH2, they might have a prognostic value in cancer.

We also found that most of the circRNAs among the seven different cancers are unique, except hsa_circ_0074817| EBF1, which was common between liver cancer and thyroid cancer

and hsa_circ_0001821| circPVT1, which was common between head and neck cancer and gastric cancer. In liver cancer, hsa_circ_0074817| EBF1 targets miR-539-5p, targeting CDK4 and SPAG5. The upregulation of CDK4 triggers the development of non-alcoholic fatty liver disease (NFALD), which leads to the phosphorylation of C/EBPα on Ser193 and the formation of C/EBPα-p300 complexes, resulting in hepatic steatosis, fibrosis, and liver cancer. The overexpression of SPAG5 promoted tumor growth and metastasis, as SPAG5 interacts with CEP55 to trigger the phosphorylation of AKT at Ser473, causing liver cancer. In thyroid cancer, hsa_circ_0074817 targets miR-27a-3p, targeting MET, ABCA1, MMP13, and PLAG1, promoting cell proliferation, invasion, and metastasis in thyroid carcinoma. The circRNA hsa_circ_0001821| circPVT1 has common miRNA targets in HNSCC and gastric cancer, except miRNAs, i.e., hsa-miR-361-3p and hsa-miR-497-5p that are specific in HNSCC and hsa-miR-125 in gastric cancer.

This study acknowledges the fact that these findings are based on computational analysis and remain predictive until validated. Although we have tried to provide information about the mRNAs that are significantly translated into proteins (DEPs), yet the utilization of proteomics data might provide additional information to understand the behavior of the proteins in the regulatory triad. However, the strength of this study lies in the fact that it is the first that specifies the circRNA–miRNA–mRNA triad that might play a role in regulating the downstream process in different cancers based on (1) the positive and negative correlations among the regulatory elements, (2) mRNAs translated to proteins (DEPs), (3) the driver and tumor suppressor triads, and (4) the cluster-specific triads classifying the circRNAs into specific biological processes and functions. The future aspect would be to validate the circRNA–miRNA–mRNA axis and the possible functional roles experimentally. The circRNA sequencing from cell lines and patient samples would validate the *in silico* findings in this study and indicates the abundance of novel circRNAs. We also planned to find the function of circRNAs as miRNA sponges using various assays, including immunoprecipitation, miRNA pull-down assays, and luciferase activity analysis (Wu et al., 2018; Zhang et al., 2019; Lin et al., 2020). This study provides essential pathways enriched in different cancers. A study based on how the circRNAs influencing the cancer pathways, e.g., apoptosis, epithelial-mesenchymal transition (EMT) pathways, and angiogenesis, would help understand their roles in the pathogenesis. We also planned on working toward the development of circRNA-based therapeutic approaches (Holdt et al., 2018) by manipulating the circRNA expression, either knocking down (Wang et al., 2016) or overexpressing it (Zeng et al., 2017). Besides the understanding of circRNAs and work done in this field, which is still minimal at present, there are many other things about circRNA, including circRNA structure, degradation, biogenesis, and interaction with other RNAs, which remain undiscovered. This study is a step toward understanding the world of non-coding RNAs and their mechanisms which remain unexplored.

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

AD, AP, and SK conceptualized the study. SK and AJ performed the investigation and curated the data. AD and AP supervised the workflow. AD and SK prepared the original draft. All authors reviewed, edited, and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.671309/full#supplementary-material

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PBX2-Mediated circTLK1 Activates JAK/STAT Signaling to Promote Gliomagenesis via miR-452-5p/SSR1 Axis

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Glioma is considered one of the most lethal brain tumors, as the aggressive blood vessel formation leads to high morbidity and mortality rates. However, the mechanisms underlying the initiation and progression of glioma remain unclear. Here, we aimed to reveal the role of circTLK1 in glioma development. Our results revealed that circTLK1 is highly expressed in glioma tumor tissues and glioma cell lines. We then conducted a series of experiments that showed that circTLK1 was involved in the progression of gliomas. Mechanistically, investigation of the factors downstream of circTLK1 revealed that circTLK1 activated JAK/STAT signaling in glioma cells. Furthermore, AGO2-RIP, RNA-pull down, and luciferase reporter gene assays led to the identification of the novel circTLK1/miR-452-5p/SSR1 axis. Moreover, we investigated the upstream regulator of circTLK1 and found that circTLK1 expression in glioma cells could be regulated by the transcriptional factor PBX2. Taken together, our findings show that circTLK1 mediated by PBX2 activates JAK/STAT signaling to promote glioma progression through the miR-452-5p/SSR1 pathway. These results provide new insights into glioma diagnosis and therapy.

Keywords: circular RNA, circTLK1, miR-452-5p, SSR1, JAK/STAT signaling, glioma, PBX2

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INTRODUCTION

Glioma is one of the most common subtypes of malignant brain cancers and accounts for approximately 27% of central nervous system tumors (Louis et al., 2016). It is characterized by aggressive blood vessel formation, which leads to high morbidity and mortality (Van Meir et al., 2010; Khasraw et al., 2014). Despite clinical treatment of glioma, such as via surgery, combined chemotherapy, and radiotherapy, which have considerably improved in recent years, outcomes for patients remain unfavorable (Mangiola et al., 2010; Chen and Xu, 2016). Therefore, uncovering the mechanism underlying development of glioma is highly needed.

Circular RNAs (circRNAs) are 200–2,000 bp endogenous RNAs, which are transcribed by RNA polymerase II (Zhang et al., 2018) and forms a covalently closed cyclic structure (Li et al., 2018a). CircRNAs have been found to participate in the progression of multiple pathophysiological

processes, including cancers (Kristensen et al., 2018; Patop and Kadener, 2018; Di Agostino et al., 2020). They play critical roles in many biological processes of tumorigenesis, such as cell viability (Chen et al., 2017), invasion (Song et al., 2019), migration (Ren et al., 2019), and angiogenesis (Jia et al., 2016). Recently, the role of circRNAs in gliomagenesis progression has also been studied. Yang et al. (2018) found that circRNA FBXW7 suppresses glioma tumorigenesis, Xiong et al. (2019) revealed that circRNA circMAN2B2 mediates glioma progression by regulating S100A8 expression through sponging of miR-1205. Barbagallo et al. (2018) demonstrated that circSMARCA5 affects the migration of glioblastoma multiforme cells by facilitating SRSF1/SRSF3/PTB axis activity. These studies suggest an essential role of circRNA in glioma progression.

CircRNA circTLK1, derived from TLK1 messenger RNA (mRNA), was first identified in a renal cell carcinoma study as an oncogene. CircTLK1 upregulates CBX4 expression to promote renal cell carcinoma development by acting as a molecular sponge for miR-136-5p (Li et al., 2020). Subsequently, Wu F. et al. (2019) revealed the promotive effects of circTLK1 in ischemic stroke. The function of circTLK1 in myocardial ischemia/reperfusion injury was demonstrated by Song Y.F. et al. (2020). However, whether circTLK1 participates in glioma progression remains unclear.

Here, we aimed to uncover the role of circTLK1 in the initiation and progression of glioma. We found that circTLK1 expression is upregulated in glioma tissues and cell lines. Furthermore, downregulation of circTLK1 inhibited glioma cellular progress and suppressed cell growth *in vivo*. Investigation of the mechanisms upstream and downstream of circTLK1 revealed that circTLK1 mediated by PBX2 aggravates glioma progression by activating JAK/STAT signaling via the miR-452-5p/SSR1 axis.

MATERIALS AND METHODS

Clinical Samples

Thirty pairs of glioma tissues and normal marched tissues were harvested from patients who were diagnosed with glioma in Huai'an Hospital Affiliated to Xuzhou Medical University, Second People's Hospital of Huai'an City from January 2017 to June 2018 and were stored at −80°C. These patients did not receive radiotherapy or chemotherapy. Informed consent was obtained from all patients. Sample status was confirmed by two pathologists. This study was approved by the Ethics Committee of the Huai'an Hospital Affiliated to Xuzhou Medical University, Second People's Hospital of Huai'an City (No. HEYLL 201928).

Cell Culture and Treatment

Glioma cell lines (T98G, LN229, CRT, U251, M059J, and M059K), normal human astrocytes (NHAs), and 293T cells were purchased from the Cell Bank of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, United States) with 10% FBS (Gibco, Waltham, MA, United States) in a 37°C, 5% CO_2 environment. All plasmids, Sh-NC, Sh-circTLK1#1, Sh-circTLK1#2, OE-NC, and OE-circTLK1#1, were purchased from RiboBio (Guangzhou, China). All transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions.

Animal Experiment

Eight-week-old nude mice were separated into three groups (n = 6), subcutaneously inoculated with U251 cells (10^6 per mouse), which were preinfected with Sh-NC, Sh-circTLK1#1, and Sh-circTLK1#2. After 21 days, tumor samples from mice were harvested. Tumor volumes or end weights were recorded. The animal experiments were approved by the Ethics Committee of the Huai'an Hospital Affiliated to Xuzhou Medical University, Second People's Hospital of Huai'an City.

Quantitative Real-Time Polymerase Chain Reaction

All RNAs from cells or human tissues were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The PARIS^{TM} Kit (Invitrogen, Carlsbad, CA, United States) was use to perform nuclear and cytoplasmic RNA fractionation. The Reverse Transcription Kit (Invitrogen, Carlsbad, CA, United States) was used to reverse transcribe circRNA, mRNA, and RNA into complementary DNA (cDNA). An internal reference was applied using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative real-time PCR (qRT-PCR) assays were conducted using the SYBR-Green Real-Time Kit (Takara, Tokyo, Japan) on a Bio-Rad CFX96 system. The fold expression changes were analyzed using the $2^{-\Delta\Delta Ct}$ method. All primers used in the current study were as follows: circTLK1, F, 5′-ACAGTTTTGGAAGCTTGGGATCT-3′ and R, 5′-TGCTCCCACTTGCAACTCCA3′; miR-452-5p, F, 5′-TCGGCAATCATGATGGGCTCCTC-3′ and R, 5′-CTCA ACTGGTGTCGTGGAGTC-3′; SSR1, F, 5′-AAGAACTACAAA ACCGCCCC-3′ and R, 5′-ATCCCAGGCTGAGACCCAT-3′; PBX2, F, 5′-CCCATGTCATGAACCTGCTG-3′ and R, 5′-GC GCTGAACTTTCGATGGAT-3′; GAPDH, F, 5′-AAGGT CGGAGTCAACGGATTT-3′ and R, 5′-ACCAGAGTTAAAAG CAGCCCTG-3′.

Fluorescence *in situ* Hybridization Assay

The Cy3-labeled circTLK1 probe was synthesized and commercially obtained from RiboBio (Guangzhou, China) to localize circTLK1 expression in NHAs. A fluorescence *in situ* hybridization kit (Geneseed, Guangzhou, China) was used to perform fluorescence *in situ* hybridization (FISH) assay following the manufacturer's instructions. The cells were imaged using a fluorescence microscope (Leica, Wetzlar, Germany).

Western Blot

All proteins were isolated using radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China). Bicinchoninic acid (BCA) (Beyotime, Shanghai, China) was used to quantify

the proteins. The proteins were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, United States). The membranes were blocked with 5% skim milk at 37°C for 90 min. Next, membranes were incubated at 4°C overnight with primary antibodies as follows: JAK1 (CST; 1:1,000; 29261S), p-JAK1 (CST; 1:1,000; 74129S), STAT1 (CST; 1:1,000; 14994S), p-STAT1 (CST; 1:1,000; 9167S), STAT3 (CST; 1:1,000; 8768S), p-STAT3 (CST; 1:2,000; 9145S), SSR1 (Sigma; 0.4 μg/ml; HPA017062), and GAPDH (CST; 1:1,000; 5174S). Subsequently, the membranes were washed with Tris-buffered saline with Tween 20 (TBST) and incubated with secondary antibodies for 2 h. Protein bands were visualized using ECL (Beyotime, Shanghai, China).

Hematoxylin and Eosin Staining

Mouse tumor tissues were immersed in 10% neutral formalin for 1 day under sterile conditions. Next, the tumor samples were dehydrated, embedded in paraffin, and cut into 4-μm-thick sections. H&E staining was performed via a standard procedure.

CCK-8 Assay

Cell proliferation assays were conducted using a cell counting kit 8 (CCK-8) solution (Dojindo, Kumamoto, Japan). Cells were cultured in a 96-well plate and incubated with the solution for 3 h. Absorbance at 450 nm was measured using a Thermomax microplate reader (Molecular Devices, CA, United States). All assays were performed at least three times.

Transwell Assay

Cell migration was measured using a Transwell chamber (Corning Inc., Corning, NY, United States) with an 8.0-μm pore size polycarbonate membrane. Cells (10^5 per chamber) were cultured in up-chambers (matrix for invasion assay) for 24 h, after which cells were removed from the chamber. The cells on the reverse side of the chamber were fixed using 4% paraformaldehyde and then stained with crystal violet. The migrated cells were visualized using an IX71 inverted microscope (Olympus, Tokyo, Japan).

Scratch Wound Healing Assay

U251 and CRT cells, upon indicated transfections, were seeded in a six-well plate for culturing at 37°C in a 5% CO_2 environment. The fine end of a 10-μl pipette tip was used to create a scratch wound. The cells were imaged using phase-contrast microscopy at the indicated times.

Biotinylated RNA Pull-Down

Biotinylated circTLK1 probes, miR-452-5p probes, and normal control probes were obtained from RiboBio (Guangzhou, China). Coimmunoprecipitation buffer (Beyotime) was used to lyse the cells. Probe-coated beads were constructed using the coincubating biotinylated probe and C-1 magnetic beads (Life Technologies, CA, United States) for 120 min. The probes were then incubated with cell lysates overnight. TRIzol reagent was used for RNA isolation, and qRT-PCR assays were performed to analyze the RNA complexes.

AGO2-RNA Binding Protein Immunoprecipitation

RIP assays were performed using a Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Germany) with anti-AGO2 and anti-IgG following the manufacturer's instructions. The complexes were evaluated using qRT-PCR assays.

Chromatin Immunoprecipitation

First, glycine was used to terminate the crosslinking reaction in cells with 1% formaldehyde. Subsequently, the bound RNA-protein was subjected to sonication for fragment production. Next, the antibodies were added, and the proteinated A-Sepharose beads were applied to the immunoprecipitated fractions. The results were analyzed using qRT-PCR analysis.

Luciferase Reporter Assays

The wild-type (WT) or mutant-type (Mut) sequences of circTLK1, circTLK1 promoter, or SSR1 were subjected to PmirGLO dual-luciferase vectors and then transfected into U251 and 293T cells with NC mimics miR-452-5p mimics or PcDNA 3.1/PcDNA 3.1-PBX2. After 24 h, the luciferase activities in U251 or 293T cells were detected using a dual-luciferase reporter assay system (Promega, WI, United States). Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical Analysis

Statistical analyses were performed using the SPSS 20 software (SPSS, Chicago, IL, United States). Results are shown as mean ± SD. Results in two different groups were calculated using unpaired Student's *t*-test, and results among three or more groups were analyzed using one-way ANOVA. All assays were conducted at least thrice. Spearman analysis was used to evaluate the association of expression in the samples. Statistical significance was set at $p < 0.05$.

RESULTS

Measurement of circTLK1 in Gliomas

First, to uncover the role of circTLK1 in glioma, we assessed circTLK1 expression levels in 30 pairs of human glioma tissues. It was found that circTLK1 expression in glioma tumors was markedly higher than in normal matched tissues (**Figure 1A**). As shown in **Figure 1B**, circTLK1 expressed in T98G, LN229, CRT, U251, M059J, and M059K cells was higher than that in NHAs, and circTLK1 was abundantly expressed in U251 and CRT cells. Furthermore, we assessed the circRNA characteristics of circTLK1. The linear TLK1 (mTLK1) expression was significantly downregulated in U251 and CRT cells treated with RNase R; however, the expression of circTLK1 remained stable (**Figure 1C**). Subsequently, circTLK1 was found to be more stable than mTLK1 in U251 cells treated with the transcription inhibitor actinomycin D (**Figure 1D**). Random hexamer and oligo (dT)18 primers were used to amplify the TLK1 RNAs. As shown in **Figure 1E**, TLK1 mRNA expression was significantly upregulated but not that of the circular RNA. Moreover, nuclear-cytoplasmic fractionation assays (**Figure 1F**) and FISH analysis

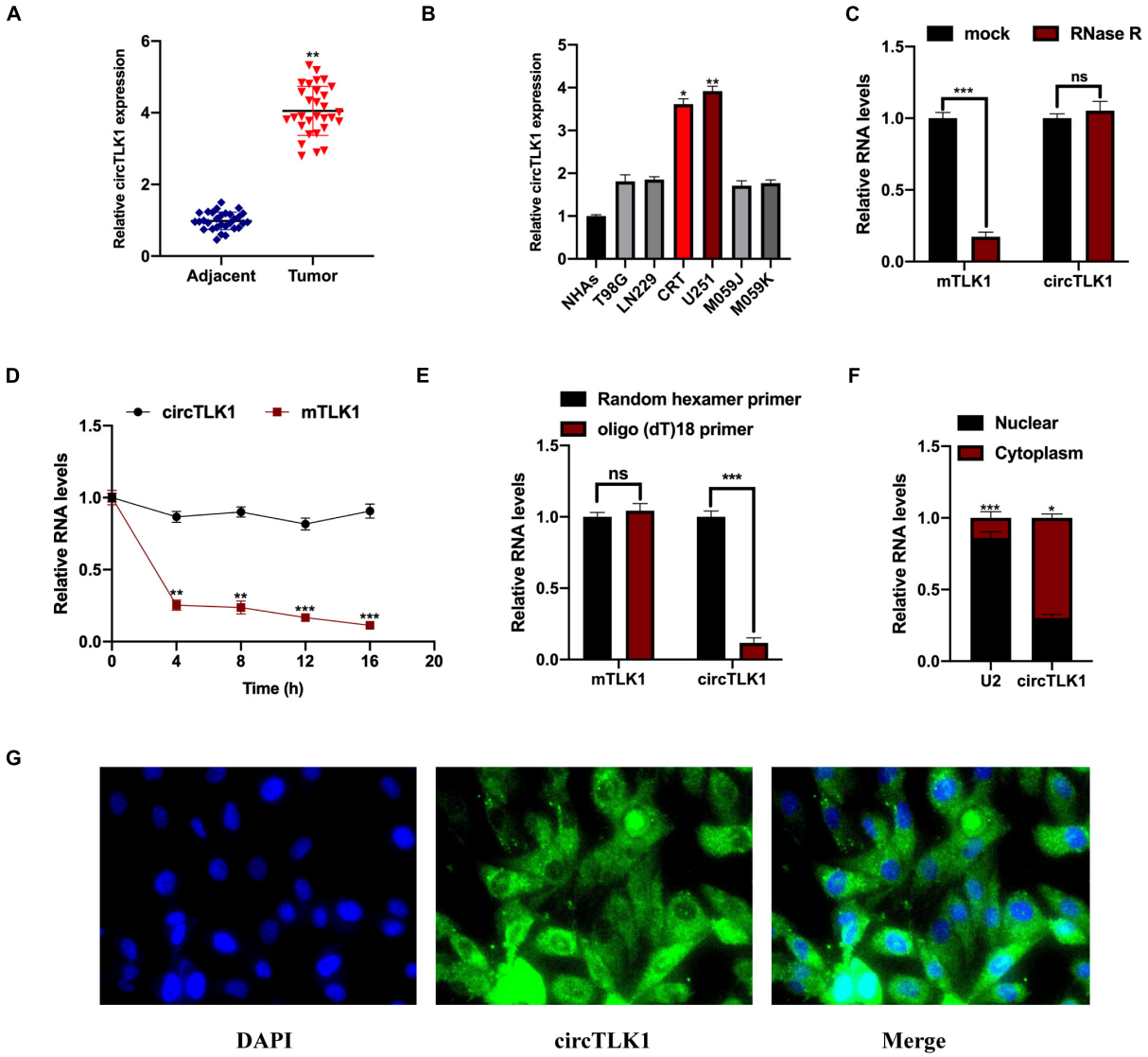

FIGURE 1 | Measurement of circTLK1 in gliomas. **(A)** Determination of the circTLK1 expression in 30 pairs of glioma tumor tissues and matched normal tissues using qRT-PCR. **(B)** qRT-PCR was used to measure circTLK1 expression in glioma cells T98G, LN229, CRT, U251, M059J, M059K, and normal human astrocytes NHAs. **(C)** Relative RNA levels were analyzed using RT-qPCR; the mock group was used for internal normalization. **(D)** U251 cells were treated with the transcription inhibitor actinomycin D to evaluate mTLK1 and circTLK1 stability. **(E)** Relative RNA levels analyzed using RT-qPCR in U251 cells; random hexamer primers were used for internal normalization. **(F)** The level of circTLK1 in the nuclear and cytoplasm of NHAs was measured using a nuclear-cytoplasmic fractionation assay. **(G)** FISH analysis was conducted to detect the distribution of circTLK1 in NHAs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figure 1G**) revealed that circTLK1 was primarily distributed in the cytoplasm. These findings suggests that circTLK1 may be involved in glioma development.

Downregulated circTLK1 Inhibits Glioma Cell Proliferation, Migration, and Invasion

Here, we aimed to uncover the biological function of circTLK1 in glioma progression. Sh-NC, Sh-circTLK1#1, and Sh-circTLK1#2 were constructed and transfected into U251 and CRT cells. The relative expression of circTLK1 in U251 and CRT cells was measured (**Figure 2A**), and it was found that downregulated circTLK1 significantly inhibited cell proliferation (**Figure 2B**). Furthermore, our results showed that downregulated circTLK1 suppressed glioma cell migration and invasion (**Figures 2C–E**). Taken together, the results show that circTLK1 was involved in glioma progression by mediating increased cell viability, migration, and invasion of glioma cells *in vitro*.

Downregulated circTLK1 Inhibits Glioma Cell Growth *in vivo*

Next, we evaluated the function of circTLK1 in a xenograft nude mouse model. U251 cells (10^6 per mouse), which were

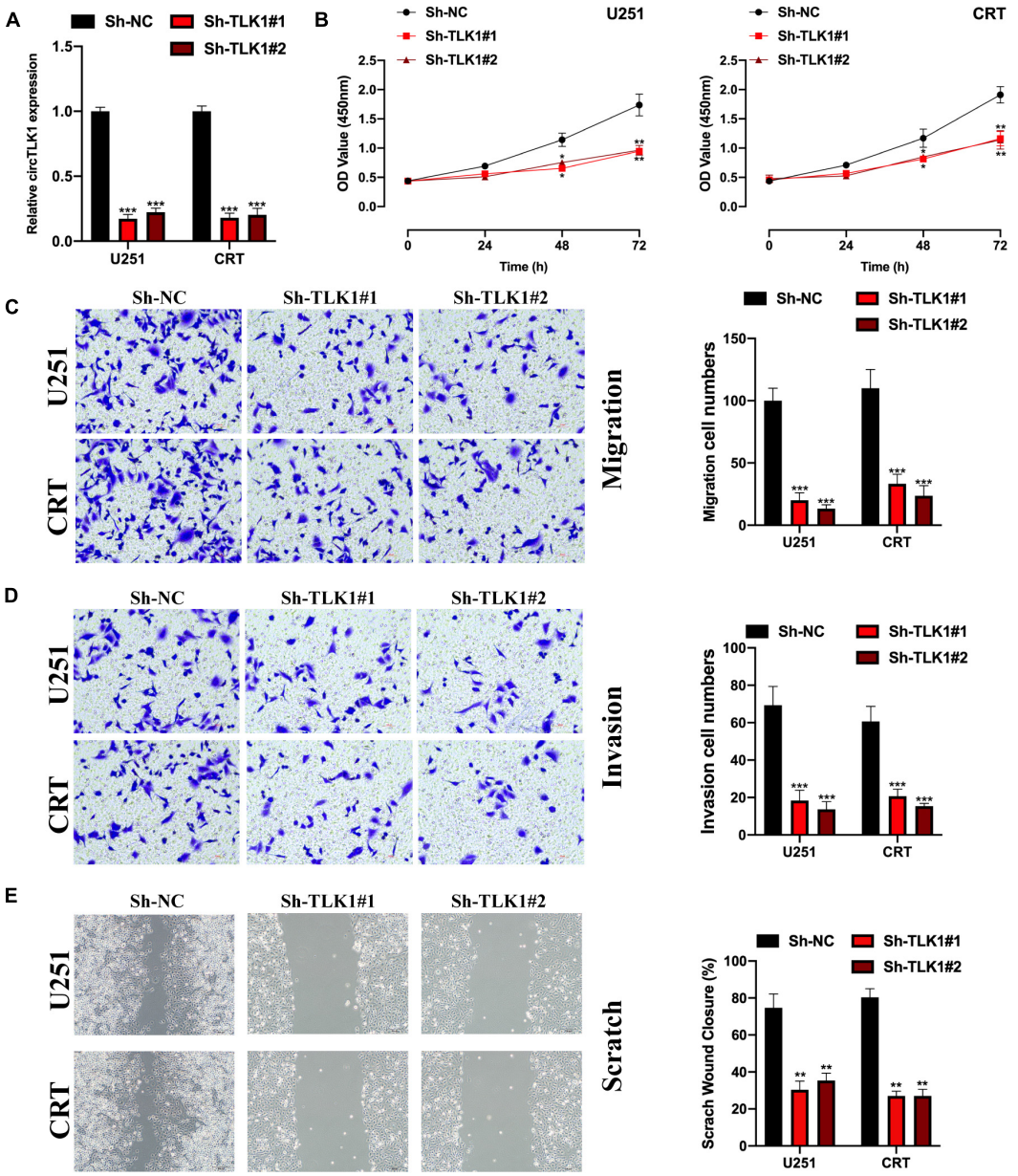

FIGURE 2 | Downregulated circTLK1 inhibits glioma cell proliferation, migration, and invasion. **(A)** Transfection efficiencies of Sh-NC, Sh-circTLK1#1, Sh-circTLK1#2 in U251, and CRT detected using qRT-PCR. **(B)** CCK-8 assay assessing the cell proliferation abilities. **(C)** Transwell migration assay was performed to measure the cell migration levels. **(D)** Transwell invasion experiment was applied to detect cell invasion abilities. **(E)** Scratch test was applied to evaluate the migration ability of transfected cells. Comparative statistics are shown here. $^{**}p < 0.01$, $^{***}p < 0.001$.

preinfected with Sh-NC, Sh-circTLK1#1, and Sh-circTLK1#2, were injected into nude mice. As shown in **Figure 3A**, representative images of subcutaneous tumors indicated that knockdown of circTLK1 suppressed tumor growth *in vivo*. Tumor weights and volumes were recorded, and comparative statistics were analyzed (**Figures 3B,C**). HE staining assays were performed, and the expression of proliferation biomarker Ki67 was assessed in mouse tumor tissues by immunohistochemistry (IHC) staining. The level of Ki67 was obviously decreased in circTLK1 knockdown tumors (**Figure 3D**). These results indicated that downregulation of circTLK1 suppressed glioma cell growth *in vivo*.

circTLK1 Modulates Gliomagenesis via Activating JAK/STAT Signaling

Previous studies have suggested that JAK/STAT signaling plays a role in glioma progression (Tu et al., 2011; Zhang et al., 2019; Swiatek-Machado and Kaminska, 2020). To explore whether circTLK1 regulates glioma progression through

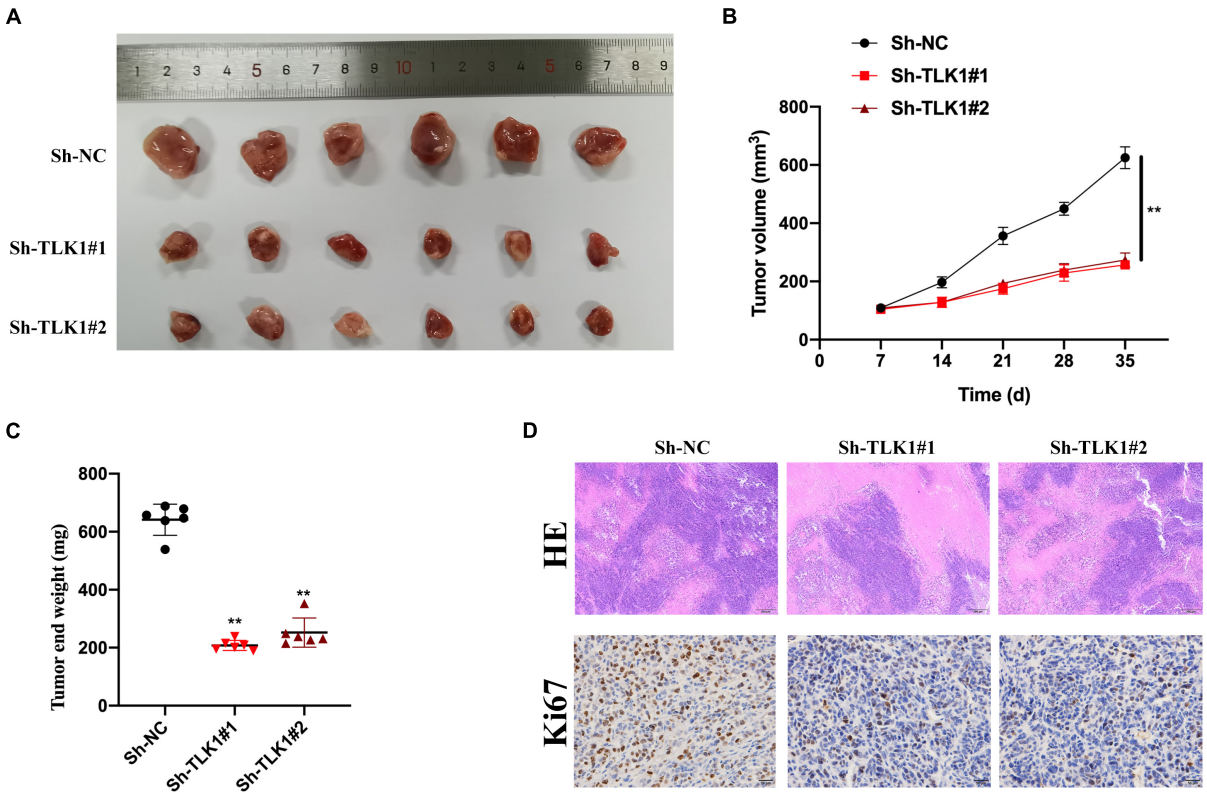

FIGURE 3 | Downregulated circTLK1 inhibits glioma cell growth *in vivo*. Mice were separated into three groups (n = 6) and then subcutaneously injected with U251 (10^6 per mouse) cells preinfected with Sh-NC, Sh-circTLK1#1, and Sh-circTLK1#2. **(A)** Representative image of subcutaneously incubated tumors (n = 6). **(B)** Tumor volumes were recorded every 3 days, and statistical results are presented. **(C)** Tumor end weights were recorded, and comparative statistics are shown. **(D)** Tumor histology was assessed using H&E staining (scale bar, 200 μm), and the proliferation marker Ki67 was detected using IHC staining (scale bar, 50 μm). $^{**}p < 0.01$.

JAK/STAT signaling, we constructed OE-NC and OE-circTLK1 and transfected them into M059K and M059J cells. CircTLK1 expression was measured in the transfected cells (**Figure 4A**). Western blot analysis revealed that p-JAK1, p-STAT1, and p-STAT3 expression were significantly increased upon circTLK1 overexpression, while JAK1, STAT1, and STAT3 expression remained unchanged (**Figures 4B,C**). Moreover, glioma cell models were generated by transfecting OE-NC, OE-circTLK1, and JAK/STAT inhibitor cyt387 as indicated. Overexpression of circTLK1 in M059K and M059J cells promoted cell proliferation (**Figures 4D,E**), migration (**Figures 4F,G,J,K**), and invasion (**Figures 4H,I**), but these phenomena were rescued by treatment with the JAK/STAT inhibitor cyt387. The above results indicate that circTLK1 promotes gliomagenesis by regulating JAK/STAT signaling.

circTLK1 Regulates SSR1 Expression Through Sponging miR-452-5p

First, we assessed the mRNA binding ability of circTLK1 in U251 cells. AGO2-RNA binding protein immunoprecipitation (AGO2-RIP) assays were performed, and it was found that circTLK1 was abundantly enriched in AGO2 antibody complexes in comparison with anti-IgG (**Figure 5A**), which showed that circTLK1 acts as a competitive endogenous RNA (ceRNA) in gliomagenesis. Then, to understand the mechanisms underlying circTLK1 in gliomagenesis, downstream factors were investigated via bioinformatics analysis. Three putative mRNAs were selected, and biotinylated RNA pull-down assays were performed. As shown in **Figure 5B**, in comparison with bio-NC, miR-452-5p expression was found to be significantly higher than that of other putative mRNAs, suggesting that miR-452-5p might bind to circTLK1. The predicted binding sites between miR-452-5p and circTLK1 (WT or MUT) were synthesized (**Figure 5C**). The correlation between miR-452-5p and circTLK1 was assessed using a luciferase reporter gene assay. Luciferase activities in 293T and U251 cells preinfected with miR-452-5p mimic and vectors harboring circTLK1 WT sequences were evidently decreased (**Figures 5D,E**). Subsequently, miR-452-5p expression levels in U251 and CRT cells, infected with Sh-NC, Sh-circTLK1#1, and Sh-circTLK1#2, were detected. Knockdown of circTLK1 upregulated miR-452-5p expression (**Figure 5F**). Moreover, miR-452-5p expression was inhibited by circTLK1 (**Figure 5G**) and downregulated in glioma tumor tissues (**Figure 5H**). The miR-452-5p expression in glioma tumor tissues was significantly correlated with circTLK1 (**Figure 5I**).

Next, we explored the target mRNA of miR-452-5p using the PITA and DIANA-microT databases. The expression of

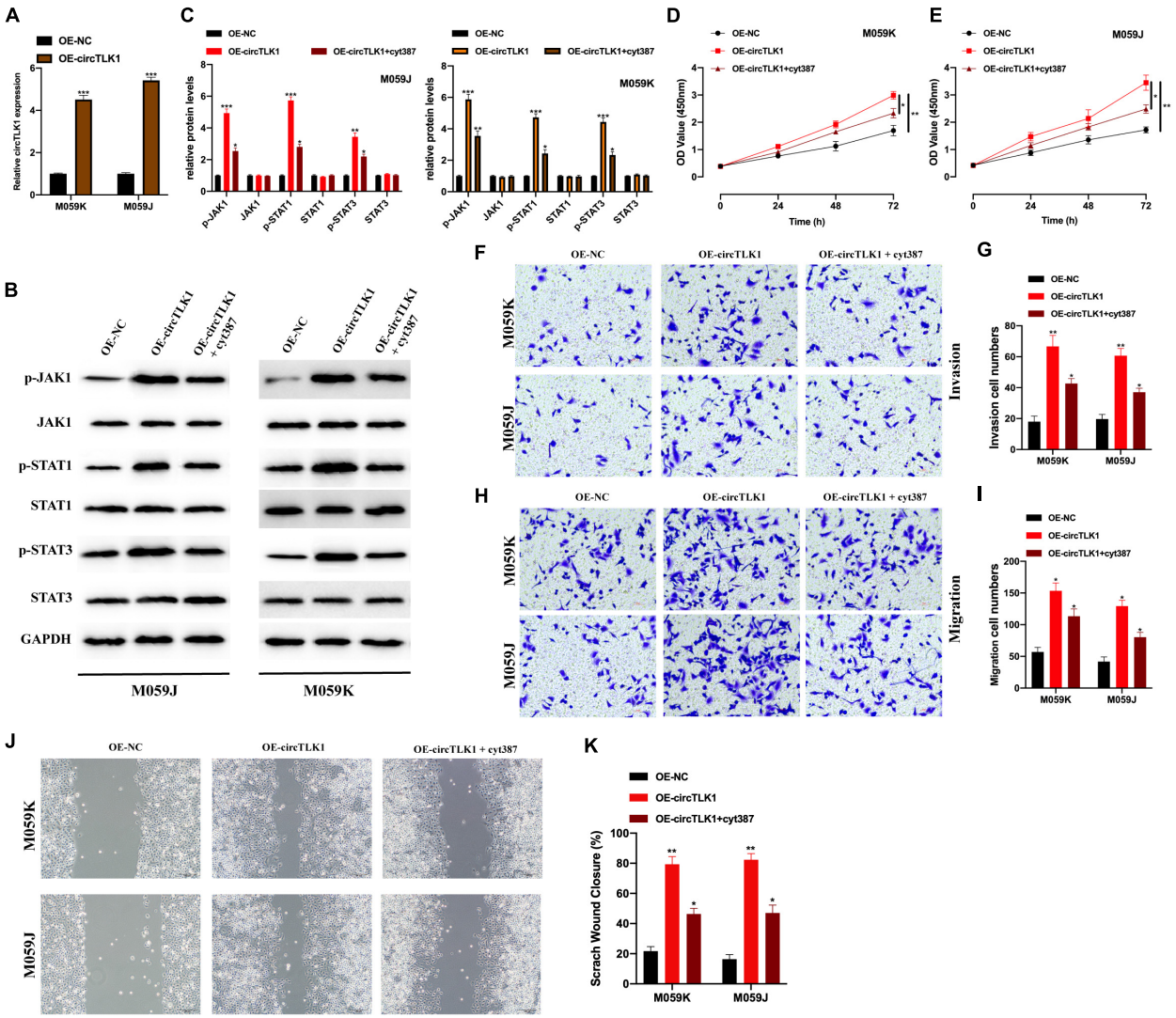

FIGURE 4 | CircTLK1 modulates gliomagenesis by activating JAK/STAT signaling. M059K and M059J cells were transfected with OE-NC or OE-circTLK1. **(A)** M059K and M059J cells were subjected to qRT-PCR to detect transfection efficiencies. **(B,C)** The expression of JAK/STAT pathway proteins in M059K and M059J cells, which were pretransfected with OE-NC, OE-circTLK1, and JAK/STAT inhibitor cyt387 as indicated, measured using Western blot, and comparative statistics are presented. **(D,E)** CCK-8 assays were conducted to measure the proliferation levels. **(F,G)** Treated M059K and M059J cells were subjected to a Transwell migration experiment to measure the cell migration level. **(H,I)** Transwell invasion assays were applied to detect cell invasion levels of treated M059K and M059J cells. **(J,K)** Scratch test was applied to evaluate the migration ability of transfected cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

potential mRNA targets in biotinylated probes transfected into U251 cells was assessed; the SSR1 expression level was significantly higher in these cells than in the other groups (**Figure 6A**). The binding sites between SSR1 (WT or MUT) and miR-452-5p are presented in **Figure 6B**. Subsequently, we found that luciferase activity in 293T and U251 cells preinfected with miR-452-5p mimic and a vector containing SSR1 WT sequences were markedly decreased (**Figures 6C,D**). The SSR1 expression level was significantly inhibited by circTLK1 knockdown but reversed by miR-452-5p inhibition (**Figures 6E–G**). This indicated that SSR1 was a downstream target of miR-452-5p and positively mediated by circTLK1. Furthermore, we found that SSR1 expression in glioma tumor tissues was downregulated and correlated with miR-452-5p or circTLK1 expression (**Figures 6H–J**).

circTLK1 Promotes Gliomagenesis via miR-452-5p/SSR1/JAK/STAT Pathway

We further explored whether circTLK1 mediates JAK/STAT signaling via the miR-452-5p/SSR1 axis. For this purpose, we constructed glioma knockdown cell models and evaluated the expression of SSR1, and JAK/STAT pathway proteins in these cell models were measured (**Figures 7A,B**). Subsequently, the biological functions of each cell model were assessed. It was found that overexpression of SSR1 inhibited the suppressive

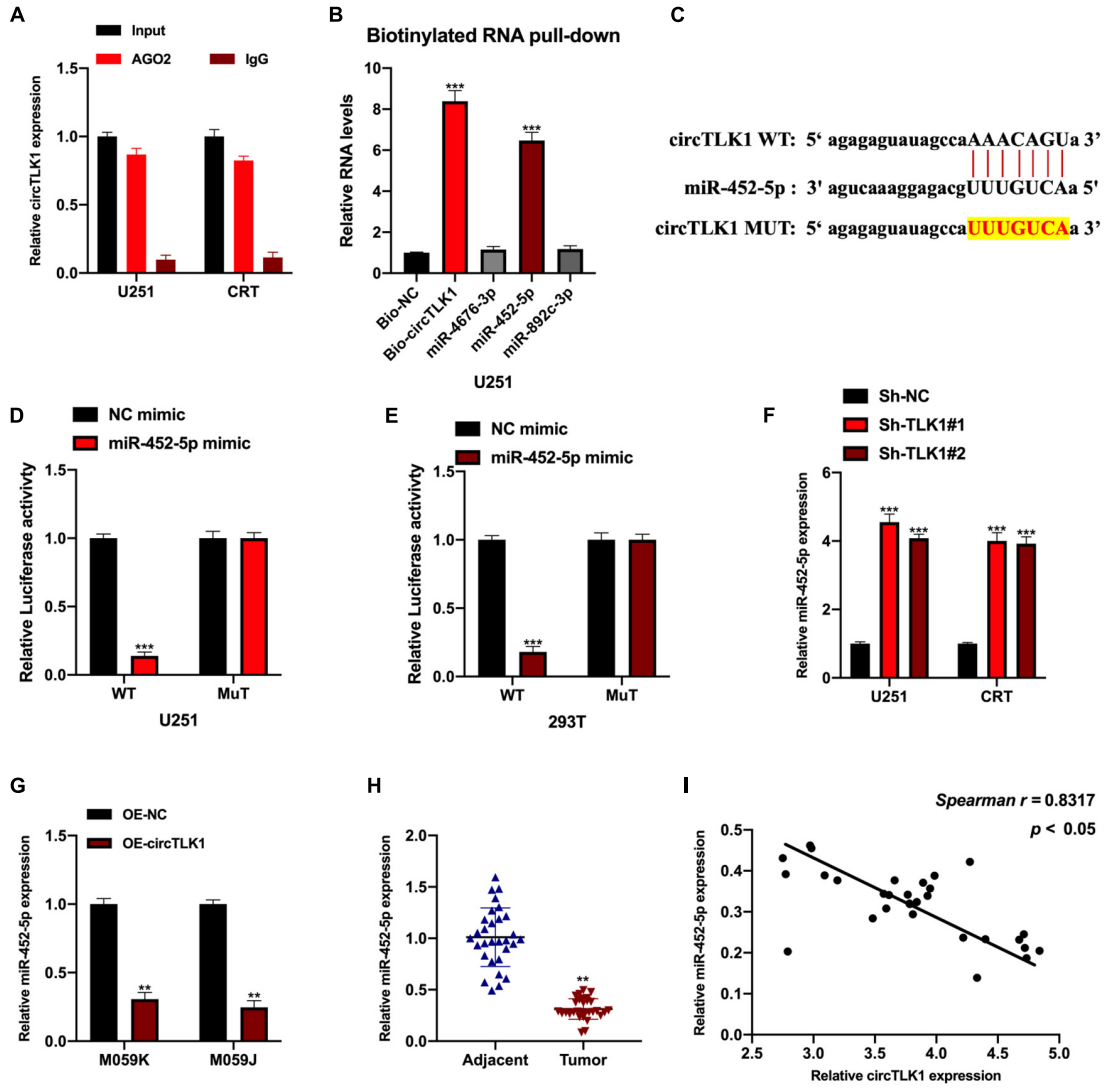

FIGURE 5 | CircTLK1 sponges to miR-452-5p. ENCORI database (http://starbase.sysu.edu.cn/index.php) with CLIP data, strict stringency (≥ 5), and degradome data, medium stringency (≥2) **(A)** mRNA binding ability of circTLK1 was detected using AGO2-RIP assays in U251 cells. **(B)** qRT-PCR was performed to assess relative RNA expressions in U251 cells. **(C)** The binding sites between miR-452-5p and circTLK1 (WT or MUT). **(D,E)** Relative luciferase activities in NC mimic, miR-452-5p mimic, and reporter vectors containing circTLK1 WT and Mut sequences transfected into 293T and U251 cells were measured. **(F)** Relative miR-452-5p expression in U251 and CRT cells pretreated with Sh-NC, Sh-circTLK1#1, and Sh-circTLK1#2 was measured using qRT-PCR. **(G)** miR-452-5p expression in U251 and CRT cells pretreated with OE-NC and OE-circTLK1 was evaluated using qRT-PCR. **(H)** The miR-452-5p expression level in glioma tissues was detected using qRT-PCR. **(I)** The circTLK1 and miR-452-5p expression association was calculated using Spearman analysis. $^{**}p < 0.01$, $^{***}p < 0.001$.

effects of Sh-circTLK1 on cell proliferation (**Figure 7C**), migration (**Figures 7D,E,H,I**), and invasion (**Figures 7F,G**), but these phenomena were rescued by treatment with the JAK/STAT inhibitor cyt387. This suggested that circTLK1/miR-452-5p/SSR1 participates in glioma progression by mediating JAK/STAT signaling.

circTLK1 Expression Is Mediated by PBX2

CircRNA expression could be regulated by transcriptional factors (Wang et al., 2018; Wu L. et al., 2019; Lv et al., 2020), which is an important mechanism in circRNA functional patterns. Here, we further investigated the mechanisms upstream of circTLK1 in gliomagenesis. Using the JASPAR[1] dataset, we identified three transcriptional regulators (ZNF460. ZNF135, and PBX2) that may mediate circTLK1 expression. Subsequently, we constructed dysregulation cell models of each regulator and measured the expression levels of circTLK1 in these cells. As shown in **Figures 8A–C**, circTLK1 expression was upregulated in pre-B-cell leukemia transcription factor 2 (PBX2)-overexpressing cells and downregulated in PBX2 knockdown cells. Furthermore,

[1]http://jaspar.genereg.net/

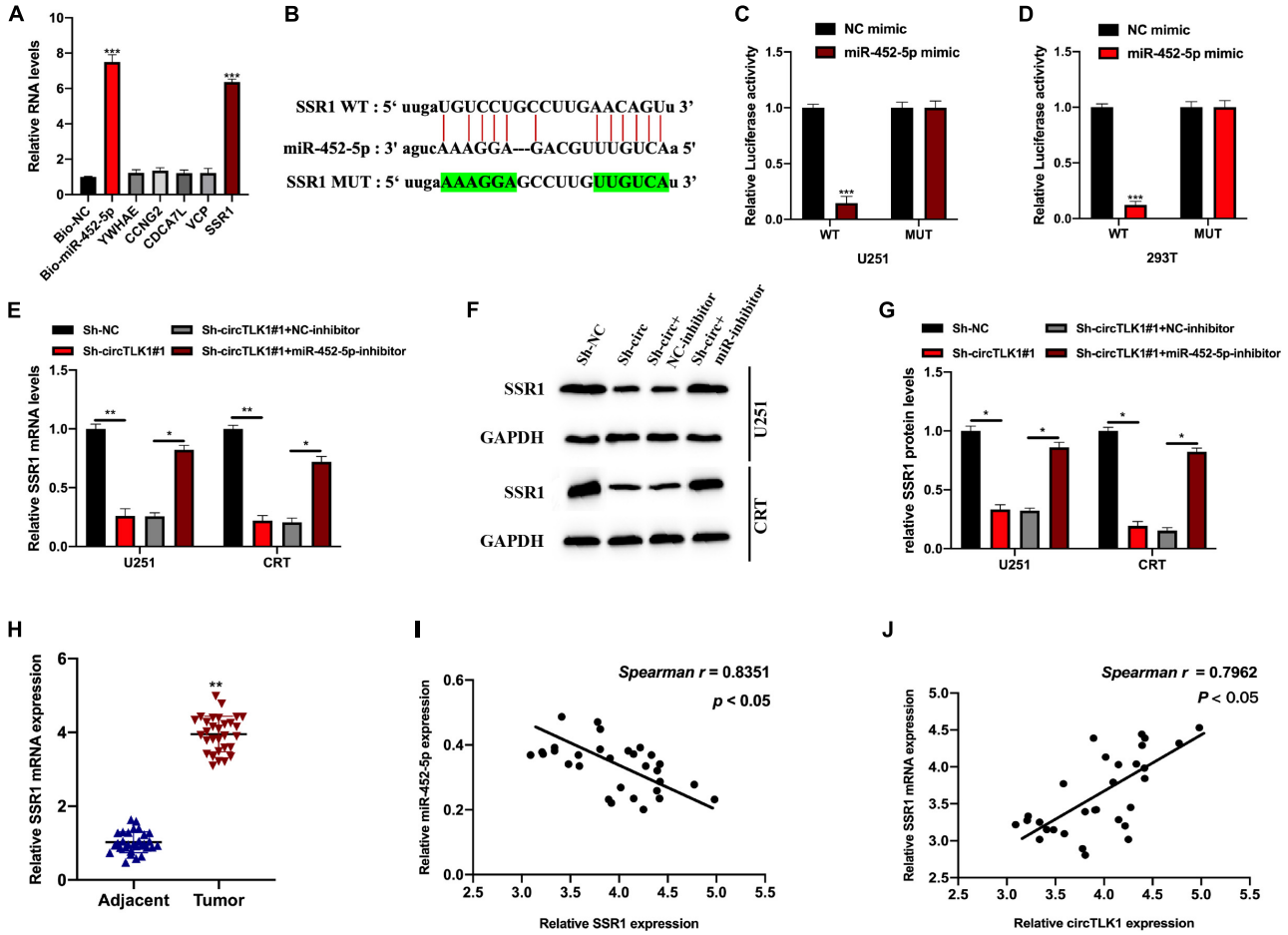

FIGURE 6 | CircTLK1 regulates SSR1 expression through sponging miR-452-5p. PITA database (http://genie.weizmann.ac.il/pubs/mir07/mir07_dyn_data.html) and DIANA-microT (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) with CLIP Data, strict stringency (≥5), and degradome data, high stringency (≥3), was used. **(A)** qRT-PCR was used to assess potential binding mRNAs expression and bio-miR-452-5p expression in U251 cells. **(B)** The WT and MUT binding sites between SSR1 and miR-452-5p. **(C,D)** Relative luciferase activities in NC mimic, miR-452-5p mimic, and reporter vectors containing SSR1 WT and Mut sequences transfected into 293T and U251 cells were measured. **(E–G)** The protein and mRNA expressions of SSR1 in U251 and CRT pretreated with Sh-NC, Sh-TLK1#1, Sh-TLK1#1 + NC-inhibitor, and Sh-TLK1#1 + miR-452-5p-inhibitor were measured using qRT-PCR and Western blot. All experiments were performed in triplicates. **(H)** SSR1 expression in glioma tissues was assessed using qRT-PCR. **(I,J)** Spearman analysis was used to calculate the association between SSR1 and circTLK1 or miR-452-5p. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

chromatin immunoprecipitation (ChIP) assay results showed that TLK1 was markedly enriched after treatment with anti-PBX2 antibody as to isotype control (**Figure 8D**). Our results suggest that PBX2 might be at functional upstream regulator of circTLK1 in glioma cells. The predicted binding sequences between the TLK1 promoter and PBX2 were obtained from the JASPAR dataset (**Figures 8E,F**). The interaction between the TLK1 promoter and PBX2 was assessed using a luciferase reporter assay. As shown in **Figures 8G,H**, PBX2 overexpression promoted the activity of the wild type (WT) of the TLK promoter, while this phenomenon was attenuated in each mutant type (Mut) of the TLK promoter. Furthermore, the promotive effect of PBX2 overexpression on the TLK1 promoter was completely abrogated when the site 1 and 2 binding sequences were mutated. Our results indicated that circTLK1 expression in glioma cells could be mediated by the transcriptional regulator PBX2.

DISCUSSION

The findings of this study revealed that circTLK1 mediated by PBX2 regulated JAK/STAT signaling to promote glioma development by facilitating miR-452-5p/SSR1. First, it was found that that circTLK1 was expressed at higher levels in glioma tissues than in matched normal tissues. The circular RNA characteristic of circTLK1 was assessed. CircTLK1 is abundantly expressed in glioma cells. These results suggest that circTLK1 may be involved in glioma development.

Subsequently, the biological functions of circTLK1 in glioma cell lines were assessed. It was found that circTLK1 knockdown suppressed glioma cell progression by performing CCK-8, Transwell, and scratch assays. Then, animal models were constructed, and our results suggested that downregulated circTLK1 suppressed tumor cell growth *in vivo*. We noticed that

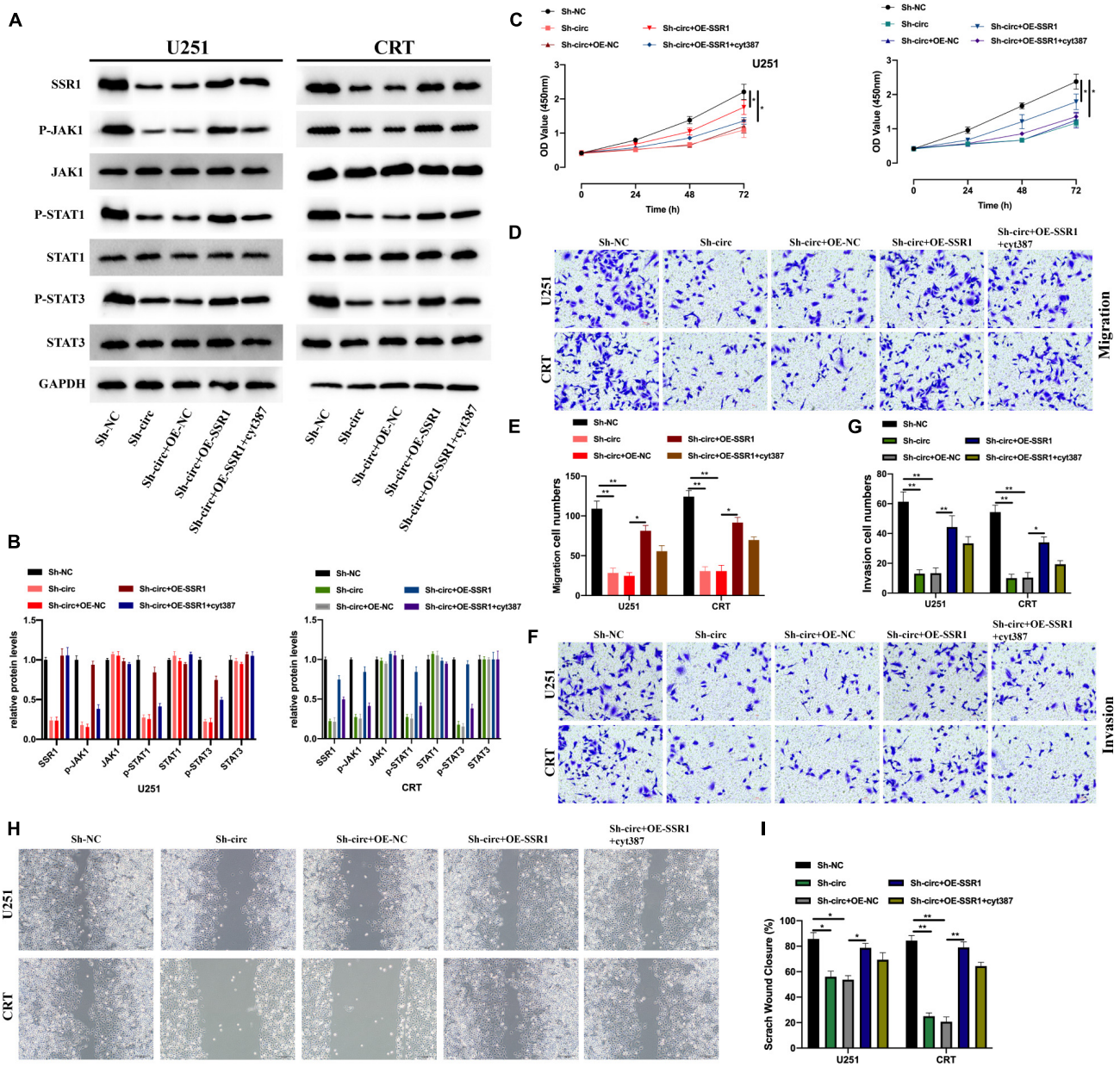

FIGURE 7 | CircTLK1 promotes gliomagenesis via the miR-452-5p/SSR1/JAK/STAT pathway. **(A,B)** The expressions of SSR1 and JAK/STAT pathway proteins in Sh-NC, Sh-circTLK1#1, Sh-circTLK1#1 + OE-NC, Sh-circTLK1#1 + OE-SSR1, and Sh-circTLK1#1 + OE-SSR1 + cyt387 transfected into U251 and CRT cells were detected using Western blot, and comparative statistics were analyzed. **(C)** CCK-8 was used to measure the proliferation levels. **(D,E)** Transwell migration assay was applied to measure migration abilities of treated U251 and CRT cells. **(F,G)** Transwell invasion assay was applied to assess cell invasion abilities of treated U251 and CRT cells, and comparative statistics are presented. **(H,I)** The migration level of transfected cells was detected using the scratch test, and results were analyzed as indicated. $^{*}p < 0.05$ and $^{**}p < 0.01$.

JAK/STAT signaling plays a crucial role in glioma progression, and we presumed that circTLK1 plays a role in mediating JAK/STAT signaling in glioma progression. The expressions of p-JAK1, p-STAT1, and p-STAT3 in circTLK1-overexpressing cells were upregulated, suggesting that JAK/STAT signaling was activated by circTLK1. Moreover, the promotive effects of circTLK1 on glioma cellular progress were rescued by treatment with the JAK/STAT signaling inhibitor cyt387. Our results imply that circTLK1 participates in glioma progression by activating JAK/STAT signaling.

Previous studies have demonstrated that miR-452-5p participates in multiple biological processes, such as colorectal cancer (Yan et al., 2020b), gastric cancer (Zhu et al., 2020), hepatocellular cancer (Yang et al., 2020), prostate cancer (Song X. et al., 2020), and renal cancer (Zhai et al., 2018). However, the role of miR-452-5p in gliomas has not been previously reported.

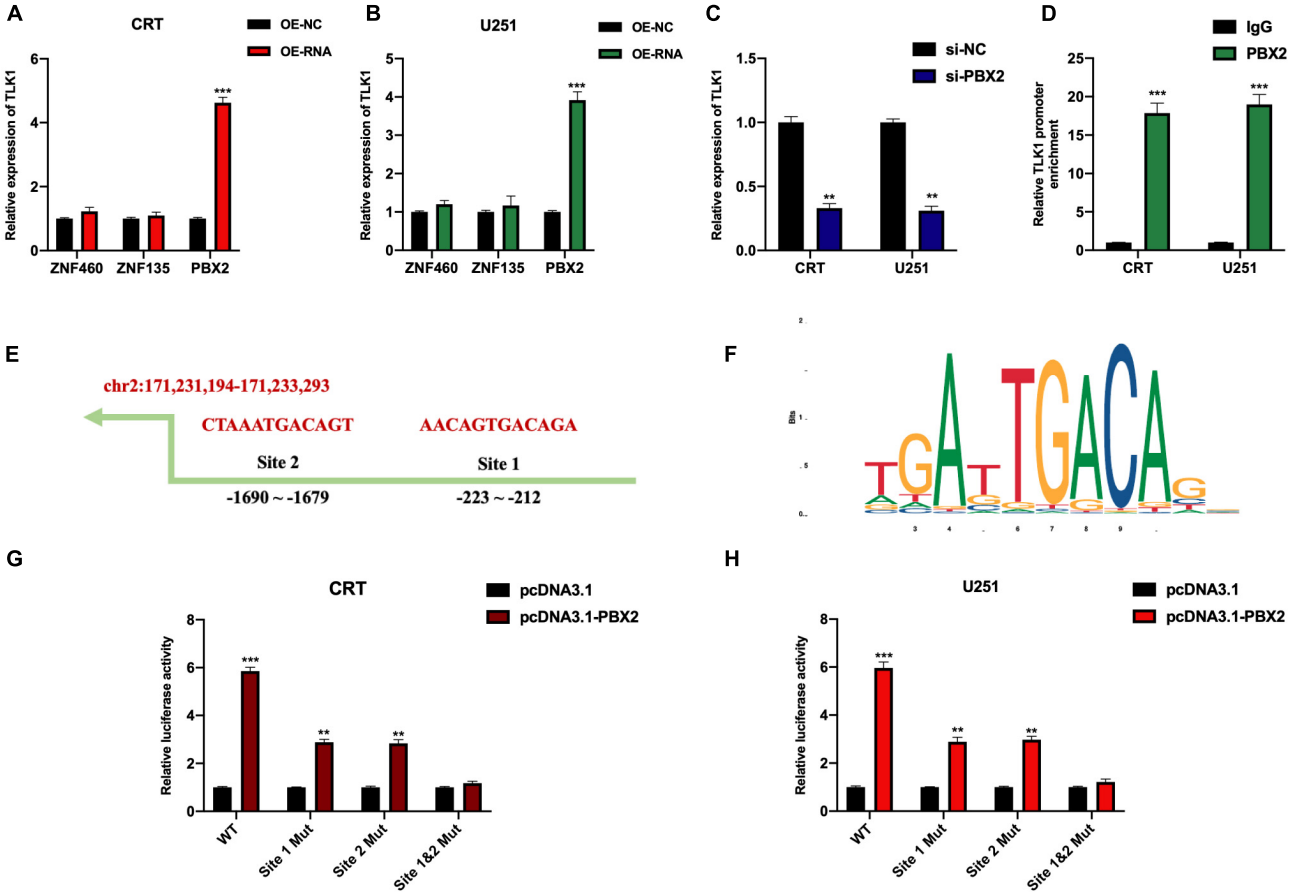

FIGURE 8 | CircTLK1 expression is mediated by PBX2. CRT and U251 cells were stably infected with OE-NC, OE-RNAs, si-NC, and si-PBX2, as indicated. **(A,B)** Relative expression of circTLK1 in **(A)** CRT and **(B)** U251 cells pretreated with OE-NC and OE-RNAs was measured using qRT-PCR. **(C)** Relative expression of circTLK1 in CRT and U251 cells pretransfected with si-NC and si-PBX2 was detected using qRT-PCR. **(D)** ChIP assay was performed using anti-IgG and anti-PBX2; results were detected using the qRT-PCR assay. **(E)** The predicted binding sites of circTLK1 promoter were obtained from the JASPAR dataset. **(F)** The predicted binding sequence of PBX2 was collected from the JASPAR dataset. **(G,H)** The impact of PBX2 on circTLK1 transcription in **(G)** CRT and **(H)** U251 cells was assessed using luciferase reporter assay. $^{**}p < 0.01$, $^{***}p < 0.001$.

Our results revealed that circTLK1 sponges miR-452-5p and negatively mediates miR-452-5p expression. Signal sequence receptor subunit 1 (SSR1) was found to be a downstream target of miR-452-5p in glioma cells. SSR1 was found to be associated with hypopharyngeal squamous cell carcinoma (Yan et al., 2020a) and breast cancer (Funakoshi et al., 2019). However, whether SSR1 participates in glioma progression remains unclear. Our results revealed that miR-452-5p directly targeted SSR1 and suppressed its expression in glioma cells. Furthermore, JAK/STAT signaling was found to be mediated by the circTLK1/miR-452-5p/SSR1 axis.

The expression of circRNAs can be regulated by transcription factors. Lv et al. (2020) demonstrated that circ-MMP2 expression in lung adenocarcinoma cells is induced by FOXM1 and that the transcription factor c-FOS could bind to the promoter region of circPVT1 and promote circPVT1 expression in non-small cell lung cancer cells (Li et al., 2018b). Wang et al. (2018) demonstrated that circ-4099 expression in intervertebral disk degradation is regulated by TNF-α-induced GRP78. Transcription factor-induced expression is an important pattern of circRNAs in various biological processes. In this study, we have partially revealed the downstream mechanisms affected by circTLK1 in gliomagenesis, and we further investigated the upstream regulator of circTLK1. By utilizing the JASPAR dataset, ChIP, and luciferase reporter assays, we identified that PBX2 could bind to the circTLK1 promoter and mediate circTLK1 expression in glioma cells.

Although we partially demonstrated the existence of the novel PBX2/circTLK1/miR-452-5p/SSR1 axis in glioma progression, this will require extensive investigation. Our clinical results require additional samples for further confirmation. Moreover, the relationship between SSR1 and JAK/STAT signaling needs to be verified.

In conclusion, we partially elucidated the role of circTLK1 in glioma progression. CircTLK1 mediated by PBX2 regulates JAK/STAT signaling to promote glioma development via the miR-452-5p/SSR1 axis. Our results provide novel diagnostic and therapeutic targets for treating glioma.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Huai'an Hospital Affiliated to Xuzhou Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of the Huai'an Hospital Affiliated to Xuzhou Medical University. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JL, YW, and RY: conception, design, supervision, resources, manuscript writing, and revising. ZZ and XW: experiments conduction and data analysis. QM and HJ: data visualization and statistical analysis. All authors contributed to the article and approved the submitted version.

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