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Circular DNAs Participate in Tumorigenesis and Its Mechanisms

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KEYWORDS

Circular DNAs;
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ABSTRACT

Chromosome-derived circular DNAs have different characters from linear DNAs and RNAs, which exert distinctive functions, and participate in a widespread range of physiological and pathological processes. Although, the composition, structure and genome-wide frequency of circular DNAs have not yet been illuminated systemically, circular DNAs are identified to act as unanticipated major sources of somatic rearrangements, which is an important genomic feature in tumorigenesis and represents a multihit and ongoing mutagenic process. And, this process remarkably contributes to oncogenic remodeling and oncogenes amplification through chimeric circularization and reintegration of circular DNAs into the linear genome, which is closely associated with high cancer mortality and morbidity. It has been found that circular DNAs-stimulated oncogenes amplification and overexpression implicate in diverse cytopathological processes during cancer progression, including cell proliferation, apoptosis, autophagy, epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) stiffness and angiogenesis. Herein, we comprehensively introduced circular DNAs' formation and characteristics, and emphatically elaborated circular DNAs-induced oncogenes amplification and overexpression and their functional mechanisms regulating cancer behaviors. Lastly, some mysteries in oncogenic circular DNAs' study were introduced, and the perspectives were also discussed in cancer diagnosis and treatment.

INTRODUCTION

Genomic amplification is responsible for complicated chromosomal rearrangements, which is triggered by double-strand break (DSB) events, like tandem duplication[1], breakage–fusion–bridge cycles[2] and chromothripsis[3]. Chromosomal rearrangements frequently produce extrachromosomal DNA particles that have been confirmed in many eukaryotic species, such as caenorhabditis elegans[4], drosophila melanogaster[5], yeast[6] and humans[7], and majority of them reside outside of cell nucleus. There are two kinds of extrachromosomal DNA particles, containing covalently closed circular and linear. Impressively, increasing studies have showed that diverse types of extrachromosomal circular DNAs possess explicit functions, while the roles of extrachromosomal linear DNAs remain to be explored.

Extrachromosomal circular DNAs were first discovered in 1964, and had been highly conservative[8]. Circular DNAs are divided into three classes based on size and copy number, composed of small extrachromosomal circular DNAs (eccDNAs)[9], large extrachromosomal circular DNAs (ecDNAs), also referred to as double-minute chromosomes (DMs)[6] and neochromosomes[10]. Among these, eccDNAs are usually less than 1 kb size and visible under light microscopy, which mainly comprise microDNAs[7], telomeric circles[11] and small polydisperse circle DNAs[12]. ecDNAs are often in 1–3 Mb size or larger, copy number–amplified and microscopically visible[13]. Compared with eccDNAs, ecDNAs contain one

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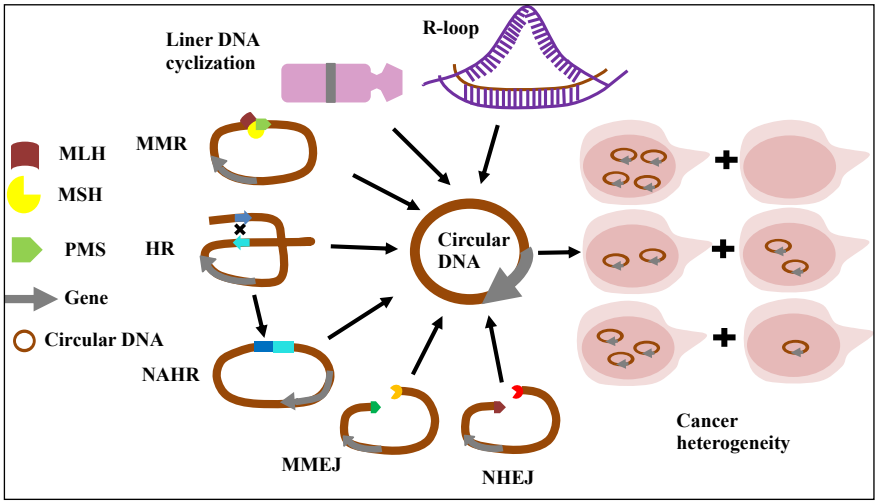

Figure 1 | Oncogenic circular DNAs' formation

Anomalous cyclization of linear chromosomes constitutes circular DNAs. R-loop appears as an attractive structure for inducing circular DNAs biogenesis. Intrachromosomal DNA-repair mechanisms of double-stranded DNA, including HR, NHEJ, MMEJ, NAHR and MMR, constructively contribute to the circulation of DNA and form extrachromosomal circular DNAs. MMR occurrence is dependent on MSH/MLH/PMS complex, NHEJ needs abundant Ku protein, and MMEJ is enriched in MHs. Circular DNAs are unequally segmented into daughter cells due to lack of centromeres, resulting in cancer heterogeneity when cells divide.

or multiple full genes and regulatory regions and employ the same replication patterns as chromosomes[9]. In coincidence, "chromothripsis" (chromosome shattering) can give rise to ecDNAs owing to genomic instability, the event is observed particularly in cancer and germline[14]. Neochromosomes appear as a supernumerary chromosome with a ring topology, the ring size and number of them are variable in different cells[10]. Conventional methods for characterizing circular DNAs are frequently semi-quantitative and biased, and can't be performed with single-cell analysis. To overcome these limitations, some sensitive and advanced technologies are urgently to be developed, such as circular DNAs enrichment sequencing (CIDER-seq)[15], rolling circle amplification (RCA)[16], whole-genome sequencing (WGS)[17], circle sequencing (Circle-seq)[18] and ultrahigh-throughput sequencing[19]. These technologies may be used to reconstruct the fine architecture of locus-specific amplicons when in conjunction with computational algorithms, such as Ampliconn Architect[17].

Circular DNAs had formerly been considered as non-functional. Recently, it has been defined as independent loops that can't only boost gene expression in adjacent and distant cells, but affect their functions, further mediating various physiological and pathological activities[20]. For instance, extrachromosomal circular DNAs significantly implicate in central nervous system (CNS) aging and neurodegeneration. Further, circular DNAs have been proved to involve in the pathology of cancer by permitting rapid and extensive oncogenes copy number variation[9]. Oncogenes amplification or overexpression is one of the most common molecular alterations in cancer. Thereby, the formation of circular DNAs-carrying oncogenes is a potent and frequent mechanism for inducing genome remodeling and oncogenes anomalous expression, which powerfully drives cancer heterogeneity and adaptive evolution via mediating cell proliferation, autophagy, apoptosis, EMT, ECM remodeling and angiogenesis. To comprehensively clarify the functional mechanisms of circular DNAs in cancer progression, we systematically introduced circular DNAs' generation and characteristics, and their molecular mechanisms modulating oncogenic processes. Further, we also introduced some mysteries to be solved in circular DNAs' research and declared perspectives in the fields of cancer diagnosis and treatment.

ONCOGENIC CIRCULAR DNAS' FORMATION

Currently, the pathogenesis of circular DNAs formation is being explored. DNA damage and repair, or DNA replication and transcription processes lead to the release of DNA segments. Then, DNA segments automatically merge into circular DNAs, which is based on both single- or double-stranded DNA circularization. Next, the circular DNAs start to replicate, and the circular DNAs copy number would rapidly accumulate due to the disjointed feature of circular DNAs replication[21]. Lastly, circular DNAs are unevenly apportioned to daughter cells due to lack of centromeres, which could rapidly lead to heterogenetic counts of circular DNAs in cells and enable daughter cells to achieve higher circular DNAs copy number than mother cells. Of note, the genomic composition of circular DNAs is actually different in various cancer types. Hence, the generation of circular DNAs may be a multistep procedure evolving from a single-segment structure to a complex multi-segment structure[21].

Undoubtedly, genomic instability is essential for inducing circular DNAs formation. For one thing, the anomalous cyclization of linear chromosomes can constitute circular DNAs (**Figure 1**), which obtains the increased genes copy number dependent on self-amplification of the amplicon[22]. For another thing, R-loop emerges as an attractive structure in the procedure of circular DNAs genesis (Figure 1)[23]. In mechanism, the formation of R-loop during DNA replication and transcription processes functionally determines the localization of transcription-associated DNA-damaging events, which might correlate with potential single-stranded DNA-based circular DNAs generation and further structurally trigger the overexpression of transcriptionally active genes exist-

ing in circular DNAs[7, 23]. Similarly, R-loop is accepted as an interesting hallmark of C-circle-generating alternative lengthening of telomeres (ALT) process, which facilitates telomeric circles release via involving telomeric repeat-containing RNA (TERRA). Moreover, R-loop induces the formation of triple-stranded DNA-RNA hybrids that are tightly associated with the misplacement of the single-stranded non-template DNA, further dually manipulating DNA damage and DNA repair processes to assist circular DNAs production[23]. To some extent, R-loop is critical for efficient class switch recombination (CSR). CSR arising from activated B cells is considered as the isotype switch of immunoglobulin heavy-chain classes, which facilitates circular DNAs biogenesis due to the influence of DSB and deletions[24]. More importantly, programmed DSB in the switch regions is dependent on R-loop-mediated transcription and replication activities[25]. Accordingly, R-loop is beneficial for the survival of highly replicative cancer cells on the basis of permanent telomere repairment, demonstrating that targeting R-loop formation might succeed to suppress oncogenes amplification and cancer growth by interfering the formation of circular DNAs and extrachromosomal telomere repeats (ECTRs)[23]. Nevertheless, it is an overt question whether R-loop devotes to linking genic regions to circular DNAs excision.

Emerging evidence indicates that circular DNAs biogenesis alternatively relies on DNA-repair processes of double-stranded DNA break. DNA-repair mechanisms are mainly composed of homologous recombination (HR)-dependent and -independent DNA-repair (Figure 1). HR is responsible for the generation of extrachromosomal circular amplicons and circular DNAs[26], and inhibition of HR would attenuate extrachromosomal circular DNAs-induced genes amplification, but has no effect on liner DNAs-induced amplification[27]. HR-independent DNA-repair mechanisms comprise non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), non-allelic homologous recombination (NAHR) and mismatch repair (MMR). The novel role of NHEJ in DMs formation has been detected in colon cancer cells accompanied by increased DNA-PKcs protein[28]. More importantly, NHEJ and MMEJ-induced extrachromosomal DMs are simultaneously observed in glioma, meanwhile, co-amplification of epidermal growth factor receptor (EGFR) and MYC loci is present in the structure of DMs[22]. NAHR is ascribed to account for genomic rearrangements in the human genome, but its frequency relies on homology properties[29]. Thus, NAHR-induced repair may be an abundant source for circular DNAs formation. In addition, MMR also gives rise to abundant circular DNAs, which might participate in transcriptional regulation during cancer development. And deletion of the Mut S homolog 3 (MSH3) DNA mismatch repair protein significantly reduces the enrichment of microDNAs, specifically in non-CpG genomic regions[19].

THE ADVANCED APPROACHES FOR CIRCULAR DNAS DETECTION

Although, the sequences of circular DNAs have some degree of homology to chromosome DNAs in several organisms, present DNA sequencing methods can't be used to detect the comprehensive sequences of all types of circular DNAs. Thereby, sensitive and novel approaches are urgently developed to better define the nucleotide sequences of circular DNAs.

Recently, effective circular DNAs extraction and purification techniques have been developed based on the different structures between chromosome and circular DNAs, which is indispensable for detecting circular DNAs. For instance, high-speed ultracentrifugation has been employed to extract eccDNAs from the human cancer cells[30], in which the approach firstly extracts nuclei by sucrose ultracentrifugation followed by plasmid purification and several rounds of enzymatic reaction. Reportedly, RCA can exponentially amplify circular DNAs templates in vitro, which has been alternatively employed to recognize and characterize short eccDNAs in plants, mouse and human cell lines via multiple displacement amplification, the recognized eccDNAs are confirmed by electron microscopy[7, 16]. RCA procedure includes extraction, amplification, restriction digestion, electrophoretic separation, cloning and sequencing. During the process, oligonucleotides are designed to sequence across the junction and confirm the circular structure of the amplified molecule. Subsequently, the entire molecule is re-sequenced utilizing sets of specific and overlapped hexamer primers. Prevalently, RCA is sequence neutral and very robust, but it only detects known sequences using specific probes, which constrains the detection potential for newfound molecules[16].

In order to comprehensively quantify the spectrum of circular DNAs and explore the contents, combination of genomic with transcriptomic strategies has also been utilized to characterize extrachromosomal circular DNAs. Since DNA circularity can be computationally inferred from WGS data[17], conjunction of WGS with an algorithm using paired-end read orientation is applied to predict cancer-specific circular DNAs. To achieve more exact detection of circular DNAs, the Circle-seq method is properly modified to detect sequence composition and genomic origin through purifying circular DNAs, removing remained linear chromosomal DNAs, deeply sequencing and mapping. Afterwards, the predicted circular head-to-tail is verified by PCR and Sanger sequencing. In one experiment, Circle-seq validates 100% of ecDNAs and 30% of eccDNAs predicted from WGS. Although WGS shows high sensitivity (100%) for predicting ecDNAs, the Circle-seq is superior due to unique abilities to obtain a comprehensive characterization of circular DNAs in human cancers[13, 18].

In addition, ultrahigh-throughput sequencing has been exploited to characterize circular DNAs and analyze paired-end sequencing data via combining with algorithms[19]. In human ovarian and prostate cancer cells, the features of dubbed microDNAs have been defined using ultrahigh-throughput sequencing, such as prevalently enriched in exons, 5' and 3'

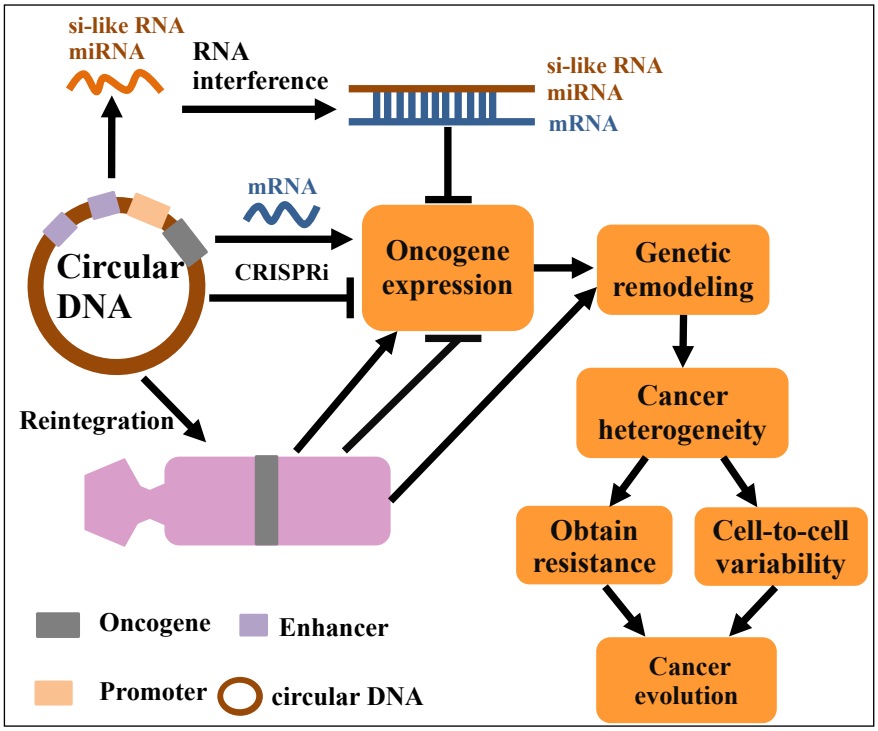

Figure 2 | Circular DNAs mediate oncogenes expression

Oncogene segment is inserted into circular DNAs, which enhances oncogenes transcription via contacting with distal enhancers elements. RNA interference also inhibits oncogenes expression through designing miRNA and si-like RNA sequences derived from circular DNAs. Meanwhile, circular DNAs may even conversely transfer oncogenes back onto the cells' linear DNAs to regulate oncogenes expression and support genetic remodeling. Oncogenes overexpression-triggered genetic remodeling promotes cancer heterogeneity, which is responsible for maintaining cell-to-cell variability and obtaining resistance to therapy, subsequently facilitating cancer evolution.

UTRs, high GC contents, DNase hypersensitivity sites, CpG islands and affluent short direct repeats. Nevertheless, the high cost of this technology restricts its use[19]. CIDER-seq is emerged as an accurate and simple method to enrich and detect full-length sequences of circular DNAs without requiring polymerase chain reaction amplification, cloning, and computational sequence assembly. The approach relies on randomly primed circular DNAs amplification, enzymatic DNA repair processes, long-read sequencing and a sequence deconcatenation algorithm termed as DeConcat[15]. The greatest accuracy of CIDER-seq-generated circular DNAs sequences is less than 10 kb due to the limitations of long-read single-molecule sequencing.

CIRCULAR DNAS MEDIATING ONCOGENES EXPRESSION IN CANCER

Circular DNAs are rarely found in normal tissues and cells, but they are abundant in cancer cells, and markedly vary from cell to cell accompanied with different degree of oncogenes expression[20]. Of crucial note, circular DNAs can recruit and integrate additional oncogenes segments, which enhances oncogenes transcription via contacting with distal enhancers elements[31]. Therefore, using CRISPRi technology to attenuate enhancers activity would restrict oncogenes expression. In addition, RNA interference also inhibits oncogenes transcription through designing miRNA and si-like RNA sequences originated from circular DNAs. Meanwhile, the circular DNAs may conversely transfer oncogenes into the cells' linear DNAs to regulate oncogenes expression[32] (**Figure 2**).

Oncogenes overexpression is considered as one of frequent features in cancer mutation, which can occur in either chromosomes or nuclear circular DNAs elements, dominantly including DMs. The mutations in DMs are named as amplification-linked extrachromosomal mutations (ALEMs) that are distributed in different cancer types[33]. Interestingly, DMs are enveloped by a nuclear-like membrane (micronuclei) to support DNA replication and transcription[34]. The comprehensive analysis of various cancers demonstrates that DMs are primarily distributed in glioblastomas and low-grade gliomas with co-enrichment of EGFR and platelet—derived growth factor receptor α (PDGFRA) mutations[33]. In addition, other circular DNAs-derived oncogenes have also been identified in glioblastoma, such as MYC, MET, and cyclin-dependent kinase (CDK4)–MDM2 gene cluster[35]. Strikingly, it has been verified that amplification of MYC gene doesn't only locate in the paired DMs, but also jump onto aberrant chromosomal homogeneously staining regions (HSRs) to achieve a more stable form of amplification, showing the unstable gene amplification in circular DNAs[36].

Even if amplicons of circular DNAs are less stable circular DNAs-induced oncogene amplification can reach much higher copy number in cancer compared with on a chromosome. As a consequence, heterogeneity of cancer with regard to oncogenes copy number would rise more high and be maintained much longer, but be more likely to emerge relatively slowly if it resides on a chromosome[9]. Particularly, increasing observations have shown that genetically heterogeneous cancer is inclined to maintain cell-to-cell variability, facilitate cell proliferation and survival, and obtain resistance to therapy[33] (Figure 2). Taken together, circular DNAs are the constructive structure for oncogenes amplification and overexpression in vivo, which is essential for accelerating cancer evolution and genetic remodeling through strong mitotic potential and unique mechanisms of inheritance[37]. Moreover, alterations of spatial architecture, including disruption of

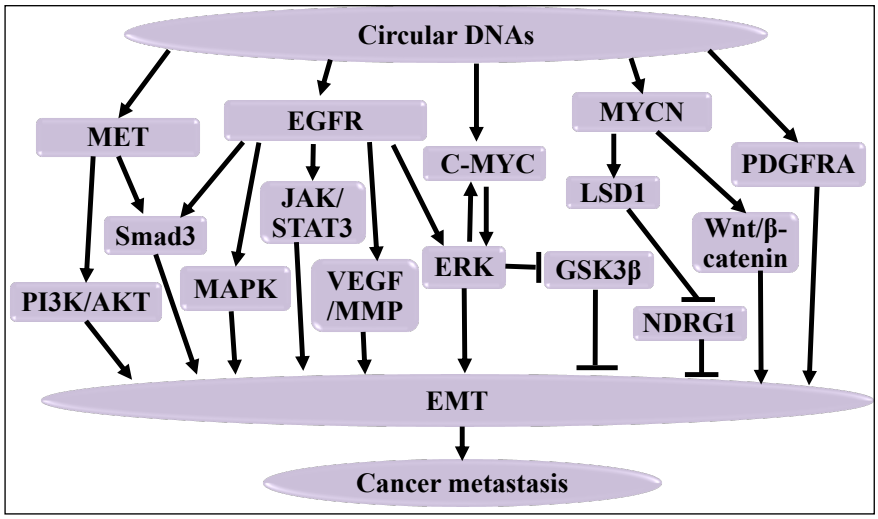

Figure 3 | Circular DNAs mediate cell EMT process

Circular DNAs-induced EGFR activates Smad3, JAK/STAT3, VEGF/MMP, MAPK and ERK pathways. The DMs-induced C-MYC overexpression inhibits GSK3β activity through activating ERK, subsequently facilitating EMT. C-MYC expression is inversely boosted by ERK. There is a positive feedback between C-MYC and ERK activity. DMs-stimulated MYC gene, MYCN, it can't only enhance Wnt/β-catenin signaling, but inhibit suppressor NDRG1 gene expression by interacting with the lysine-specific demethylase 1 (LSD1), consequently leading to the strengthened EMT. Circular DNAs-boosted MET is also capable of promoting Smad3 phosphorylation, as well as boosting PI3K/AKT signaling to promote EMT. DMs amplification of mutant PDGFRA has also been found to increase EMT process. Activation of these pathways induced by circular DNAs has a positive effect on cancer metastasis via enhancing EMT.

topologically associating domain boundaries, have a remarkable impact on development of cancer containing circular DNAs[38]. Hence, it has been thought to be important to clarify the characteristics and functional mechanisms of circular DNAs manipulating oncogenes expression.

THE MOLECULAR MECHANISMS OF CIRCULAR DNAS REGULATING CANCER PROGRESS

Cancer cells containing circular DNAs might quickly adapt to variable environments through triggering oncogenic alterations and evading immune surveillance after a series of chemotherapies, radiotherapies and target therapies[39]. In agreement with this, circular DNAs-stimulated oncogenes amplification and overexpression, containing EGFR, PDGFR, MYC, EMT and MDM2-CDK4 gene cluster, are thought to be a potent and frequent mechanisms for facilitating tumor pathogenesis by mediating multiple oncogenic processes, including EMT, cell proliferation, angiogenesis, apoptosis, autophagy and ECM remodeling. These provide clues for Figuring out the molecular mechanisms of circular DNAs-derived oncogenes driving cancer progression.

Circular DNAs Mediate Cancer EMT Process

The EMT has been acknowledged as a phenotypic conversion from epithelial cells to mesenchymal-like properties. Circular DNAs-induced oncogenes amplification participates in promoting EMT phenotype, this plays a critical role in enhancing metastasis, invasion, and therapeutic resistance during cancer progression (**Figure 3**). DMs-induced C-MYC amplification is detected to boost EMT in human cancers[40], C-MYC overexpression promotes migration of oral cancer cells by inhibiting E-cadherin expression and promoting N-cadherin expression[41]. Mechanically, circular DNAs-induced C-MYC is positively modulated by extracellular regulated protein kinase 2 (ERK2), which further accelerates EMT process and metastasis via up-regulating Vimentin, Fibronectin, Slug and Twist2[42]. On the other side, C-MYC can inversely activate ERK to inhibit glycogen synthase kinase (GSK)3β activity, subsequently facilitating EMT[43]. The combined analysis shows that there is a positive feedback between C-MYC and ERK activity. Another kind of DMs-stimulated MYC, MYCN (also known as N-myc), is validated in neuroblastoma (NB)[13], which is associated with disease stage and prognosis[44]. In mechanism, MYCN interacts with the lysine-specific demethylase 1 (LSD1), both LSD1 and MYCN co-occupy at the promoter region of metastasis suppressor NDRG1 gene, consequently leading to the strengthened EMT, motility and invasiveness of NB cells[45]. In addition, the overexpressed lncRNA long stress-induced noncoding transcript 5 (LSINCT5) also physically interacts with NCYM to cause enhanced Wnt/β-catenin signaling and EMT activation[46].

In glioblastoma, EGFR gene located in circular DNAs is frequently amplified due to chromothripsis-originated extrachromosomal amplicons, and the copy number of the upstream enhancers of EGFR is high. Thus, both EGFR and its endogenous enhancers might show higher expression relying on circular DNAs-triggered amplification[47]. Functionally, circular DNAs-induced EGFR can activate protein kinase B (AKT) to enhance phosphorylation of Smad3 at S208 and boost its nuclear accumulation, causing up-regulated EMT-related genes. Profoundly, EGFR residing in circular DNAs also positively mediates janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3), vascular endothelial growth factor (VEGF)/matrix metallopeptidase (MMP), mitogen-activated protein kinase (MAPK) and ERK pathways, all of them consequently accelerate EMT phenotype[48-50]. Owing to the powerful and extensive functions of circular DNAs-boosted EGFR, the suppression of EGFR-mediated EMT by tyrosine kinase inhibitors (TKIs) is a frequent and effective strategy to prevent cancer invasion and metastasis. However, drug resistance emerges through some signaling bypassing EGFR after TKIs therapy[51]. For instance, circular DNAs-boosted EGFR highly activates MET amplification in EGFR-mutated cancers. Moreover, DMs-induced MET is capable of promoting Smad3/Snail activation, as well as boosting phosphatidylinositol 3-kinase (PI3K)/AKT/Snail

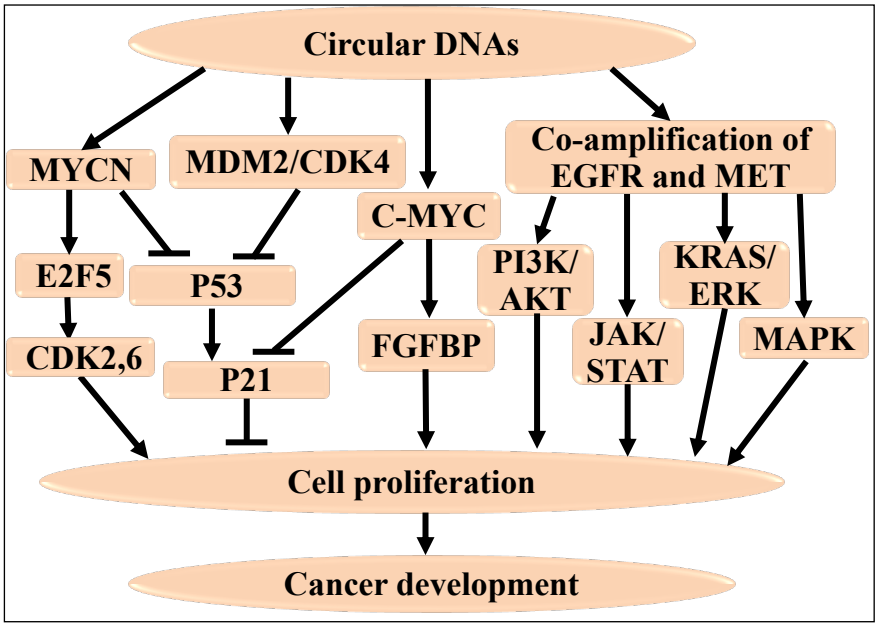

Figure 4 | Circular DNAs participate in cancer cells proliferation

Circular DNAs-induced MYCN oncoprotein positively regulates E2F5 expression, subsequently resulting in CDK2 and CDK6 expression to increase cell proliferation. Circular DNAs-stimulated MYCN and MDM2-CDK4 gene cluster amplification inhibit the anti-oncogenic functions of P53 protein. p53 further boosts p21 expression to prohibit cancer cells proliferation. Circular DNAs-induced C-MYC also directly contributes to p21 decrease and enhances cell proliferation. C-MYC up-regulates FGFBP to promote cell proliferation. EGFR and MET co-amplification triggered by circular DNAs constitutively activates MAPK, PI3K/AKT, KRAS/ERK and JAK/STAT pathways, facilitating cell proliferation and cancer development.

signaling to decrease E-cadherin and increase N-cadherin, Fibronectin, and Vimentin levels[52, 53]. Similarly, DMs amplification of mutant PDGFRA has also been found to mediate EMT process and chemotherapy resistance via up-regulating Snail, Slug and Zeb1 in glioma and sarcomas[34, 54]. Collectively, blockade of these pathways has a powerful effect on cancer treatment.

Circular DNAs Participate in Cell Proliferation

Cancer cells proliferation is a prominent activity that is regulated by various oncogenes residing in circular DNAs (**Figure 4**). p53 is an important anti-proliferative gene, which is frequently prohibited by multiple oncogenes existing in circular DNAs, like MDM2-CDK4 gene cluster and MYCN. In recent, DMs-triggered MDM2 gene amplification in NB and ecDNAs-stimulated MDM2-CDK4 co-amplification in Müllerian adenosarcomas have been detected, respectively[55, 56]. More interestingly, ecDNAs-stimulated MDM2-CDK4 gene cluster can bind to p53 protein and inhibit its anti-oncogenic functions[57]. Furthermore, co-administration of CDK4 inhibition with a MDM2 antagonist can stabilize p53, allowing p21 accumulation and arresting cell cycle[58]. Hence, co-targeting CDK4 and MDM2 locating in circular DNAs via suppressing circular DNAs would exert remarkably anti-proliferative function. In addition, DMs-stimulated MYCN in NB also directly represses p53 transcription, further facilitating MYCN-dependent cell proliferation[59]. In agreement with this, MYCN oncoprotein directly targets the E2F transcription factor 5 (E2F5) gene promoter, and positively regulate E2F expression, subsequently resulting in overexpressed CDKs, including CDK2 and CDK6. These data suggest that DMs-stimulated MYCN is essential for accelerating the proliferation of NB cells via triggering E2F5 up-regulation[60].

C-MYC acts as another type of circular DNAs-induced MYC, which promotes cell proliferation by enhancing glutaminase and glutamine synthetase activity, or mediating Warburg effect[41, 61]. Besides, C-MYC located in circular DNAs directly binds to the promoter region of fibroblast growth factor binding protein (FGFBP) to up-regulate FGFBP expression, then the up-regulated FGFBP promotes cell proliferation in pancreatic cancer[62]. It is worth noting that circular DNAs-activated C-MYC might be manipulated by the interaction of apolipoprotein E2 (ApoE2) with lipoprotein receptor related protein8 (LRP8). Detailly, the ApoE2/LRP8 complex phosphorylates ERK1/2 to activate C-MYC and then contributes to the reduced p21 activity, accompanied with up-regulation of cyclin D1, cdc2, and cyclin B1. Thus, C-MYC-down-regulated p21 is responsible for cancer cells proliferation[63]. Taken together, targeting the oncogenes-associated axis residing in circular DNAs might suppress cancer recurrence and progression, providing novel treatment strategies for various cancers.

Profound research has reported that there is a positive crosstalk between circular DNAs-stimulated EGFR and MET. Activation of MET/EGFR signaling functions as an important mediator for facilitating cancer cells proliferation[64]. Moreover, both EGFR and MET amplification in circular DNAs constitutively activate multiple proliferation-associated signaling, including MAPK[65, 66], PI3K/AKT[67, 68], KRAS/ERK[69] and JAK/STAT[67, 70] pathways. Further, activation of these pathways promotes G/S transition and increases cyclin D1 expression, and their corresponding inhibitors or co-inhibition of MET and EGFR may be a promising novel therapeutic strategy for suppressing cancer growth.

Circular DNAs Regulate Cancer Angiogenesis

Angiogenesis is defined as the formation of new blood vessels from preexisting vessels, which is important for delivering oxygen and nutrients to growing cancers. Circular DNAs participate in the regulation of several angiogenic molecules, such as VEGF and hypoxia-inducible factor-1α (HIF-1α), and blocking these signaling would slow the growth of many types of cancers (**Figure 5**). It has been identified that activation of PI3K/AKT signaling and GSK3β can increase MYCN

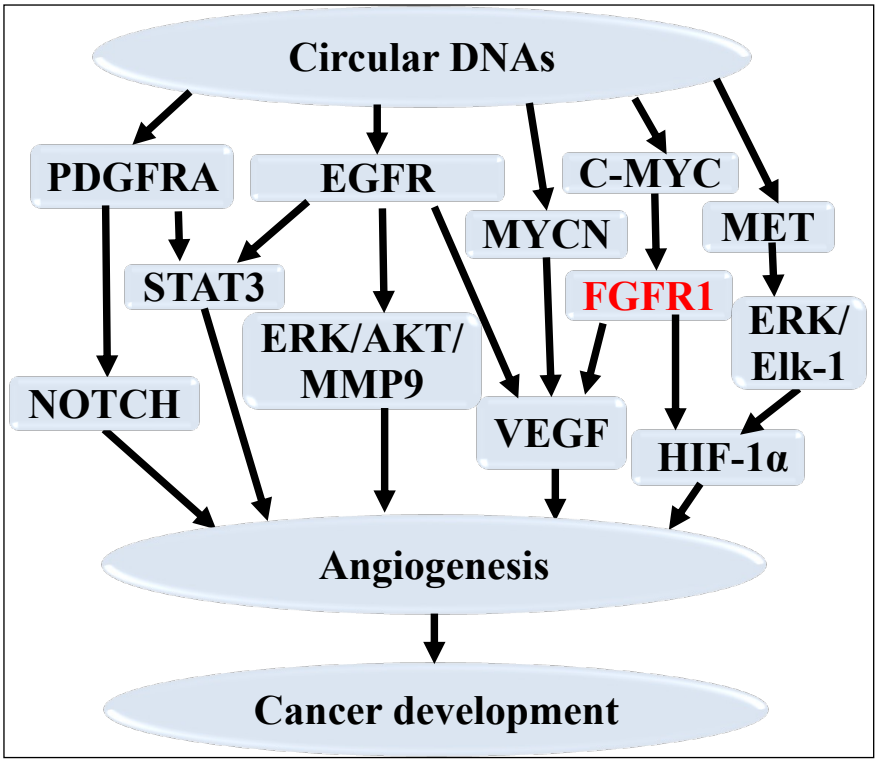

Figure 5 | Circular DNAs regulate cancer angiogenesis

Circular DNAs boost PDGFRA, EGFR, MYCN, C-MYC and MET expression. Circular DNAs-boosted PDGFRA activates NOTCH and STAT3 pathways, and mediates angiogenesis. Circular DNAs-mediated EGFR mediates angiogenesis through activating STAT3, ERK/AKT/MMP9 and VEGF signaling pathways. Circular DNAs-mediated MYCN triggers angiogenesis through VEGF. Circular DNAs-activated C-MYC mediates angiogenesis via activating FGFR1/VEGF and FGFR1/HIF-1α signal pathways. Circular DNAs-triggered MET mediates angiogenesis via activating ERK/HIF-1α signal pathway.

protein expression and further promote VEGF secretion, showing that circular DNAs-stimulated MYCN amplification could boost VEGF-induced angiogenesis[71, 72]. Further, inhibitors of PI3K/AKT and GSK3β signaling might exert an anti-angiogenic effect in vivo with decreased MYCN expression. HIF-1α is also considered as a significant regulator for angiogenesis. The reports have demonstrated that overexpression of C-MYC prevents HIF-1α degradation and increases mitochondrial ROS production in circular DNAs, which ultimately promotes formation of angiogenesis[73]. Consistently, C-MYC in circular DNAs positively correlates with pro-angiogenic molecules including VEGF and HIF-1α by associating with fibroblast growth factor receptor 1 (FGFR1) activation[74]. In addition, VEGF and HIF-1α are also induced by MET dependent on the activation of ERK/Elk-1 pathway[75]. Significantly, dual inhibition of c-MET kinase and VEGF receptor (VEGFR) shows great potential for overcoming HGF-induced EGFR-TKIs resistance in EGFR mutant lung cancer[76].

EGFR has been accepted as a multifunctional gene, which is extremely important for cancer angiogenesis via stimulating VEGF and ERK/AKT/MMP9 signaling pathways[77, 78]. In addition, STAT3 is a significant marker for cancer angiogenesis, which is markedly mediated by EGFR and PDGFRA residing in circular DNAs. The studies have found that salidroside-triggered downregulation of EGFR/STAT3 signaling pathway would inhibit angiogenesis through MMPs in breast cancer cells[79]. Of crucial note, in circular DNAs, PDGFRA activation can't only trigger STAT3 phosphorylation, but also result in NOTCH pathway activation companied with the up-regulation of PDGFs, ultimately leading to angiogenesis[80, 81].

Circular DNAs Participate in Immune Response

Effective immune response is indispensable for anticancer immunotherapy, noteworthy, circular DNAs-derived oncogenes have a remarkable impact on the functions of immune cells and the expression of immune checkpoints, further modulating immune response and immune evasion of cancer cells (**Figure 6**). Strikingly, circular DNAs-triggered MYCN overexpression occurs in different human malignancies, but majority of research about MYCN is mainly focused on high-risk NB. During NB progression, MYCN exerts immunosuppressive function through dampening the expression of ligands for NKG2D and DNAM1 activating receptors expressed on NK cells[82]. Similarly, another study has also demonstrated that MYCN participates in immunosuppressive pathways characterized by inhibition of T cells activation and increase of T cells inhibitory gene transcripts. Therefore, investigating effective DNA vaccination against MYCN would enhance antigen specific immune response[83].

Notably, programmed death ligand 1 (PD-L1) has been an attractive immune checkpoint that is partially governed by several oncogenes locating in circular DNAs, comprising C-MYC, EGFR and MET. C-MYC protein expression prominently affects immune response, and inactivation of C-MYC in non-small cell lung cancer (NSCLC) has the capability to reverse PD-L1-mediated immune escape[84]. Conversely, immune components would influence C-MYC amplification by blocking circular DNAs formation, causing tumor growth arrest and innate immune response activation[85]. As well, circular DNAs-boosted EGFR activation induces up-regulation of PD-L1 and triggers immune escape in EGFR-driven NSCLC, suggesting the prospective of optional immune targeted therapy for cancer patients with EGFR mutation[86].

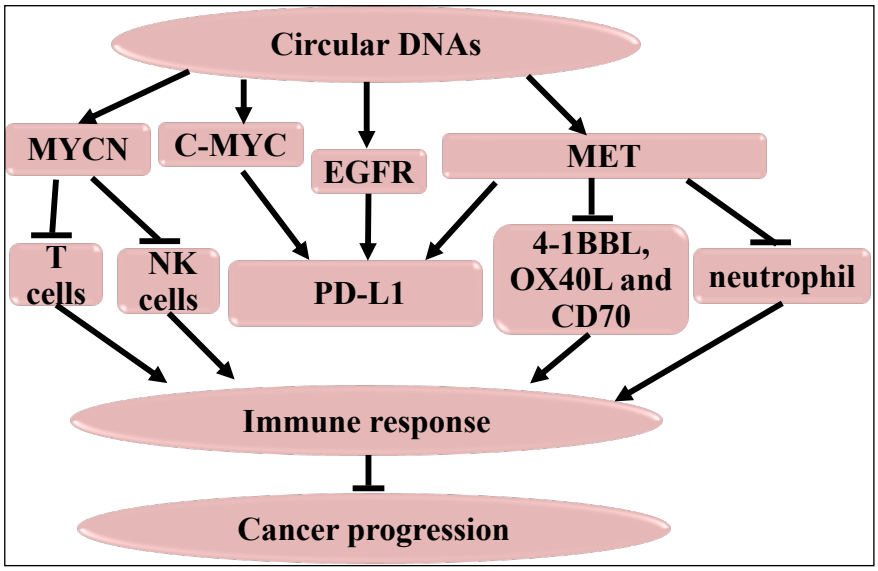

Figure 6 | Circular DNAs mediate tumor immune evasion

Circular DNAs activate MYCN, C-MYC, EGFR, and MET. Circular DNAs-activated MYCN attenuates T and NK cells, and mediates tumor immune evasion. Circular DNAs-activated MYCN, EGFR and MET mediate PD-L1 expression, and then induces tumor immune evasion. MET located in circular DNAs markedly down-regulates co-stimulatory molecules including 4-1BBL, OX40L and CD70, and represses neutrophil-dependent immune response.

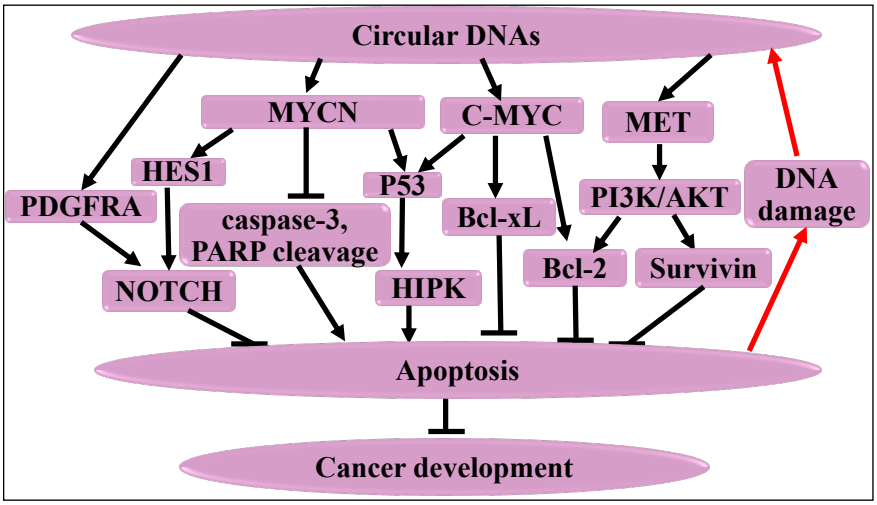

Figure 7 | Circular DNAs manipulate cell apoptosis

Circular DNAs activate NOTCH pathway through triggering PDGFRA and MYCN activation, further attenuating apoptosis. Circular DNAs-activated MYCN inhibits apoptosis through decreasing caspase-3 and PARP cleavage, but induces apoptosis through activating p53/HIPK signal pathway. Circular DNAs-activated C-MYC also induces apoptosis through activating p53/HIPK signal pathway, while inhibits apoptosis through regulating Bcl-xL expression. Circular DNAs-mediated MET activates PI3K/AKT signal pathway, increases expressions of survivin and Bcl-2, and attenuates apoptosis. And DNP- damage by apoptosis generates Circular DNAs.

Besides, MET located in circular DNAs is involved in the regulation of immune checkpoints. MET suppression using MET inhibitor or siRNAs increases co-stimulatory molecules expression, including 4-1BBL, OX40L and CD70, and down-regulates co-inhibitory molecules, especially PD-L1, indicating that MET activation would markedly increase PD-L1 expression and suppress anticancer immune response[87]. In agreement with this, interference with c-MET tyrosine kinase receptor can boost neutrophil-dependent immune response and heighten the effectiveness of immunotherapy even in the context of c-MET-independent cancers[88]. Collectively, targeting oncogenes in circular DNAs may be an effective treatment strategy for patients with oncogenes-dependent cancer when combined with immunotherapy.

Circular DNAs Manipulate Cancer Cells Apoptosis

Cell apoptosis might be a considerable source of circular DNAs that assist oncogenes amplification and overexpression (**Figure 7**). It is well acknowledged that NOTCH pathway is an important anti-apoptotic signaling, and it functionally activated by circular DNAs-induced PDGFRA and MYCN. MYCN activates NOTCH pathway via directly promoting HES1 expression, which suppresses drug-induced apoptosis and enhances chemoresistance of small-cell lung cancer (SCLC) cells in vitro and in vivo[89]. Accordingly, the NOTCH inhibitor FLI-60 would strengthen drug-induced apoptosis in SCLC. Consistently, down-regulation of MYCN gene via inhibiting circular DNAs formation would induce apoptosis, together with activation of caspase-3, enhancement of poly ADP-ribose polymerase (PARP) cleavage and up-regulation of RAF kinase inhibitor protein (RKIP)[90]. For another thing, circular DNAs-triggered MYCN also sensitizes cancer cells to apoptosis via stabilizing p53 and activating its pro-apoptotic homeodomain interacting protein kinase (HIPK)2[91]. Similar to MYCN, C-MYC existing in circular DNAs promotes anti-apoptotic Bcl-2 and Bcl-xL expression along with significantly decreased caspase-9 and caspase-3 activity[92], as well efficiently induces p53-dependent apoptosis following DNA damage in colorectal cancer[93]. The comprehensive data demonstrate that MYC resided in circular DNAs has a dual influence on apoptosis.

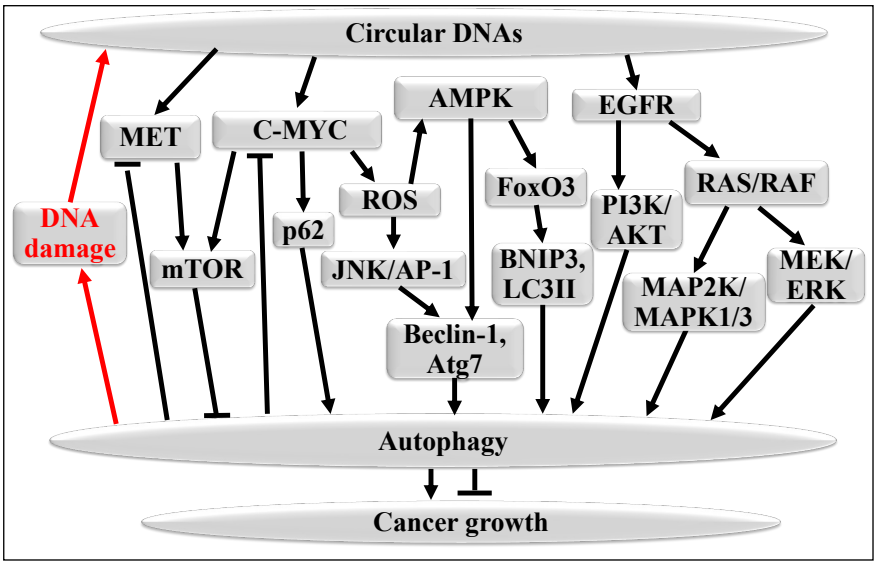

Figure 8 | Circular DNAs participate in autophagy

Circular DNAs mediate MET, C-MYC and EGFR expression, the circular DNAs-mediated MET and C-MYC activate mTOR pathway and inhibit autophagy, and the autophagy conversely attenuates MET and C-MYC expression. In other way, circular DNAs-mediated C-MYC accelerates autophagy through p16 and ROS/JNK/ Beclin-1 pathway. ROS, JNK, Beclin-1 and AMPK form a positive feedback circuit. Activated AMPK also mediates autophagy through FoxO3/ BNIP3 pathway. Circular DNAs-mediated EGFR enhances autophagy through PI3K/AKT, RAS/RAF/ MAPK and RAS/RAF/MEK pathways. Autophagy facilitates circular DNAs generation via inducing DNA damage, which further negatively or positively modulates tumor growth.

Additionally, oncogenic MET existed in circular DNAs exerts anti-apoptotic function via activating PI3K/AKT signaling accompanied by up-regulated Survivin and Bcl-2, which would represent radio-resistance with increased aldehyde dehydrogenase (ALDH) expression[94]. Thus, inhibition to circular DNAs-mediated MET can facilitate apoptosis and enhance treatment sensitivity. Combination treatment with a MET inhibitor and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) effectively heightens apoptosis in dedifferentiated liposarcoma patient-derived cells[95].

Circular DNAs Involve in ECM Remodeling

ECM components play a key role in tumor microenvironment (TME), which controls the overall cancer progression and therapeutic sensitivity. The interplay between ECM and circular DNAs is worthy of exploring. It has been reported that circular DNAs-induced C-MYC overexpression promotes ECM remodeling by triggering MMPs secretion during cancer progression, including MMP-2,7[43, 96]. In addition, cell free circular DNAs activate PDGF/PDGFR signaling, further profoundly stimulating cancer-associated fibroblasts (CAFs) activation. CAFs represent the most important stromal cells, which is responsible for deposition and remodeling of ECM components, as well as for the release of cytokines and growth factors in TME[97]. Theoretically, targeting of TME at the level of PDGF/PDGFR presents a prospective approach for future anticancer therapies.

The aberrant activation of circular DNAs containing MET has been implicated in ECM stiffness in a variety of human cancers, and the crosstalk between ECM components and MET activity is worth concerning. In circular DNAs, ECM-derived hepatocyte growth factor (HGF) in cancer and stromal cells acts as a multifunctional cytokine, which binds into c-MET receptor expressed in different cancer cells. Then, activated HGF/c-MET axis ultimately strengthens cancer cells proliferation, motility, angiogenesis, invasion and metastasis[98]. Of particular, highly selective c-MET kinase inhibitors may demonstrate promising potential for c-MET-driven cancer treatment, but efficiency of treatment is influenced by TME, especially HGF[99].

Circular DNAs Participate In Autophagy Regulation

Autophagy, as an evolutionarily conserved cellular recycling process, represses cancer initiation at the early stage and exacerbates cancer development at the later stage. Interestingly, autophagy might facilitate circular DNAs generation via inducing autophagic cells death and DNA damage, and circular DNAs comprising oncogenes inversely govern autophagy (**Figure 8**). It has been identified that oncogenes could negatively or positively regulate autophagy. On the one side, circular DNAs-stimulated C-MYC amplification enhances endoplasmic reticulum (ER) stress and triggers reactive oxygen species (ROS) generation, which stimulates autophagy via boosting CaMKII-mediated adenosine 5'-monophosphate-activated protein kinase (AMPK) phosphorylation and activating c-jun N-terminal kinase(JNK)/activator protein (AP)-1 pathway together with Beclin-1 and Atg7 up-regulation[100]. Likewise, another observation has noted that circular DNAs-mediated overexpression C-MYC increases AMPK phosphorylation and p62 expression. The boosted AMPK is found to stimulate the activity of FoxO3, then favoring the up-regulation of autophagy-related proteins BNIP3 and microtubule-associated protein light chain 3 II (LC3II) as a consequence of mitochondrial stress[101]. On the other side, oncogenes located in circular DNAs suppress autophagy by up-regulating mammalian target of rapamycin (mTOR) that is considered as a common autophagy regulator. Circular DNA-mediated MYC directly binds to regulatory regions of the eukaryotic initiation factor 4E-binding protein 1 (4EBP1) gene, a downstream molecule of mTOR, then increasing its expression and prohibiting autophagy[102]. Similarly, circular DNAs-mediated c-MET also contributes to AKT/mTOR signaling pathway activation to repress autophagy[103]. Autophagy could conversely regulate circular DNAs-induced oncogenes expression. It has verified that autophagy mediates c-MET inactivation and diminishes CRC malignancy at mTORC2-dependent manner[104]. Additionally, the pro-au-

tophagic protein AMBRA1 inversely contributes to protein phosphatase 2A (PP2A)-induced C-MYC dephosphorylation and degradation, which is regulated by mTOR[105].

The current emerging evidence indicates that circular DNAs-boosted EGFR can mediate RAS/RAF/mitogen-activated extracellular signal-regulated kinase (MEK)/ERK and PI3K/AKT/mTOR signaling pathways, these pathways play a critical role in the induction of autophagy in various cancers. Circular DNAs-induced EGFR doesn't only activate autophagy, but also involves in several other anti-proliferative events such as apoptosis and senescence[106]. Obviously, there is a closely relationship between autophagy and apoptosis, and both of them are frequently modulated by the same pathways. EGFR located in circular DNAs can induce activation of RAS/RAF1/MAP2K/MAPK1/3 signaling, it can't only stimulate autophagy with up-regulated LC3II, but also trigger apoptosis with increased cleavage of PARP1, caspase-3 and caspase-8. Moreover, the inhibition of MAPK1/3 pathway with U0126 or treatment with specific autophagy inhibitors prominently attenuates caspase-dependent apoptosis[106]. The combined data indicate that co-targeting of EGFR or circular DNAs and autophagy signaling is feasible for anticancer treatment. The current observation has proved that combination of anti-EGFR monoclonal antibodies (MoAbs) and autophagy inhibitors results in autophagic cells death and exerts anticancer efficiency[107].

CHALLENGE AND PROSPECTIVE

Currently, the potent functions of circular DNAs are attracting extensive attention since they have a fundamental impact on epigenetic regulation, chromatin accessibility and gene transcription[108]. Notably, circular DNAs can help intercellular communication, even over long distance or extracellular communication via transporting gene fragments to other cells, which suggests that RNA transcribed from circular DNAs may modulate oncogenes activity and protein production[20]. Thus, oncogenes existed in circular DNAs might show unique amplification advantages. In order to corroborate specificity, further studies will have to probe how far circular DNAs arises from DSB and elucidate how circular DNAs are transmitted from tissues into the circulation, which could depend on the death of cells or endosomal transport processes[109]. Although circular DNAs are free in cancer cells, it is worth deliberating that how circular DNAs reconcile with other forms of rearrangements, such as breakage–fusion–bridge, chromothripsis and chromoplexy formation.

Circular DNAs have a variable frequency in different cancer types[110]. Furthermore, the topology, location and biological functions of circular DNAs are markedly distinctive from chromosome DNAs, hence, alternative circular DNAs extraction and detection methods are urgently needed. New avenues are expected to visualize the chromatin structure, transcriptional landscape and functional distribution of circular DNAs, which will distinctly characterize their pathogenic roles in cancer and enlighten us to Figure out the impact of circular DNAs on oncogenes expression during cancer initiation, progression and resistance formation in chromosomal or extrachromosomal context.

It has been described that multiple parallel circular DNAs could simultaneously carry several different oncogenes, like the co-amplification of MYC and EGFR in circular DNAs. And intranuclear location and spatial organization of amplified oncogenes may have important consequences for the pathogenesis of cancer. However, it is unclear whether an individual cell may harbor multiple circular DNAs or whether these DNAs reflect co-existing subclones[21]. In general, extrachromosomal circular DNAs are considered to be more transient and in low amounts when patient-derived cancer cells are brought into culture due to lack of centromeres and potential absence of DNA synthesis at replication, which brings immense challenges to the clinical study of circular DNAs[33].

In clinical, the fragments of cell-free plasma DNAs have been applied for noninvasive prenatal testing and cancer testing[111, 112], this kind of DNAs demonstrates higher stability and longer survival than linear chromosomal DNAs due to the resistance to digestion by exonucleases and RNases. Likewise, circular DNAs also carry with higher stability and stronger amplification ability, as well as distinct dual-direct-repeat patterns over linear DNAs[113]. Hence, circular DNAs are being explored for sensitive and noninvasive diagnostic biomarkers of cancers due to their presence in blood, which brings a new insight into malignancy checking with non-delay, low expenses and low risks at the early stage. Besides the diagnostic value, the levels of circular DNAs are dynamically influenced by cancer treatment, and their genetic changes have extraordinary potential for quickly reflecting therapy response, detecting resistance and forecasting cancer recurrence[114]. In consequence, monitoring the generation of circular DNAs using liquid biopsy could exhibit distinctive diagnostic, prognostic and therapeutic perspectives for oncogenes-associated cancers.

CONCLUSION

In summary, the functions of newfound circular DNAs in eukaryotic cells have been identified, especially in cancer. DNA damage and repair, DNA replication and transcription processes are important sources for circular DNAs formation. The direct consequence of circular DNAs genesis remarkably increases copy number and expression of oncogenes residing on the extrachromosomal DNA elements, which is exhibited as a frequent genomic alteration involvement in genome remodeling and cancer heterogeneity. More significantly, circular DNAs-induced oncogenes amplification and overexpression promote cancer progression and evolution via accelerating diverse pro-oncogenic processes, containing EMT, cancer cells proliferation, angiogenesis, apoptosis, autophagy and ECM remodeling. Therefore, it is required for understanding the underlying molecular mechanisms of oncogenes amplification existing in circular DNAs, which might provide fundamental information for targeting circular DNAs or oncogenes inhibition. Although, there are many open issues to be

solved in circular DNAs' studies, detecting circular DNAs in the blood via liquid biopsy may be a novel and effective strategy for diagnosis, prognosis and treatment of cancers.

List of Abbreviations ALT, alternative lengthening of telomeres; **ALDH,** aldehyde dehydrogenase; **ALEMs**, amplification-linked extrachromosomal mutations; **ApoE2**, apolipoprotein E2; **AMPK**, adenosine 5'-monophosphate-activated protein kinase; **CIDER-seq**, circular DNAs enrichment sequencing; **Circle-seq**, circle sequencing; **CNS**, central nervous system; **CSR**, class switch recombination; **DSB**, double-strand break; **DMs**, double-minute chromosomes; **EMT**, epithelial-mesenchymal transition; **ECM**, extracellular matrix; **eccDNAs**, small extrachromosomal circular DNAs; **ecDNAs**, large extrachromosomal circular DNAs; **ECTRs**, extrachromosomal telomere repeats; **EGFR**, epidermal growth factor receptor; **4EBP1**, eukaryotic initiation factor 4E-binding protein 1; **GSK**, glycogen synthase kinase; **HR**, homologous recombination; **HIPK**, homeodomain interacting protein kinase; **HGF**, hepatocyte growth factor; **HIF-1*α***, hypoxia-inducible factor-1α; **LSINCT5**, long stress-induced noncoding transcript 5; **LSD1**, lysine-specific demethylase 1; **LC3II**, microtubule-associated protein light chain 3 II; **MMEJ**, microhomology-mediated end joining; **MSH3**, Mut S homolog 3; **MMR**, mismatch repair; **mTOR**, mammalian target of rapamycin; **MEK**, mitogen-activated extracellular signal-regulated kinase; **MoAbs**, monoclonal antibodies; **NHEJ**, non-homologous end joining; **NAHR**, non-allelic homologous recombination; **NB**, neuroblastoma; **NSCLC,** non-small cell lung cancer; **PDGFRA**, platelet—derived growth factor receptor α; **PI3K**, phosphatidylinositol 3-kinase; **LRP8**, lipoprotein receptor related protein8; **PD-L1**, programmed death ligand 1; **PP2A**, protein phosphatase 2A; **RCA**, rolling circle amplification; **STAT3**, signal transducer and activator of transcription 3; **SCLC**, small-cell lung cancer; **TERRA**, telomeric repeat-containing RNA; **TKIs**, tyrosine kinase inhibitors; **TRAIL**, tumor necrosis factor-related apoptosis-inducing ligand; **TME**, tumor microenvironment; **VEGF**, vascular endothelial growth factor; **WGS**, whole-genome sequencing.

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