# A comprehensive review of circRNA: from purification and identification to disease marker potential

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**Subheadings**

Disease marker potential; methods of detection and characterization

**Abstract:** Circular RNA (circRNA) is an endogenous noncoding RNA with a covalently closed cyclic structure. Based on their components, circRNAs are divided into exonic circRNAs, intronic circRNAs and exon-intron circRNAs. CircRNAs have well-conserved sequences and often have high stability due to their resistance to exonucleases. Depending on their sequence, circRNAs are involved in different biological functions, including microRNA sponge activity, modulation of alternative splicing or transcription, interaction with RNA-binding proteins, and rolling translation, and are a derivative of pseudogenes. CircRNAs are involved in the development of a variety of pathological conditions, such as cardiovascular diseases, diabetes, neurological diseases and cancer. Emerging evidence has shown that circRNAs are likely to be new potential clinical diagnostic markers or treatments for many diseases. Here we describe circRNA research methods and biological functions as well as discusses the potential relationship between circRNAs and disease progression.

## 1. Introduction

Circular RNA (circRNA) was considered as a class of endogenous noncoding RNA (ncRNA) (Wilusz & Sharp 2013), but it is now considered that circRNA can be translated into functional polypeptides (Legnini et al. 2017; Pamudurti et al. 2017; Yang et al. 2017b; Yang et al. 2018b). Unlike linear ncRNA, circRNA is formed with different combinations of sequences and domains, and it can be divided into three categories, namely exonic circRNA (ecRNA) (Zhang et al. 2014), circular intronic (ciRNA) (Zhang et al. 2013)and exon-intron circRNA (ElciRNA) (Li et al. 2017b)(Table 1 and Fig. 1). Similar to other ncRNAs, the sequence and structure of circRNA determine its biological functions. CircRNA is mainly located in the cytoplasm and is highly stable compared to other ncRNAs (Danan et al. 2012). In addition, recent research has shown that the lengths of mature circRNA dictate the mode of nuclear export (Huang et al. 2018). CircRNA is abundantly expressed and evolutionarily conserved across eukaryotic organisms (Morris & Mattick 2014; Wang et al. 2014), and it plays critical roles in many diseases, including nervous system disorders, cardiovascular diseases (CVDs), diabetes and cancer (Burd et al. 2010; Wang et al. 2017b). CircRNA governs gene expression through guiding a number of other

64    molecules, such as splicing factors, RNA polymerase II (Jeck et al. 2013), nuclear small ribose

65    nucleoprotein (snRNP) (Huang & Shan 2015; Li et al. 2017b) and miRNAs (Li et al. 2015a). These

66    interactions promote or inhibit the transcription of the corresponding mRNA.

67

68    **Survey Methodology**

69    Analysis: Through extensive literature searches, the role of circRNA in diseases and the

70    methods of circRNA detection and characterization were analyzed, indicating the importance of

71    circRNA and its research prospects, including our previous research results combined with

72    other research results.

73    **2. Biogenesis of CircRNA**

74        CircRNA does not have terminal structures, such as a 5' end cap and 3' end poly (A) tail,

75    which are covalently closed to form a circular structure (Jeck et al. 2013). Jeck and colleagues

76    proposed two different models of exon circularization. One model is intron-pairing-driven

77    circularization (Fig. 2a), and the other model is lariat-driven circularization (Fig. 2b) (Jeck et al.

78    2013). In the first mechanism, the two introns that flank the exon or exons of the incipient

79    circRNA have a complementary structure to bind to each other. The pairing of the flanking

80    introns brings the splice sites close to each other, shaping a secondary structure that makes

81    back-splicing possible. In the second mechanism, a pre-mRNA is spliced, and two transcripts are

82    produced as follows: an mRNA from which one or more exons are missing; and a lariat

83    consisting of the skipped exons, which makes the circularization possible. The exon lariat is

84    spliced one more time to generate two other elements, namely a circRNA and an intron lariat.

85    Typically, many lasso structures are formed by introns, but they are degraded by the branching

86    enzyme (Rodriguez-Trelles et al. 2006). In these two typical models of circularization, ALU

87    complementary flanking elements (retrotransposons characterized by the action of the

88    Arthrobacter luteus (Alu) restriction endonuclease) repeated in intronic regions compete with

89    canonical linear-RNA splicing and act as an inevitable accelerator in the formation of circRNA by

90    reverse complementary matches (Ashwal-Fluss et al. 2014; Hansen et al. 2013a; Ivanov et al.

91    2015; Jeck et al. 2013). DExH-Box Helicase 9 (DHX9) is an RNA helicase that specifically binds to

92    reverse Alu elements (IRAlu) to guide the formation of circRNA (Aktas et al. 2017). IRAlu has

93    already become a significant basis of analyzing and forecasting the formation mechanism of

94    circRNA (Zhang et al. 2014). In addition, the genomic structure of long exons flanked by long

95    introns harboring inverted repeat elements facilitates RNA circularization (Jeck et al. 2013).

96    Many proteins are involved in circRNA biogenesis. In normal growing cells, NF90/NF110 binds

97    to A/U-rich elements (including base paired Alu elements) in the introns flanking many exons

98    that yield circular RNAs, promoting back-splicing events (Li et al. 2017a). HNRNPL promotes

99    circRNA formation via back splicing (Fei et al. 2017). The RNA-binding proteins, such as MBL

100   (muscleblind) (Ashwal-Fluss et al. 2014) and QKI (RNA-binding protein quaking I) (Conn et al.

101   2015), also participate in the back-splicing process and cyclization of RNA. Interestingly, high

102   levels of MBL bind to its own pre-mRNA and determine its back-splicing, leading to the

103   inhibition of canonical splicing, decreasing MBL levels and upgrading circMBL (Ashwal-Fluss et al.

104   2014). Monomeric QKI binds to both ends of intron flanking sites and combines to form cyclic

105   exons by bringing the two cyclic shear sites close (Conn et al. 2015). FUS regulates circRNA

106   biogenesis by binding the introns flanking the back-splicing junctions (Errichelli et al. 2017).

107   CircRNA production is further controlled by FUS (Errichelli et al. 2017) and by multiple hnRNP

108   (heterogeneous nuclear ribonucleoprotein) and SR (serine-arginine) proteins (Fei et al. 2017;

109   Kramer et al. 2015; Liang et al. 2017). In contrast, the RNA-editing enzyme, ADARs (Adenosine

110   deaminases acting on RNA) block circRNA formation by binding to complementary double-

111   stranded areas of flanking introns and abolishing the interaction of double-stranded chains

112   (Ivanov et al. 2015). Recent research has found that inhibition or slowing of pre-mRNA

113   processing mechanisms, such as spliceosomes, leads to profound increases in circRNA

114   production by extending read through to downstream genes and production of circRNA (Liang

115   et al. 2017).

**3. Properties of CircRNA**

117   CircRNA has several unique features and properties when compared to other linear RNAs

118   and ncRNAs. Most of the unique features are generated from exons, while few others are

119   generated from introns or intron fragments (Cocquerelle et al. 1993). Several circRNAs possess

120   microRNA response elements (MREs), which enable them to interact with miRNAs to govern

121   target gene expression (Hansen et al. 2013a; Yang et al. 2016). Many circRNAs are derived from

122   pre-mRNA and regulate their own gene expression predominantly at posttranscriptional levels

123   (Salzman et al. 2012). Generally, circRNAs show tissue-specific and/or developmental stage-

124   specific expression patterns similar to those of corresponding linear mRNA targets, and their

125   expression level is >10 times higher than that of the linear mRNA (Jeck et al. 2013; Memczak et

126   al. 2013). CircRNA exists and has been detected in many types of extracellular body fluids, such

127   as saliva, blood and urine (Jeck et al. 2013; Qu et al. 2015). More than 400 circRNAs have been

128   found in human cell-free saliva (CFS) from healthy individuals (Bahn et al. 2015). CircRNAs have

129   evolutionary conserved sequence features across different species (Rybak-Wolf et al. 2015).

130   The covalently closed loop structures lacking 5'-3' polarity and without poly-adenylated tail

131   favor resistance to RNA exonuclease degradation (Suzuki & Tsukahara 2014). CircRNA plays

132   stable biological roles because the average half-life of circRNA in most species is much longer

133   than its linear counterpart (Bahn et al. 2015; Memczak et al. 2013).

134   **4. Function of CircRNA**

135   CircRNA has a variety of functions, including modulation of alternative splicing or

136   transcription, regulating the expression of parental genes, interacting with RNA-binding

137   proteins (RBPs), altering RBP activity, miRNA sponge activity, rolling circle translation and

138   generating pseudogenes.

139   *4. 1 CircRNA Modulates Alternative Splicing or Transcription*

140   CircRNA participates in the regulation of alternative splicing and transcription, thereby

141   controlling gene expression (Fig. 3a). For example, circMbl is generated from the second exon

142   of the splicing factor MBL, which competes with canonical pre-mRNA splicing, while circMbl and

143   its flanking introns have conserved MBL-binding sites to allow binding to MBL. Interestingly, the

144   alteration of MBL level significantly affects circMbl formation, and this effect depends on MBL-

145   binding sites in the flanking intronic sequences (Ashwal-Fluss et al. 2014). Studies have shown

146   that several circRNAs are abundantly found in the nucleus where they regulate transcriptional

147   activity by interacting with polymerase II and homeopathic reactions. For instance, EIciRNA

148   interacts with small nuclear ribonuclease proteins (snRNPs) to regulate the transcription of

149  parental genes in a homeopathic manner (Chen 2016). Li et al. found that circRNA-ITCH

150  interacts with miR-7, miR-17 and miR-214 as well as upregulates the expression of ITCH (Li et al.

151  2015a). During embryogenesis, sisR-4 promotes transcription of its host gene by activating an

152  enhancer present in the intron where sisR-4 is encoded, which is essential for development (Tay

153  & Pek 2017). HNRNPL directly regulates the alternative splicing of RNAs, including encoding the

154  androgen receptor, the key lineage-specific prostate cancer oncogene (Fei et al. 2017).

155  *4. 2 CircRNA Interacts with RBPs*

156      Apart from miRNA regulation, circRNA can sequester RBPs and thus control the

157  intracellular localization and transport of RBPs and associated mRNAs (Hentze & Preiss 2013;

158  Jeck & Sharpless 2014) (Fig. 3b). Some circRNAs combine with RBPs and ribonucleoprotein

159  complexes, thereby inhibiting their activity. However, circRNA functions as stores of RBPs and

160  ribonucleoprotein complexes. EcRNA acts as a scaffold by specifically binding to protein

161  molecules to provide an interaction platform for RNA-binding proteins, RNA and DNA. For

162  example, in HEK293 cells, CDR1as contains a region near perfectly complementary to miR-671,

163  which causes the circRNA to be endonucleolytically cleaved by argonaute 2 (AGO2) in a miR671-

164  dependent manner (Hansen et al. 2011). CDR1as is derived from an antisense long noncoding

165  RNA (Barrett et al. 2017) and is expressed several orders of magnitude higher than cerebellar

166  degeneration-related protein 1 (CDR1) gene from the opposite strand (Hansen et al. 2011;

167  Piwecka et al. 2017). Chen et al. found that there are cellular differential mechanisms in the

168  recognition of internal and external circRNAs as follows: external circRNA induces activation of

169  RIG-I-mediated cellular autoimmune effector pathways, and endogenous circRNA does not

170  induce this pathway due to binding of RBPs (Chen et al. 2017d).

171  *4. 3 CircRNA as MiRNA Sponge*

172      CircRNAs act as competing endogenous RNAs (ceRNAs) that contain shared MREs by which

173  they sequester miRNAs and prevent their interactions with target mRNAs (Fig. 3c).

174  Systematically validated circRNAs, such as ciRS-7 (CDR1as) (Hansen et al. 2013a; Memczak et al.

175  2013) and Sry circRNA (circSry) (Hansen et al. 2013a; Zhao & Shen 2015), are produced from the

176  mRNAs of cerebellar degeneration associated protein 1 (CDR1) and dysregulated rat testis SRY,

177 respectively. During the embryonic developmental process in zebrafish, the expression of

178 CDRlas reduces brain volume, thereby hampering brain development. However, exogenous

179 delivery of miR-7 reverses the brain volume reduction and reinstates normal brain

180 development, indicating that CDRlas blocks miR-7 by sponging functions (Hansen et al. 2013a;

181 Hansen et al. 2013b). New research has found that the CDR1as sequence overlaps the lncRNA

182 LINC00632 sequence (Barrett et al. 2017). In general, there are only several circRNAs containing

183 enough miRNA-binding sites to function as strong sponges, and other circRNAs are exceptional

184 cases (Chen 2016; Tay & Pek 2017). Knockdown of circHIPK2 expression significantly inhibits

185 astrocyte activation via regulation of autophagy and endoplasmic reticulum (ER) stress through

186 targeting MIR124-2HG and SIGMAR1 (Huang et al. 2017). CircHECW2 plays a role in the

187 epithelial-mesenchymal transition (EMT) pathway by competitively inhibiting miR-30D, which

188 releases ATG5, thereby promoting the Notch1 signaling pathway (Yang et al. 2018a).

189 *4. 4 Rolling Circle Translation*

190 In eukaryotic cells, cyclic mRNA can be translated by typical translation machineries

191 because it contains an internal ribosome entry site (IRES) sequence, and it can bind directly to

192 the ribosome (Thompson 2012) (Fig. 3d). In prokaryotic cells, such as E. coli, circRNA contains a

193 well-conserved infinite open reading frame (ORF) system, which enables the translation of

194 circularized RNA (Abe et al. 2015). In eukaryotic systems, some circRNAs have binding sites for

195 ribosomal 40S subunits, thus initiating translation, which has been demonstrated both in vivo

196 and in vitro studies (Holdt et al. 2017). In an E. coli system, circRNA with green fluorescent

197 protein (GFP) inserted in the open reading frame can successfully translate GFP (Wang & Wang

198 2015). Interestingly, circRNA also drives protein translation by methylation of adenosine N6

199 (m6A) (Yang et al. 2017b). Protein translated by circRNA can act synergistically with the protein

200 expression products of the parent gene and function together. For example, circ-FBXW7

201 translates a new protein that inhibits glioma (Yang et al. 2018b). Circ-ZNF609 directly translates

202 into proteins that participate in muscle formation (Legnini et al. 2017). In prokaryotic cells,

203 proteins are generated from circRNA by means of rolling circle amplification (RCA) analogous to

204 a polymerase reaction in the eukaryotic translation system, which reveals that there is no need

205 for multiple binding of translational machinery to the RNA template (Rodriguez-Trelles et al.

206  2006). The circular amplification not only produces long and repetitive peptide sequences but

207  also increases the productivity of the linear counterpart (Thompson 2012).

208  *4. 5 Generate Pseudogenes*

209  Studies have shown that stable circulatory molecules can be reverse transcribed and

210  integrated into the genome to form circRNA-derived pseudogenes (Dong et al. 2016) (Fig. 3e).

211  Bioinformatics analysis of the mouse genome using computational pipeline (CIRCpseudo) found

212  that at least 33 pseudogenes are possibly derived from the same circRNA at the ring finger and

213  WD repeat domain 2 (RFWD2) locus (circRFWD2) and that 9 of the pseudogenes are from exons

214  (exons 2 to 4 or 5) of circRFWD2. It is well documented that pseudogenes play an important

215  role in cell differentiation and in cancer progression (Kalyana-Sundaram et al. 2012).

216

217  **5 Methods of CircRNA Detection and Characterization**

218  *5. 1 Preliminary Purification and Identification*

219  5. 1. 1 Molecular biology method

220  The loop structure of circRNA has high stability compared to linear RNA, and it is resistance

221  to enzyme digestion (You & Conrad 2016). Therefore, an enzymatic digestion method can be

222  used for the preliminary purification and identification of circRNA (Jeck & Sharpless 2014).

223  First, the processing of extracted RNA with exonucleases, such as RNase R, nicotinic acid

224  phosphatase and 5' end exonuclease, destroys most linear RNA, but circRNA remains intact due

225  to no open ends in circRNA for these enzyme reactions. A circRNA-specific divergent primer can

226  be used to amplify abundant circRNA in which linear RNAs do not amplify (Jeck & Sharpless

227  2014; Suzuki et al. 2006). Second, the migration velocity of circRNA is slower than that of long

228  linear RNA due to lack of polarity at the end. Particularly, circRNA migration is much slower

229  than RNA from homologous gene transcription in weak crosslinked gels, and this difference

230  helps to detect circRNAs easily through Northern blot analysis (Tabak et al. 1988). Third, the

231  fluorescence in situ hybridization technique can locate circRNA at a subcellular level (Li et al.

232  2017b; Zhang et al. 2013). As circRNA does not have a poly (A) structure, the traditional oligo dT

233   enrichment method using a Ribo-Zero kit to remove rRNA is not effective. The removal of linear

234   RNA using RNase R is the most effective step for the enrichment of circRNA and generating a

235   circRNA library (Ebbesen et al. 2016; Jeck & Sharpless 2014).

236   5. 1. 2 High-throughput sequencing

237   The traditional RNA-Seq technique does not distinguish circRNAs from linear RNAs (You &

238   Conrad 2016). As a result, improvements have been made to detect and validate circRNA. First,

239   as the intergenic exon rearrangement has different forms, generation of divergent primers with

240   boundary combinations can form circRNA candidate sequence, which can be used to compare

241   to sequencing data (Salzman et al. 2013). Second, bioinformatics analysis of whole genome

242   sequence and assessment of sequence data through different sequence alignment algorithm

243   can be used to identify circRNAs (Jeck et al. 2013). Third, templates designed with multiple

244   sequence splice joints can directly detect circRNAs from cDNA sequence (Hoffmann et al. 2014).

245   Currently, many algorithms are available for the prediction and study of circRNAs, including

246   Acfs (You & Conrad 2016), FUCHS (Metge et al. 2017) and CIRI2 (Gao et al. 2017). Acfs allows

247   accurate and fast identification of circRNA, and it also determines the abundance of circRNAs

248   from single- and paired-ended RNA-Seq data. Acfs is well suitable for a wide spectrum of

249   applications, including characterizing the landscape of circRNA from a variety of organisms. The

250   FUCHS system is based on long sequencing reads (> 150 bp/reads), which detects circRNA

251   within the variable shear and provides other information for more accurate interpretation.

252   CIRI2 uses the maximum likelihood estimate based on multiple seed matches to identify reverse

253   splice junctions, and it filters out false positives and mapping errors derived from the repetitive

254   sequence. CIRI2 has a significant balance of sensitivity, reliability, duration and RAM usage

255   (Ebbesen et al. 2016; Jeck & Sharpless 2014).

256   5. 1. 3 Gene chip

257   The human genome array, U133plus2.0 tool, can detect mRNA but cannot detect ring RNA

258   because the probe is designed for linear RNA (Lu et al. 2017). Therefore, the human genome

259   array cannot effectively distinguish circRNA and linear RNA when a normal probe is used.

260   However, if the probe is designed based on the reverse splice site of the circRNA, this array tool

261  can specifically detect circRNA because there is no reverse splice site sequence in linear RNA,

262  thus effectively distinguishing circRNA and mRNA (Li et al. 2018).

263  5. 1. 4 Primer Design

264   Recently, the field of circRNA research is gaining more attention because circRNAs

265  contribute to many physiological and pathological processes. Unlike conventional PCR primers,

266  the design of circRNA primers should consider certain criteria. For the detection of ecRNA,

267  primers should be designed for the cross-cut site (back-splice). In the case of ciRNA, primers

268  targeting cross-cleavable sites should be used. Primers can also be designed around intron

269  regions. Moreover, the length of the amplified product should not be more than 100 bp.

270  Sequence position transformation is also important (Panda & Gorospe 2018). The differences in

271  the selection of primers for linear RNA and circRNA are listed in Fig. 4.

272  The actual amplification effect after primer design needs to be experimentally determined.

273  If the quantification of circRNA is performed by qPCR, the length of amplification should be

274  identified according to the experimental requirements of qPCR. Thus, qPCR remains the most

275  widely used technique to assess the expression level of circRNA.

276  *5. 2 CircRNA Research Database*

277  In recent years, increasing numbers of circRNA research tools with different aspects and

278  improved functional analysis have been generated. The currently available online databases for

279  the detection and characterization of circRNA, which contain GenBank annotations or circRNA

280  from published articles, are presented here. Each database analyzes circRNA with different

281  characteristic features for detection, and each database provides abundant information for

282  circRNAs. There are several free online databases available for circRNA research as shown in

283  Table 2.

284  **6. CircRNA as a disease marker in disease progression**

285  The best-known circRNA, CDR1as, is the inhibitor of miR-7, and it is the critical ncRNA

286  known to be involved in various diseases, including cancer, neurodegenerative diseases,

287  diabetes, and atherosclerosis (Peng et al. 2015). In addition more functions of circRNAs being

288  revealed, the underlying relationships between circRNAs and various diseases have been

289  rapidly elucidated. The great specificity and conservation of circRNAs in various tissues add a

290  further dimension to the discovery of these disease biomarkers. CircRNAs involved in diseases

291  are listed in Table 3.

292  *6.1 CircRNA in Cardiovascular Diseases (CVD)*

293  CVD poses an increasing threat to human health. According to a report from the World

294  Health Organization (WHO), nearly 17.5 million people die of CVD each year (Mendis et al.

295  2015). CircRNA is highly specific and is exclusively expressed in different tissues, including

296  vascular and heart tissue (Fan et al. 2017).

297  6.1.1 Pathological hypertrophy and heart failure (HF)

298  MiR-223 is an endogenous regulator of hypertrophy in cardiomyocytes, which can induce

299  cardiac hypertrophy and heart failure (Wang et al. 2015b). In cardiac hypertrophy, ARC

300  (apoptosis repressor with CARD domain) is a miR-223 downstream target (Wang et al. 2016).

301  The heart-related circRNA (HRCR) can function as an endogenous miR-223 sponge to inhibit

302  miR-223 activity, subsequently increasing the expression of ARC (Wang et al. 2016). However,

303  the expression of HRCR is decreased during cardiac hypertrophy and heart failure. Thus, it is

304  speculated that increased expression of HRCR attenuates the development of cardiac

305  hypertrophy and heart failure, and it may be an attractive therapeutic target for cardiovascular

306  disorders associated with pathological hypertrophy (Wang et al. 2016).

307  6.1.2 Atherosclerosis

308  Circular ANRIL RNA (circular antisense noncoding RNA in the INK4 locus, cANRIL) is an

309  antisense transcript from the INK4A/ARF (cyclin-dependent kinase 4 inhibitor, INK4a;

310  alternative reading frame, ARF) locus (Salzman et al. 2013), which inhibits the expression of

311  INK4/ARF (Burd et al. 2010). Another research group found that cANRIL prevents rRNA

312  prebinding and exonuclease-mediated rRNA maturation by binding to the C-terminal lysine-rich

313  domain of PES1, inducing an increase in the expression and activity of p53 and subsequent

314  decrease in apoptosis. This pathway inhibits atherosclerosis by eliminating hyperproliferative

315    cell types in atherosclerotic plaques, indicating that cANRIL may be associated with the

316    prevention or treatment of atherosclerosis (Holdt et al. 2016).

317    6.1.3 Cardiac senescence

318        CircFoxo3 is generated from Foxo3, a member of the forkhead family of transcription

319    factors that is highly expressed in aged heart samples from elder patients. The expression of

320    circFoxo3 is highly correlative with markers of cellular senescence (Du et al. 2016a).

321    Experimental studies have found that cells expressing high levels of circFoxo3 are unable to

322    transition to S phase, revealing that circFoxo3 represses cell cycle progression and cell

323    proliferation (Du et al. 2016b). CircFoxo3 is mainly distributed in the cytoplasm where it

324    interacts with several transcription factors (E2F1, FAK, and HIF1a) and antisenescence proteins,

325    such as ID-1, thus preventing their nuclear entry. In fact, the nuclear entry of FAK and HIF1a is

326    essential for their antisenescence role. Thus, circFoxo3 repress their antiaging effects. In

327    addition, circFoxo3 also positively correlates with cellular senescence (Du et al. 2016a).

328    Together, these studies suggest that circRNA originating from FOXO genes may be a promising

329    target for repositioning of ID-1, E2F1, FAK and HIF1a from the cytoplasm to nucleus, ultimately

330    attenuating cellular senescence in aging hearts (Du et al. 2016a).

331    6.1.4 Myocardial Infarction (MI)

332        MI is a fatal disease worldwide (Mozaffarian et al. 2015). Due to the lack of available

333    biomarkers, MI cannot be predicted effectively (Vausort et al. 2016). To date, some studies

334    have made significant progress in solving this issue.

335        Wang et al. found that MFACR (mitochondrial fission and apoptosis-related circRNA) plays

336    a protective role in the heart through attenuating mitochondrial fission in cardiomyocytes by

337    directly targeting miR-652-3p and increasing the expression of its target, MTP18, which

338    promotes cell survival in cardiomyocytes. Thus, MFACR-dependent inhibition of miR-652-3p

339    increases MTP18 and mitochondrial fission, which results in a reduction in cardiomyocyte

340    apoptosis and extension of MI injury (Wang et al. 2017a). Cdr1as play detrimental roles in MI by

341    acting as a miR-7 sponge and inhibiting its activity (Geng et al. 2016; Memczak et al. 2013; Xu et

342    al. 2017a). It is well documented that miR-7a/b plays a protective role through negatively

343    regulating the expression of PARP and decreasing apoptosis in myocardial cells (Fan et al. 2017;

344   Zhao et al. 2009). SPI and PARP (poly ADP-ribose polymerase) play proapoptotic roles during MI

345   development (Li et al. 2014a; Read et al. 2014). Hypoxia-induced increases in SP1 and PARP

346   expression cause apoptotic cell death, and SP1 and PARP are target genes of miR-7, which can

347   decrease cell apoptosis (Geng et al. 2016; Li et al. 2014a; Read et al. 2014). Collectively, the

348   ciRS-7-miR-7-PARP/SP1 axis may offer new biomarkers for the diagnosis of MI, while additional

349   diagnostic markers remain to be discovered (Fan et al. 2017).

350   *6.2 CircRNA in Neurodegenerative Diseases*

351   There are thousands of circRNAs expressed in brain tissue (Rybak-Wolf et al. 2015; You et

352   al. 2015). As the central nervous system ages, age-related circRNAs accumulate in the brain and

353   have been identified as promising indicators of aging (Kumar et al. 2017). This section focuses

354   on aging diseases, such as nerve injury, Alzheimer's disease (AD) and Parkinson's disease.

355   In hippocampal neural cells (HT22), Lin et al. (Lin et al. 2016) found that oxygen-glucose

356   deprivation/reoxygenation (OGD/R) injury significantly regulates the expression of 15 circRNAs

357   compared to normal cells, suggesting the involvement of circRNA in nerve injury. Moreover,

358   CDRlas plays a protective role by inhibiting miR-7, which directly regulates the expression of a-

359   synuclein and ubiquitin protein A. Importantly, a-synuclein and ubiquitin protein A are

360   associated with the occurrence of AD (Lukiw 2013) and Parkinson's disease (Hancock 2014).

361   However, the disturbance of the miRNA-circRNA system in the hippocampal CAl region of

362   disseminated AD causes the adsorption of miR-7, leading to increased expression of ubiquitin

363   protein A in the human central nervous system (Zhao et al. 2016). In lead-induced neuronal

364   apoptosis, circRar1 directly inhibits miR-671, resulting in suppression of Akt2 and increased

365   expression of caspase-8 and other apoptosis-related proteins (Nan et al. 2017). Currently, the

366   function of circRNA in the nervous system is largely unclear, and the potential of circRNA as a

367   biomarker for neurodegenerative disorder requires further study (Kumar et al. 2017).

368   *6.3 Diabetes*

369   CDRlas plays an essential role in islet cell function and insulin secretion (Xu et al. 2015).

370   Thus, CDRlas may gain importance in the diagnosis and treatment of diabetes mellitus. It is well

371   known that the impairment of islet β-cell function leads to absolute or relative insulin

372   deficiency (insulin resistance), which increases blood sugar level and diabetes (Kim et al. 2017;

373  Xu et al. 2015). MiR-7 negatively regulates islet β-cell proliferation. MiR-7 overexpression

374  damages the dedifferentiation ability of β cells, leading to downregulation of insulin secretion

375  and ultimately diabetes. MiR-7 targets multiple components of the mammalian (mTOR)

376  signaling pathway, which are involved in pancreatic β cell proliferation. Silencing of miR-7

377  expression in β cells increases their proliferative activity, indicating that miR-7 affects

378  pancreatic β-cell renewal and is associated with diabetes mellitus. Together, these findings

379  reveal that CDR1as/miR-7 may be a potential therapeutic target for treating and managing

380  diabetes (Wang et al. 2013).

381  *6.4 CircRNA in Cancer*

382      Accumulating evidence has shown that circRNAs affect the invasive characteristics of

383  tumors in various ways, including competition with miRNAs, translation into proteins, activity as

384  miRNA reservoirs, and formation of fusion circRNAs (f-circRNAs) (Han et al. 2017; Memczak et

385  al. 2013). Genomic alterations, particularly aberrant chromosomal translocations, are

386  responsible for the onset of many types of cancers and solid tumors (Jeck et al. 2013). F-

387  circRNA, which is produced from transcribed exons of translocated chromosomes, promotes

388  carcinogenesis by increasing cell viability and resistance to therapy. Abnormal f-circRNA is

389  functionally related to cancer progression in many types of malignancies (Guarnerio et al.

390  2016). The specific roles of circRNAs in various tumors are described in the following sections.

391  6.4.1 CircRNA in Gastric Cancer (GC)

392      GC is the fourth most common gastrointestinal malignant neoplasm and is the third leading

393  cause of cancer-related deaths worldwide (Doi et al. 2015). Numerous circRNAs are abnormally

394  expressed in GC. Increased expression of Hsa-circ-002059 is significantly associated with the

395  tumor stage of GC (Li et al. 2015c). CircRNA_100269 suppresses gastric tumor cell growth by

396  targeting miR-630. However, circRNA_100269 expression is downregulated during GC and can

397  be used as a biomarker to predict cancer recurrence (Zhang et al. 2017). The expression levels

398  of hsa_circ_0003159 (Tian et al. 2017), hsa_circ_0001895 (Shao et al. 2017) and

399  hsa_circ_0000190 (Chen et al. 2017c) are down-regulated in GC. Another research group has

400  found that the expression of circPVT1 is often upregulated in GC tissue (Chen et al. 2017a). Sui

401  et al. found that the expression of tumor-associated genes, such as CD44, CXXC5, MYH9, and

MALAT1, is regulated through different mechanisms of circRNA-miRNA-mRNA interactions (Sui et al. 2017). Together, all studies have shown that circRNA plays a crucial role during gastric cancer development and that circRNA expression levels can be used as potential biomarkers for clinical prognosis prediction, sensitivity and specificity (Li et al. 2015b).

6.4.2 Colorectal Cancer (CRC)

In CRC, the downregulated hsa_circ_001988 is associated with differentiation and perineural invasion (Wang et al. 2015a). Perineural invasion is a predictor of prognosis in colorectal cancer, and it is negatively correlated with survival time and local recurrence in colorectal cancer patients (Peng et al. 2011). These results suggest that circRNAs may be potential candidates for therapeutics and biomarkers for CRC (Wang et al. 2015a). Evidence has shown that circRNA is related to CRC. Cir-ITCH is also downregulated in CRC, exhibiting an anticancer effect by binding to miR-7 and miR-20a (Huang et al. 2015). Circ_001569 directly inhibits the regulatory activity of miR-145, thereby upregulating the expression of its targets, such as E2F5, BAG4 and FMNL2, which are involved in tumor proliferation and invasion in CRC (Xie et al. 2016). CircCCDC66 regulates a subset of oncogenes, which control multiple pathological processes, including cell proliferation, migration, invasion, and anchorage-independent growth, in CRC (Hsiao et al. 2017). In addition, other circRNAs, such as hsa_circ_000984 (Xu et al. 2017b) and hsa_circ_001988 (Wang et al. 2015a), are also abnormally expressed in CRC.

6.4.3 Esophageal Squamous Cell Carcinoma (ESCC)

ESCC is one of the most prevalent and deadly types of cancers, and the prognosis of ESCC remains poor (Xia et al. 2016b). In ESCC, has_circ_0067934 is upregulated and accelerates malignant cell proliferation (Xia et al. 2016b). The expression of Cir-ITCH inhibits ESCC proliferation by suppressing the Wnt/β-catenin pathway through sponging miRNAs, such as miCH-7, miR-17 and miR-214. It is well known that ITCH mediates degradation of activated Dvl2, which is a key component of the Wnt pathway. However, the downregulation of Cir-ITH in ESCC releases the brakes on the Wnt pathway by enhancing the expression of oncogenic miCH-7, miR-17 and miR-214, consequently leading to uncontrolled proliferation of ESCC (Li et al. 2015a). Sun et al. constructed a circRNA-miRNA interaction network, in which circRNA9927-

431  NBEAL1 represents the largest node. These findings indicate that some circRNAs may be novel

432  potential biomarkers and therapeutic targets of ESCC (Xia et al. 2016b).

433  6.4.4 Hepatocellular Carcinoma (HCC)

434  Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths across

435  the world and is particularly prevalent in less developed countries [104]. Increasing evidence

436  has suggested that circRNAs may play a key role in the development of HCC. The ZKSCAN1 gene

437  and its related circRNA (circZKSCAN1) inhibit HCC cell growth, migration, and invasion by

438  blocking several signaling pathways (Yao et al. 2017). MiR-7 is a tumor-suppressing ncRNA,

439  which attenuates HCC proliferation, and it decreases the risk of microvascular invasion (MVI) by

440  suppressing the expression of its target gene, PIK3CD, and p70S6K (Xu et al. 2017a). However,

441  miR-7 activity is counteracted by the overexpression of CDRlas, which adsorbs miR-7 (Xu et al.

442  2017a). Similarly, hsa_circ_0005075 participates in cell adhesion during HCC development and

443  is considered as a biomarker for HCC (Shang et al. 2016). In contrast, the expression levels of

444  tumor suppressive circRNA, such as hsa_circ_0004018 (Fu et al. 2017), hsa_circ_0001649 (Qin

445  et al. 2016) and CircARSP91 (Shi et al. 2017), are significantly downregulated in HCC. CircRNA

446  can interact with transcription factors. Twist is a critical epithelial-mesenchymal transition

447  (EMT)-inducing transcription factor that increases expression of Vimentin, and circ-10720

448  knockdown counteracts the tumor-promoting activity of Twist1 in vitro (Meng et al. 2018).

449  More importantly, circ-ITCH not only has prognostic significance but can also be used as a

450  predictive biomarker for HCC (Guo et al. 2017).

451  6.4.5 Cervical Cancer

452  Cervical cancer is one of the most common death-causing malignancies in women

453  worldwide. Focal adhesion kinase (FAK), which is a key regulator of growth factor receptor- and

454  integrin-mediated signal pathways, promotes the proliferation, invasion and migration of

455  cervical cancer cells, and it exacerbates the progression of the disease (Lee et al. 2015). In HeLa

456  and C33A cells, the increased level of CDRlas promotes FAK expression by inhibiting miR-7,

457  which targets FAK and acts as a tumor suppressor in cervical cancer cells. This finding indicates

458  that there is a relationship between CDRlas and oncogenic transcription factors in cervical

459  cancer. Thus, the targeted therapy of CDRlas regulatory networks would provide a new

460  approach for the diagnosis and treatment of cervical cancer (Lee et al. 2015).

461  6.4.6 Breast Cancer (BC)

462  BC, the most frequently diagnosed cancer in women around the world, has been the focus

463  of major advances in the last few decades (Doi et al. 2015). Many circRNAs are differentially

464  expressed in BC and participate in cancer-related pathways mainly by sequestering tumor

465  suppressive miRNAs (Lu et al. 2017). Hippo signaling promotes BC progression by upregulating

466  the expression of AMOTL1 and favoring metastasis (Couderc et al. 2016). Interestingly, the

467  expression of circ-Amotl1 does not alter Amotl1 mRNA or protein levels. However, circ-Amotl1

468  interacts with c-myc and translocates to the nucleus, revealing that the functions of circ-Amotl1

469  are different from the conventional miRNA sponging activity of circRNA in BC cells (Yang et al.

470  2017a). Tumor metastasis is one of the most important factors for tumor death. Forkhead box

471  C1 (FOXC1), the target of miR-3607, is downregulated in circIRAK3-silenced cells, and it

472  mediates circIRAK3-induced BC cell migration (Wu et al. 2018a).

473  *6.5 Other diseases*

474  Some other diseases also show a connection with circRNAs. Hsa_circ_0005105 promotes

475  extracellular matrix (ECM) degradation by regulating the expression of the miR-26a target,

476  NAMPT, in osteoarthritis (OA) (Wu et al. 2017). Hsa_circRNA_103636 is easily detectable in

477  blood samples, and the expression pattern of hsa_circRNA_103636 is altered in major

478  depressive disorders (MDDs) (Cui et al. 2016). CircHECTD1 mediates silica-induced macrophage

479  activation via HECTD1/ZC3H12A-dependent ubiquitination in Silicosis (Zhou et al. 2018). In

480  bladder cancer, circTCF25 downregulates miR-103a-3p and miR-107 as well as upregulates

481  cyclin-dependent kinases 6 (CDK6), suggesting that circTCF25 is a new biomarker (Zhong et al.

482  2016).

483  Cancer-related circRNAs with their corresponding miRNAs form a circRNA-miRNA-mRNA

484  axis that regulates the expression of cancer-related proteins (Sui et al. 2017). Furthermore, the

485  new expression of circRNAs in tumor cells, tissue-specificity, diversity, and high stability identify

486  circRNAs as useful biological markers of cancer, thus improving the accuracy and specificity of

487  diagnostic biomarkers (Zhong et al. 2016).

488 *6.6 CircRNA as a disease marker potential*

489   Currently available reports clearly show that alterations in the expression of circRNA play

490 important roles in the development of various pathological conditions. CircRNA is emerging as a

491 novel biomarker due to its conservation, abundance, cell type-specific expression, tissue-

492 specific expression, and roles in disease progression (Meng et al. 2017). HRCR attenuates the

493 pathogenesis of cardiac diseases and has the potential to become a therapeutic target (Wang et

494 al. 2016). CircFoxo3 may be a promising target of cellular senescence in aging heart (Du et al.

495 2016a).

496   Researchers have provided evidence that ciRS-7 has the potential as a biomarker for

497 neurodegenerative disorder (Lukiw 2013), diabetes (Wang et al. 2013), or MI (Lin et al. 2018).

498 CircRNAs regulate the expression of cancer-related proteins in various types of cancer,

499 including GC (Li et al. 2015c; Shao et al. 2017), CRC (Wang et al. 2015a), ESCC (Xia et al. 2016b),

500 HCC (Yao et al. 2017), and BC (Lu et al. 2017), through the circRNA-miRNA-mRNA axis, which

501 represents their potential as diagnostic markers, prognostic indicators, therapeutic targets and

502 even drug prospects in cancer.

503 **7. Conclusion and Future Directions**

504   CircRNAs have conserved sequences, tissue specificity, high stability and high abundance,

505 thereby making them potential markers for disease screening and treatment (Westholm et al.

506 2014). The rapid development of high-throughput sequencing techniques and bioinformatics

507 analyses suggest that circRNAs are likely to become new efficient targets in the clinical settings

508 for the detection and treatment of diseases, such as diabetes, cancer, cardiovascular disease

509 and neurological diseases. circRNAs can function as miRNA sponges (Hansen et al. 2013a) and

510 regulate multiple signaling pathways in CVDs (Fan et al. 2017), different types of cancers (Lu et

511 al. 2017; Meng et al. 2018; Yao et al. 2017), neurodegenerative diseases (Kumar et al. 2017) and

512 diabetes (Zhao et al. 2017). However, further studies are needed to reveal the complete

513 biological functions of circRNA in terms of both physiological and pathological processes to

514 promote the application of circRNA in future clinical use.

515   Despite rapid advances in the detection and characterization of circRNAs, the knowledge of

516 circRNA functions is still at an early stage, which is one of the major drawbacks for the potential

517    use of circRNAs for therapeutic or diagnostic purposes. New methods, such as chip technology,

518    can be used to screen possible disease-related circRNAs in cell or experimental animal models,

519    which will increase our knowledge about the role of circRNAs in the occurrence and

520    development of pathological disorders. In addition to broadening functional aspects of circRNA,

521    the following unknowns should also be addressed: mechanisms of trigger and control of

522    circRNA formation dynamics; the link between the circRNA formation process and the

523    corresponding linear RNA generation; the relationship between different circRNA products

524    from the same gene; and regulatory mechanism of circRNA generation. The identification and

525    characterization of specific circRNA-interacting molecules are important to provide information

526    for most of these unknowns. In addition, the naming of circRNA has not yet been unified, and

527    the mechanisms of circRNA in many diseases remain unclear. By solving these unknowns,

528    circRNA may be a promising diagnostic tool and efficient therapeutic target for treatment of

529    various pathological disorders.

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925

The characteristics of different types of CircRNA

ecRNA : Exon circRNA ; ciRNA : Intron circRNA ; ElciRNA : Exon-intron circRNA

**Table 1** Characteristics of different circRNA types

| Name | Type | Location | Joint Site | Sequence Feature | Function |
|---|---|---|---|---|---|
| EcRNA(Zhang et al. 2014) | exon | cytoplasms | 3'-5' phosphodiester bond | Formed by cyclization of exons containing the reverse complementary sequence of introns and selective cyclization. | Functioning as miRNA sponges; Interact with RNA-binding proteins (RBPs); Participates in translation. |
| CiRNA(Zhang et al. 2013) | intron | nucleus | 2'-5' phosphodiester bond | 5' splice site enriched with 7 GU motifs and 3' branch site contains 11 C motifs. | Regulation of gene transcription. |
| ElciRNA(Li et al. 2017b) | exon - intron | nucleus | 3'-5' phosphodiester bond | Formed by cyclization of exons containing the reverse complementary sequence of introns and selective cyclization. | Regulation of gene transcription. |

2         EcRNA: Exon circRNA; ciRNA: Intron circRNA; ElciRNA: Exon-intron circRNA

CircRNA in disease development and progression

**Table 2** Database for CircRNA Research

| Tool Name | The latest version | URL | Remarks |
|---|---|---|---|
| circlncRNAnet(Wu et al. 2018b) | May 2017 | http://app.cgu.edu.tw/circlnc/ | It aims to broaden the understanding of ncRNA candidates by testing in silico several hypotheses of ncRNA-based functions on the basis of large-scale RNA-seq data. |
| starBase v2.0(Li et al. 2014b) | December 2013 | http://starbase. sysu. edu. cn/ | Includes microRNA, mRNA, lncRNA and other RNA information. It is a useful tool for detecting miRNA-circRNA interaction. If there is a need to retrieve all circRNA in the genome, circRNABase is useful. |
| circBase(Glazar et al. 2014) | December 2015 | http://www. circbase. org/ | Thousands of circRNAs are annotated from eukaryotic cells. |
| circ2Traits(Hancock 2014) | December 2013 | http://gyanxet-beta. com/circdb | Provides more information about the genomic positions of circRNAs and circRNA-associated diseases. |
| nc2Cancer(Cheng et al. 2015) | | http://www. Bioinfo. Tsinghua. Edu. cn/nc2Cance | |
| CircNet(Liu et al. 2016) | December 2015 | http://circnet. mbc. nctu. edu. tw/ | A database of circular RNAs derived from transcriptome sequencing data, and this tool provides the most circRNA annotation, in particular, from human. |
| DeepBase v2.0(Zheng et al. 2016) | November 2015 | http://biocenter. sysu. edu. cn/deepBase/ | This database is a platform for annotation and discovery of small (microRNA, siRNA and piRNA) and long ncRNAs from next-generation sequencing data. |
| CircInteractome(Dudekula et al. 2016) | December 2015 | http://circinteractome. nia. nih. gov/ | This database can be used only to match the circRNA with relevant RNA-binding proteins. |
| TSCD(Xia et al. 2016a) | August 2016 | http://gb. whu. edu. cn/TSCD/ | It is useful for characterizing tissue-specific circRNAs in human and mouse genomes. |
| CIRCpedia (Zhang et al. 2016) | January 2015 | http://www. picb. ac. cn/rnomics/circpedia/ | This database contains reverse splicing and variable splicing sites of circRNAs from 39 individuals and mouse samples. |

| circRNADb(Chen et al. 2016) | http://reprod. njmu. edu. cn/circrnadb | It contains a record of more than 30,000 exons with circRNA nature in the human genome |
|---|---|---|

2  Every database present has its own sphere of competence, and only the perfect
3  combination of various databases can provide accurate information.

Database for circRNA research

Every database present has its own sphere of competence, only the perfect combination of various database s can provide information accurate.

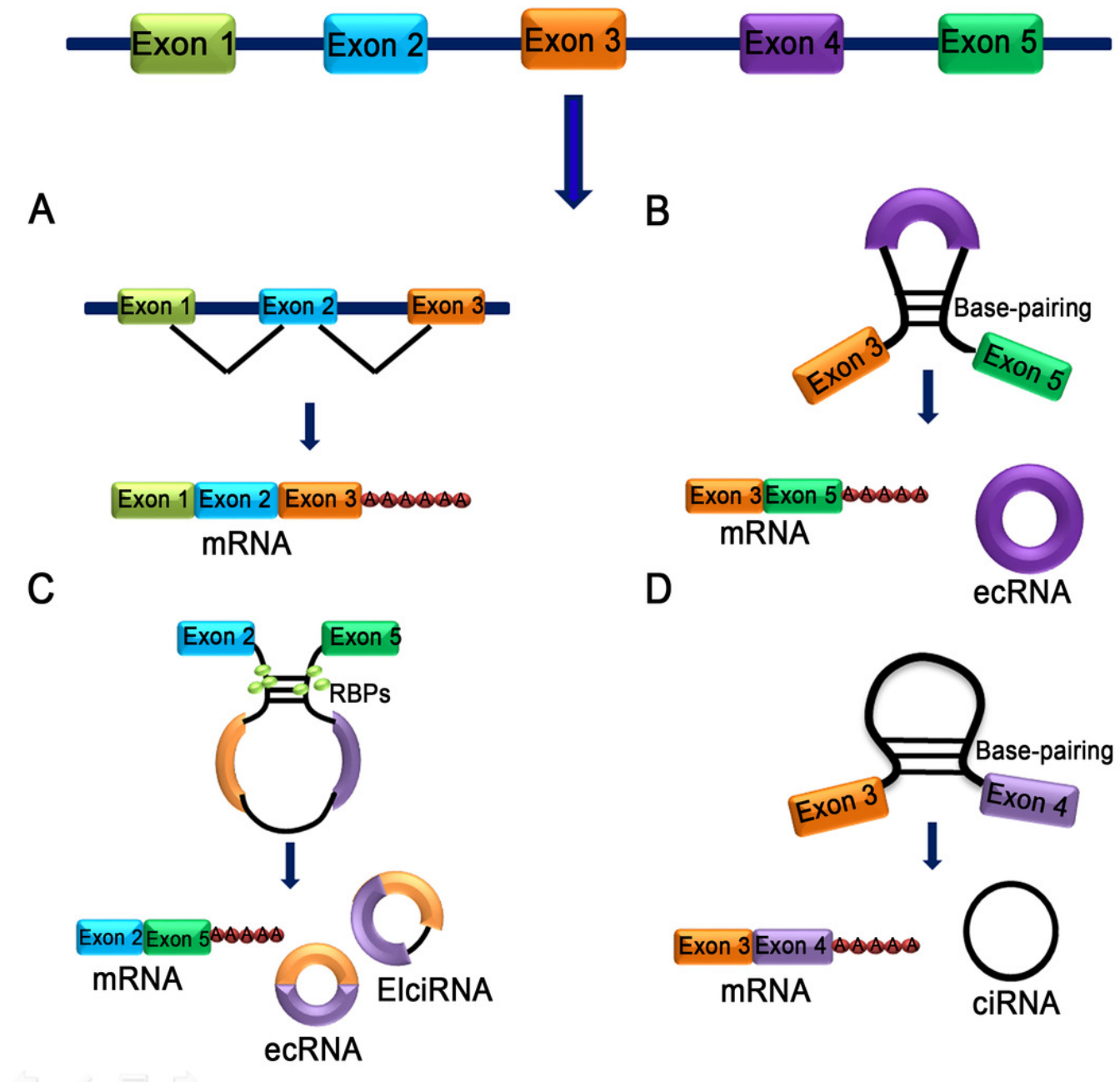
1            **Table 3** CircRNA in disease development and progression

| Diseases | | CircRNA | References |
|---|---|---|---|
| CVD | Pathological hypertrophy and HF | HRCR | (Wang et al. 2016; Wang & Wang 2015; Wang et al. 2015b) |
| | Atherosclerosis | CANRIL | (Holdt et al. 2016) |
| | Cardiac senescence | Circ-Foxo3 | (Du et al. 2016a; Du et al. 2016b) |
| | MI | CDRlas | (Geng et al. 2016; Li et al. 2014a; Read et al. 2014) |
| | | MFACR | (Wang et al. 2017a) |
| Neurodegenerative Diseases | | CDRlas | (Zhao et al. 2016) |
| | | CircRar1 | (Nan et al. 2017) |
| Diabetes | | CDRlas | (Kim et al. 2017; Wang et al. 2013; Xu et al. 2015) |
| OA | | Hsa_circ_0005105 | (Wu et al. 2017) |
| MDD | | Hsa_circRNA_103636 | (Cui et al. 2016) |
| Silicosis | | CircHECTD1 | (Zhou et al. 2018) |
| Cancer | GC | Hsa-circ-002059 | (Li et al. 2015c) |
| | | Circrna_100269 | (Zhang et al. 2017) |

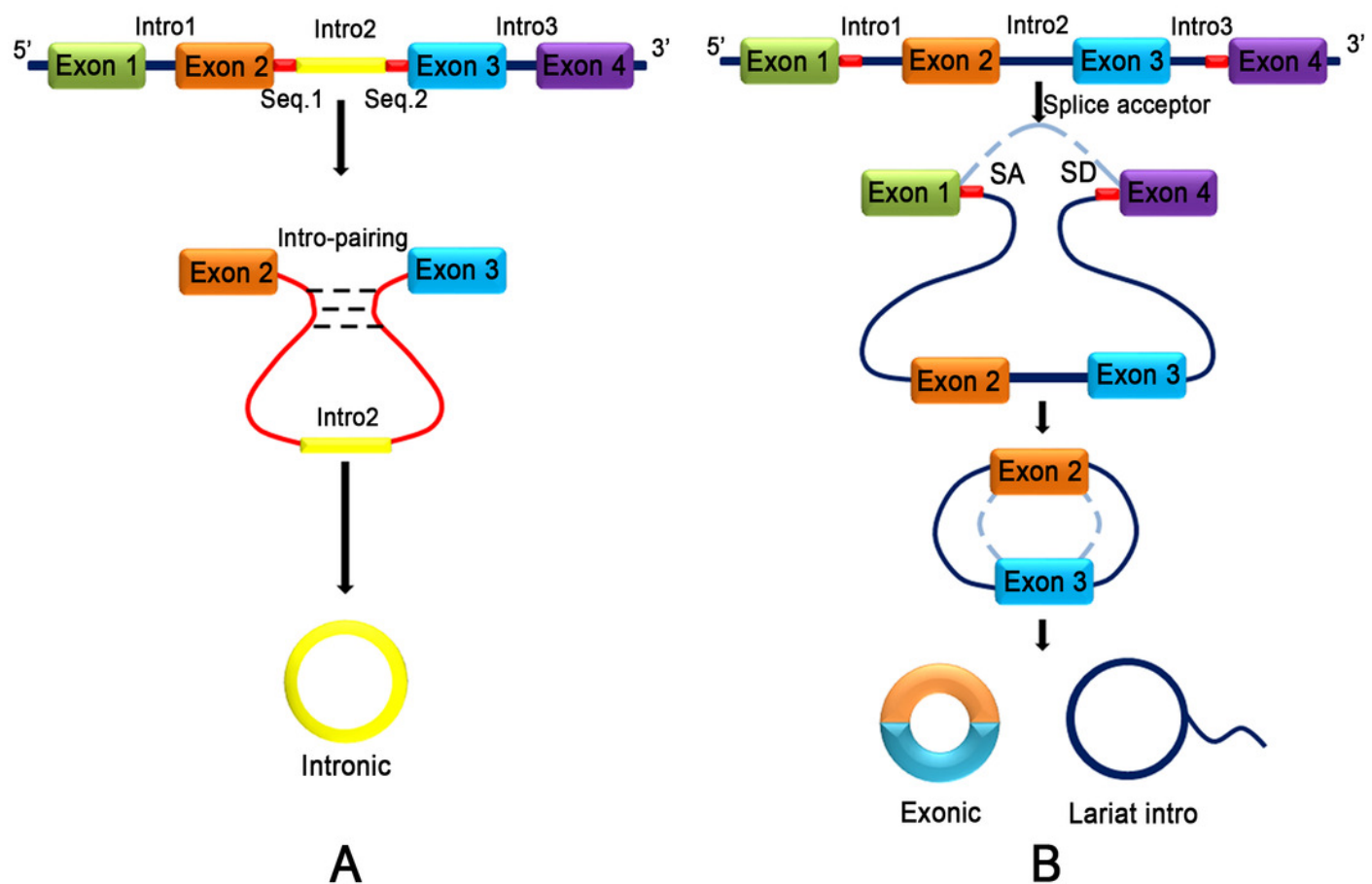
| | Hsa_circ_0003159 | (Tian et al. 2017) |
|---|---|---|
| | Hsa_circ_0000190 | (Chen et al. 2017c) |
| | CircPVT1 | (Chen et al. 2017a) |
| CRC | Hsa_circ_001988 | (Wang et al. 2015a) |
| | Circ_001569 | (Xie et al. 2016) |
| | CircCCDC66 | (Hsiao et al. 2017) |
| | Hsa_circ_000984 | (Xu et al. 2017b) |
| ESCC | Has_circ_0067934 | (Xia et al. 2016b) |
| | Cir-ITCH | (Li et al. 2015a) |
| HCC | CircZKSCAN1 | (Yao et al. 2017) |
| | CDRlas | (Xu et al. 2017a) |
| | Hsa_circ_0005075 | (Shang et al. 2016) |
| | Hsa_circ_0004018 | (Fu et al. 2017) |
| | Hsa_circ_0001649 | (Qin et al. 2016) |
| | CircARSP91 | (Shi et al. 2017) |
| | Circ-10720 | (Meng et al. 2018) |
| | Circ-ITCH | (Guo et al. 2017) |
| Cervical Cancer | CDRlas | (Lee et al. 2015) |
| BC | Circ-Amotl1 | (Yang et al. 2017a) |

| | | | |
|---|---|---|---|
| | Human oral squamous cell carcinomas (OSCC). | Circrna_100290 | (Chen et al. 2017b) |
| | Lung adenocarcinoma (LAC) | Hsa_circ_0013958 | (Zhu et al. 2017) |
| | Bladder carcinoma | CircTCF25 | (Zhong et al. 2016) |
| | | CircPTK2 | (Xu et al. 2017c) |

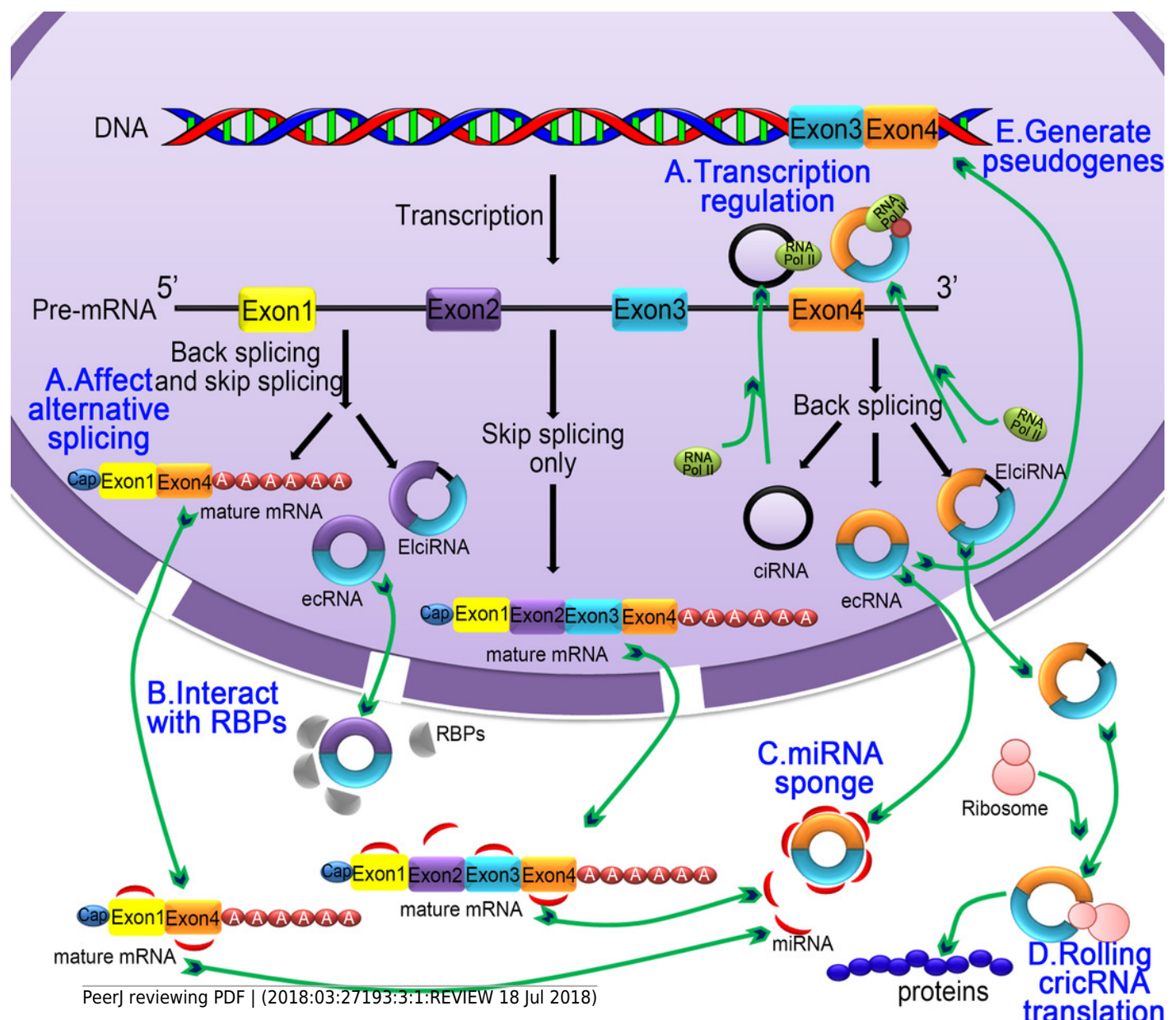
# Figure 1

Characteristics of different types of circRNA

# Figure 2

Two different models of exon circularization of circRNA

(A) intron-pairing-driven circularization : during the formation of circRNA, an intron reverse complementary motif comprising GU-rich and C-rich elements is the key component to facilitate cyclization. (B) lariat-driven circularization : t he formation of circRNA is facilitated by the lariat structure. The complementary ALU flanking element which is repeated in the intron region competing for classical linear RNA splicing and the circularization is accelerated by reverse complementarity.

# Figure 3

The five main functions of the circRNA

(A) Regulating selective splicing or transcription: Stable circRNA and EIciRNAs are located in the nucleus, where they bind to RNA polymerase and promoting transcription; circRNA competes with pre-mRNA splicing to reduce the level of linear mRNA and excludes specificity from pre-mRNA by changing the composition of processed mRNA; (B) Interaction with RBPs: circRNA binds with RBPs and ribonucleoprotein complexes and interfere with their functions. As a single circRNA can bind with multiple units of RBPs, they serve as stores of RBPs; (C) miRNA sponging activity: circRNA binds with miRNA and affecting the miRNA dependent target gene suppression; (D) Rolling Circle Translation: Some circRNA can be translated into proteins by means of a roll loop amplification mechanism; (E) Generation of Pseudogenes: Some circRNA are reverse transcribed into cDNA and integrated into the genome, however, the mechanism of integration is not yet clear.

# Figure 4

The difference between linear RNA and circRNA primer design

(A) FW is a forward primer with b chain as template. The base sequence of synthesis is the original sequence of a ; RV is a reverse primer with a chain as template, and the base sequence of synthesis is the original sequence of b , The sequence between FW and RV is high; (B) Need to reverse the original primers: The synthetic primers are FW' and RV', where FW' is the reverse complementary sequence of the RV primer, RV' is the reverse complementary sequence of FW primer.