Review Article
The biogenesis and roles of extrachromosomal oncogene involved in carcinogenesis and evolution

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Abstract: More and more extrachromosomal DNA (ecDNA) was found in human tumor cells in recent years, which has a high copy number in tumors and changes the expression of oncogenes, thus different from normal chromosomal DNA. These circular structures were identified to originate from chromosomes, and play critical roles in rapid carcinogenesis, tumor evolution and multidrug resistance. Therefore, this review mostly focuses on the biogenesis and regulation of extrachromosomal oncogene in ecDNA as well as its function and mechanism in tumors, which are of great significance for our comprehensive understanding of the role of ecDNA in tumor carcinogenic mechanism and are expected to provide ecDNA with the potential to be a new molecular target for the diagnosis and treatment of tumors.

Keywords: Extrachromosomal DNA (ecDNA), oncogene, high copy number, tumor

Introduction

Extrachromosomal DNA (ecDNA) refers to the extrachromosomal particles carrying genes originated from the chromosomal genome, which have been proven to be a kind of circular DNA molecule and participate in physiological or pathological processes in a special way [1-6], including double minutes, single body forms and forms lacking centromere or telomere [4]. As early as the 1970s, small double minutes were observed in the process of tumor cell division [5, 7, 8], in addition to the complete structure of chromosomes, accounting for 30% of ecDNA [1]. In addition to the extrachromosomal DNA formed by the shedding of proto-oncogenes from chromosomes, the human body also contains mitochondrial DNA [9], and non-organelle extrachromosomal elements (including t-loops, 5S rDNA, spcDNA, microDNA) [5, 7, 9-12], which are called extrachromosomal circular DNA (eccDNA), which are extrachromosomal particles different from ecDNA originat-

ing from chromosomes, and the circular form is invisible under a light microscope. The size of ecDNA found in tumors is 100-to-1000-fold larger than eccDNA and is usually in the range of 1-3 Mb or larger [4, 6]. EcDNA always carries multiple copies of oncogenes that drive tumor growth and survival and plays important roles in promoting tumor occurrence and development [1, 2, 4]. This review mainly summarizes the biogenesis, regulation principles, functions and molecular mechanisms of extrachromosomal oncogene in ecDNA in tumors and analyzes its clinical value and application prospects in cancer.

EcDNA leads to the high expression of oncogenes in tumor

EcDNA is specific to tumor

In human cells, the overexpression of oncogenes leads to the formation of malignant ones [3, 4]. EcDNA, which appears much more fre-

Table 1. Oncogenes expressed on ecDNA and their functions

Name of gene	Function related to tumor	Refs.
MYC	Promote cell proliferation and inhibit apoptosis	[1-4, 6, 22-31]
MYCN	Promote G1/S phase progression and tumor invasion	[2, 4, 13-15, 32-34]
EGFR	Promote tumor growth, inhibit apoptosis, lead to tumor drug resistance	[1-4, 6, 19, 20, 24, 35-37]
PEGFRA	Promote tumor proliferation and increases the adaptive potential of tumor cells	[2, 4, 35, 38]
Mecom-PIK3CA-SOX2 gene cluster	Promote tumor metastasis and recurrence	[2, 4]
CDK4-MDM2 gene cluster	Promote drug resistance in tumors	[2, 4, 6, 36]
REL	Promote tumor cell development and proliferation	[46]
ERBB2	Promote tumor growth and proliferation	[1, 35]
MDR1	Promote multidrug resistance in tumors	[47, 48]
CAD	Promote multidrug resistance in tumors	[40, 49, 50]
DHFR	Promote tumor cell resistance to methotrexate	[39-41]
MET	Promote the proliferation of tumor cells	[2, 4, 21]
CyclinD2	Promote tumor cell cycle progression and proliferation	[39, 51]
GBAs	Maintain the malignant features of the tumor	[6]
VOPP1	Promote tumor cell survival	[6]
MRPS17	By encoding mitochondrial ribosomal proteins, promoting tumor growth	[6]
LANCL2	Promote multidrug resistance in tumors	[6]
ZNF713	Involved in transcriptional regulation and tumorigenesis	[6]
SEPT14	Regulate the proliferation or apoptosis of tumor cells	[6]
CCND1	Promote tumor cell cycle progression and proliferation	[1, 35]
CCND3	Promote tumor cell cycle progression and proliferation	[1]
CCNE1	Promote tumor cell cycle progression and proliferation	[1, 35]
ATM	Promote tumor cell cycle progression, proliferation, and tumor growth	[1]

quently than expected, may be the "culprit" exacerbating the process. High-throughput sequencing was used to find that ecDNA is present in nearly one-half of human tumors, including those in neuroblastoma [13-15], colorectal cancer [16, 17], ovarian cancer [18, 19] and glioblastoma [2, 20, 21]. Its frequency varies with tumor type [3], but ecDNA is rarely seen in normal cells [1]. Similar to DNA on chromosomes, ecDNA guides gene transcription and induces gene expression to play certain tumor-promoting functions in the formation and development of malignant tumors [13, 16].

High copy number of oncogenes in ecDNA

Increasing data indicate that the rapid increase in the copy number of oncogenes in ecDNA is an effective and frequent gene amplification mechanism. For example, more than 200 oncogenes can be copied in HL60 cell lines [22], and many kinds of oncogenes in ecDNA amplified (**Table 1**), mainly includes *MYC* [1-4, 6, 22-31], *MYCN* [2, 4, 13-15, 32-34], *EGFR* [1-4, 6, 19, 20, 24, 35-37], *PDGFRA* [2, 4, 22, 38], *MET* [2, 4, 21] and *DHFR* [39-41] and *Mecom-PIK3CA-SOX2* gene cluster [2, 4] and *CDK4-MDM2* gene cluster [2, 4, 6, 36]. In the study of

mitochondrial organelle DNA copy number, it has been found that a change of mitochondrial DNA copy number leads to mitochondrial dysfunction, thus affecting the phenotype of tumor cells, but no high copies of the oncogene similar to that in ecDNA were found in mitochondria [42-44]. In the early stage, it was found that only some tumor cells show extensive ecDNA replication and highly express oncogenes, but it is not clear whether the mRNA is generated from ecDNA transcription. Pan-cancer analyses showed that [1], the oncogenes encoded by ecDNA are among the most highly expressed genes in the transcriptome of the tumors [6]. In addition, by combining the data of whole genome sequencing and RNA sequencing, Wu and his colleague proved that a large amount of transcribed oncogene mRNA was directly derived from ecDNA, not from the original sites of these genes on chromosomes, and this finding was verified in a large number of TCGA clinical samples [1]. We propose that, compared with the oncogene amplification in normal chromosomes, extrachromosomal oncogene amplification may increase the likelihood that a subpopulation of cells express the oncogene, enabling tumors to adapt to variable environmental conditions effectively to maxi-

mize their proliferation and survival [22, 36, 45]. Therefore, with the passage of time, the tumor gradually becomes more aggressive and more difficult to treat [1], and the prognosis becomes commensurately worse, however, chromosome amplification is difficult to achieve [1, 4].

Effects of ecDNA differences on tumor heterogeneity

EcDNA is different from normal chromosomal DNA in that it has no centromere structure [5, 22], causing ecDNA to be randomly segregated in daughter cells, possibly through a binomial model [14], resulting in nonuniform inheritance [52]. Through interphase fluorescence in situ hybridization (FISH) of tumor samples and Patient-Derived tumor Xenografts (PDXs), it was observed that the number of fluorescence signals in each nucleus varies greatly, ranging from 2 to 100 [2]. This heterogeneity strongly suggests that tumor cells form different tumor cell subpopulations diverge during mitosis, and the targeted gene DNA copy number in each cell also differs. Progeny cells may inherit ecDNA with a higher copy number of the driving oncogene, thus obtaining a proliferation advantage affecting the oncogenic potential of the cells [1, 2, 35]. Scanning electron microscopy revealed the clustering of double minutes (DMs) during metaphase in some tumor cells. When chromatids are separated from the equatorial plane, DM clusters adhere to chromosomal telomeres and are transported to spindle poles through this connection [53, 54], resulting in an uneven distribution in daughter cells. In addition, the size of ecDNA differs, and for specific tumors, intercellular variation may also exist, leading to a rapid increase in genomic heterogeneity [2, 20].

The mechanism of high expression of ecDNA oncogene in tumors

In tumors, oncogenes are highly expressed in ecDNA, and the mechanism is relatively complex, not only due to the increase of copy number, but also due to the high transcriptional activity of ecDNA itself, of which the spatial structure, regulatory elements and epigenetic modification of ecDNA play a key role. In addition, the regulation of mRNA stability and pro-

tein levels also promote the expression of oncogenes in ecDNA (**Figure 1**).

Regulation of copy number

The biogenesis of ecDNA is caused by the deletion of chromosomal DNA, resulting in the corresponding deletion of chromosomal sequence, or by a replication mechanism. The mechanism that leads to a very high copy number of oncogenes in tumors is not very clear. We summarized the formation mechanism of ecDNA mainly from three aspects, including chromosomal region replication, chromosomal extra DNA replication, and extra-chromosomal DNA replication. So far, no uniform replication methods of ecDNA have been reported. In addition to semiconservative replication, rolling-circle replication is likely to be a critical mode for ecDNA replication. The study of ecDNA formation and replication has important research value and clinical significance, as it may lead to the ability to block the production of ecDNA on the source, and thus to treat tumors.

Replication of chromosomal regions: Currently, all the evidences indicate that the formation of ecDNA is largely related to DNA damage, especially DNA double-strand breaks (DSBs) [19, 24, 41, 55]. A literature reported that lagging chromosomes can cause chromosome disruption in micronuclei, and then, through non-homologous end joining (NHEJ) mediated DNA repair promotes micronucleus-derived chromosome fragmentation to establish reconnection [56], thus forming ecDNA in offspring cells (**Figure 2A**). In addition to the ecDNA produced by DSBs caused by lagging chromosome, other factors, such as oxidative stress, senescence, carcinogens, DNA replication inhibitors and protein synthesis inhibitors, can lead to the formation of eccDNA [57-60]. It is known that eccDNA is different from ecDNA, and research on ecDNA is currently very limited. The amplified regions of DNA are derived from repeated and multiple replications of DNA, resulting in multiple copies of a particular gene [61]. In tumor cells, fragments can be amplified within chromosomes or they can be amplified in a manner that leads to the formation of ecDNA [3]. Some models suggest that the generation of ecDNA may be due to the removal of corresponding fragments from chromosomes during the G1 or G2 phase, and the high copy number
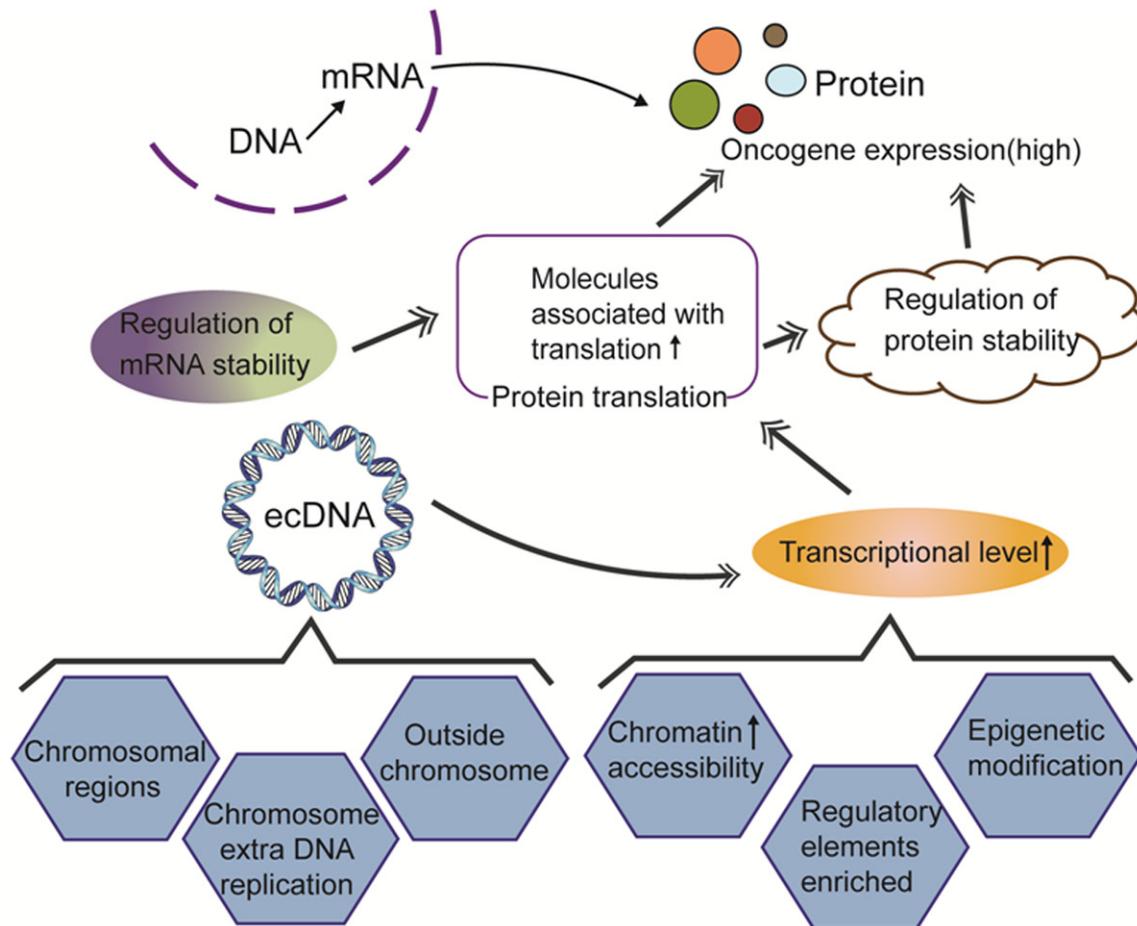
Figure 1. The mechanism of high expression of ecDNA oncogene. It is summarized from DNA level, transcriptional level, post-transcriptional level, translation level and post-translation level.

of DMs has been explained by the replication-excision model, which is based on chromosome regions during DNA replication [34, 62-65] (**Figure 2B**). The initial formation of extrachromosomal DNA molecules is caused by the cyclization of a single segment associated with intrachromosomal deletion, including the amplified but larger than this sequence, and the fusion of several syntenic or nonsyntenic DNA segments without further rearrangement of the chromosome or genetic correction of the deletion has also been observed [19, 37]. In the formation of loop amplicons, non-homologous end joining (NHEJ) [37, 57, 66, 67] and micro-homology-mediated end-joining (MMEJ) [19, 37] are the dominant mechanisms [24]. Another model suggests that circular DNA is generated by chromosome breakage on replication bubbles containing stalled replication forks [68], in which case, each circular element consists of two copies of the amplified sequence

connected in opposite directions (**Figure 2C**). Therefore, the replication of chromosome regions is the main mechanism of ecDNA production.

Chromosome extra DNA replication: A circular DNA molecule may be a product of recombination within the chromosome or extra copies formed by ectopic replication resumption (**Figure 2D**). Each replication starting point in eukaryotic cells is generally activated only once in each cell cycle, however, an extra round of DNA replication (replicon misfiring) can occur within any given chromosomal region. It has been found that extra copies of chromosomal domains produced by the misfiring of replicons can be converted into extrachromosomal chromatin fragments through the general recombinant enzyme system or special repair-type mechanisms [69]. Oncogenes are overabundant in drug-resistant cells, and some of these extra
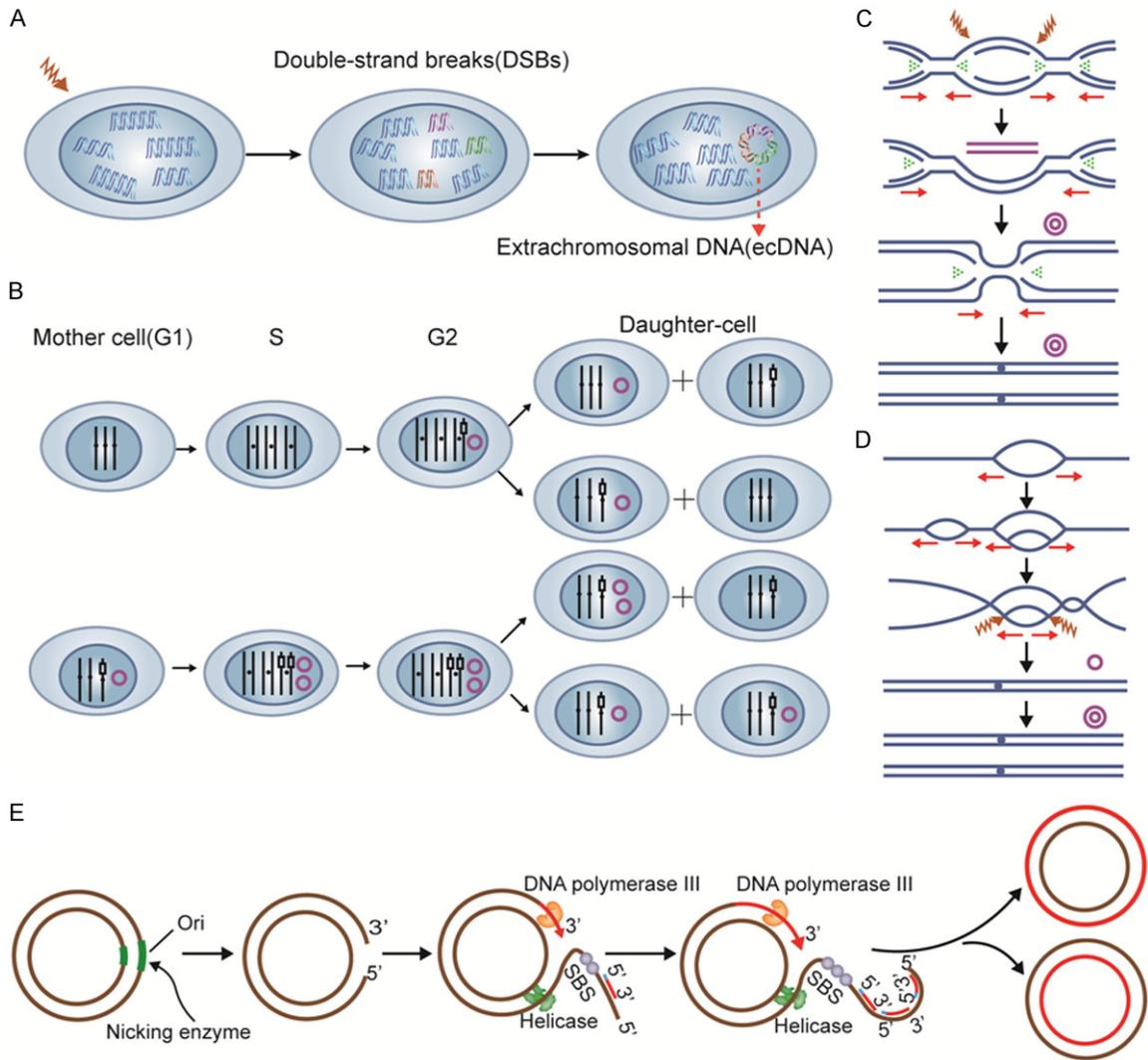
Figure 2. EcDNA production pattern. A. Under the pressure of replication, the double strands of chromosomal DNA are broken, and the extrachromosomal DNA is formed by non-homologous end joining. B. G1 or G2 chromosome segments are excised and cyclized to form ecDNA. Upper pathway: G2 chromosome fragment excision; lower pathway: G1 chromosome fragment excision. Rectangle on chromosome: deletion of chromosome segments. C. Chromosome breaks on replication bubbles form ecDNA. Arrows: replication fork movement direction; dotted triangles: replication fork position. D. Chromosome extra DNA replication (replicon misfiring). Extra copy excision produces ecDNA. E. A potential mechanism of ecDNA replication through the rolling ring model as eccDNA, however, it needs to be further confirmed in the future.

oncogenes are also present in ecDNA. These accumulated copies of oncogenes are most likely the result of frequently misfired replicons, with each misfiring resulting in an additional functional copy of the gene and its chromosomal domain [70, 71]. A similar phenomenon has been found in resistant cells [72]. Thus, chromosome extra DNA replication is also an important regulator of ecDNA high copy. Extra copies of genes in ecDNA are randomly distributed during mitosis, and as a result, so-

me cells acquire a higher number of copies [69].

Replication regulation of DNA outside chromosome: DMs constitute some of the ecDNA. Wahl and his colleagues found that DMs are composed of submicroscopic circular precursors called "episomes" [22], suggesting that "episomes" containing the *MDR1* oncogene are subject to the same replication mechanism (semiconservative replication) as regular

chromosomes, with approximately one replication per cell cycle [73]. Thus, their accumulation in tumor cells is not due to disproportionate DNA synthesis [54]. Structural studies of extrachromosomal DNA elements have shown that the initial amplification of extrachromosomal circular DNA derived from the chromosome may be proceed by various chromosomal breakage pathways. Once the initial amplicon is formed, it can be replicated autonomously [49], with an increase in copy number and a surge in tumor cells [74] (**Figure 3A**). In contrast to the chromosomal DNA copied at the chromosomal origin, plasmid DNA is independent of origin and is produced by rolling-circle replication [75]. Studies have suggested that eccDNA seems to replicate through the rolling ring mechanism [76], such as extrachromosomal t-loops [77], 5S rDNA [78], spcDNA [10, 79], and microDNA [66] (**Figure 2E**). Whether the copy number of ecDNA is increased by the rolling ring mechanism as eccDNA, which need to be further confirmed in the future.

Regulation of transcriptional level

In the study of the small circular DNA fragments (kilobase sized) edited by CRISPR-Cas9 [80], it was found that the genes encoding the enhanced green fluorescent protein were silent at the transcriptional level, which is quite different from the large fragments of ecDNA containing oncogenes and regulatory elements that have been our focus in this review. In cancer cell lines and clinical samples, the transcription levels of oncogenes amplified in ecDNA are higher than those of the same genes that have not been amplified by chromosome cyclization, which can increase the transcriptional abundance of oncogenes such that they constitute the top 1% of the whole cancer genome [1, 2, 4, 6]. Therefore, the regulation of the transcriptional level of the oncogenes in ecDNA is an important mechanism for their increased expression. Specifically, the increase of oncogene transcription level on ecDNA is mainly reflected in the following three aspects, including chromatin conformation, regulatory elements and epigenetic modification of ecDNA.

The open conformation of ecDNA chromatin: Chromatin conformation also affects the accessibility of DNA to transcriptional regulatory mechanisms [6]. Changes in the spatial structure of chromatin, including the destruction of the boundary of topological associating domains (TADs), are related to the pathogenesis of tumors [81]. The PLAC-seq/HI-ChIP method was used to prove that ecDNA also forms the same 3D functional domain as chromosome DNA, which is called topologically associating domains (TADs) [6, 81]. In addition, virtual 4C and actual 4C-seq also demonstrated that circular ecDNA can produce ultra-long-range DNA interactions, breaking the distance limit of gene relationships and enhancing gene interactions, which often have direct impact on the expression of the whole gene, further enhancing the expression of ecDNA itself (**Figure 3B**). Both ATAC-seq (inserting fragments into the "open" region of the genome using Tn5) and MNase-seq demonstrated that ecDNA is packaged as chromatin, with intact domain structures and composed of nucleosome units. In the ultrastructure of DMs, fibers composed of nucleosomes extend as loops from the chromosome core [74]. However, due to the lack of an advanced compression structure, ecDNA is more accessible than chromosomal DNA (**Figure 3C**). In addition, this chromatin openness is not dependent on the DNA sequence, only on whether it is associated with ecDNA [6]. As a result, almost all of the genes in the circular DNA can be successfully transcribed, boosting their transcription potential. Therefore, once the proto-oncogenes are no longer in the chromosome to form ecDNA, they can be expressed in large quantities.

Enhancers or other regulatory elements in ecDNA: The change in ecDNA expression can be explained by enhancer hijacking [82] or the destruction of cis-regulatory elements, and this circular rearrangement may lead to abnormal expression of tumor suppressors and proto-oncogenes [15]. The formation of ecDNA allows oncogenes to interact with adjacent enhancers (elements that enhance gene expression) to enhance their expression levels or even to establish new connections, promoting tumor development (**Figure 3D**). Under normal topological structure, the oncogene can be associated with only some enhancers (**Figure 3D**), but this relationship is significantly strengthened in ecDNA. This enhanced relationship can lead to the addition of new regulatory elements and changed the topological connections with oncogenes [81]. Abnormal enhancer activity is a
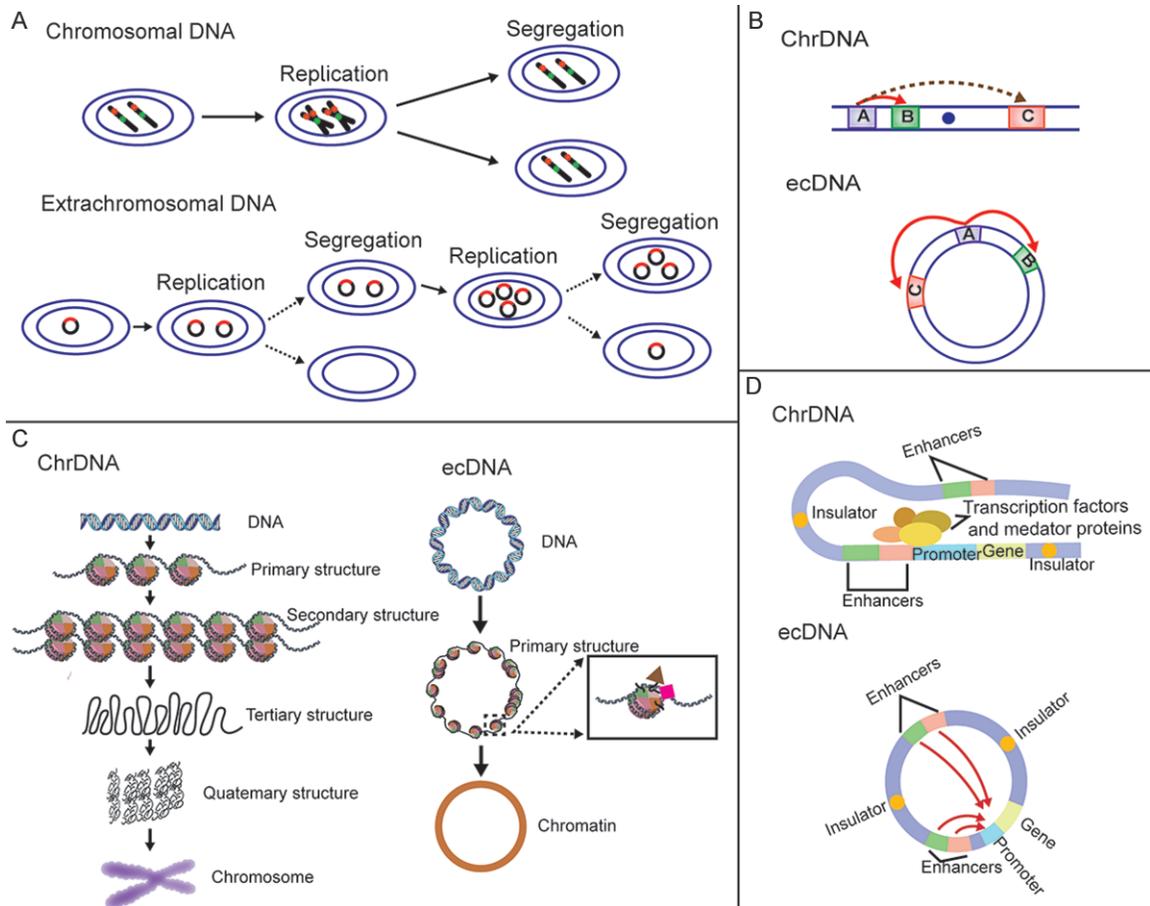
Figure 3. Expression regulation of oncogenes in ecDNA. A. Chromosome DNA is divides and equally allocated to two daughter cells during mitosis; EcDNA lacks centromeres and thus DNA is randomly assigned to daughter cells (dotted lines indicate a case of random assignment of ecDNA). B. EcDNA produces extremely long-distance DNA interactions. C. EcDNA is composed of nucleosomes and lacks the advanced compression structure of chromatin. In the dashed box, Δ and □ represent active histone modifications. D. In ecDNA, oncogenes interact with neighboring enhancers, making new connections and reinforcing existing expression patterns.

key driver of gene expression programs that promote tumor formation, maintenance, and progression. In addition, other regulatory elements are enriched in ecDNA. Matrix attachment regions (MARS) have been found in DMs. They are more often located in the noncoding regions of DNA and regulate the expression of target genes in vivo. Therefore, MARS can activate gene expression, determine which genes are transcribed, and have a strong influence on gene expression level [83, 84].

Epigenetic modification: Methylation is an important modification of proteins and nucleic acids that regulates the expression and suppression of genes and is closely related to the occurrence and development of tumors. It is one of the important research topics of epi-

genetics. The most common methylation modifications include DNA methylation and histone methylation [85]. CpG islands usually contain promoters of active genes and must therefore be accessible to transcription factors [86]. DNA methylation generally occurs at CpG site. Hypomethylated CpG islands are sensitive to nucleases added to the nucleus, while methylated CpG islands are not sensitized. Razin and his colleagues proposed that, in nuclease hypersensitive regions, *myc* gene amplification is increased [87], the CpG methylation level of DMs is generally low [83], and transcription activity is increased. Some histone residues can be methylated to inhibit or activate gene expression, forming epigenetic marks. In ecDNA, the activation and repression of histone marks in cancer cells in metaphase were analyzed by
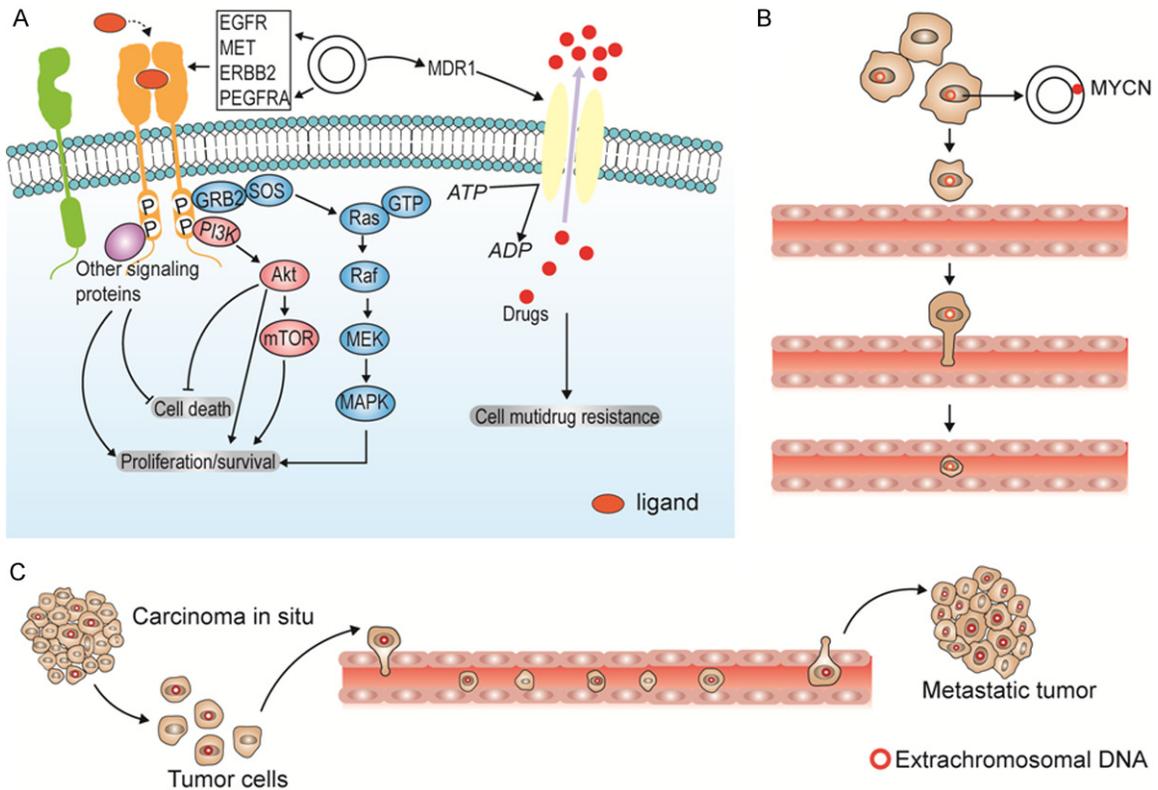
Figure 4. Diagram of the mechanism by which ecDNA plays a role in tumors. A. Some of the oncogenes in ecDNA have kinase activity. After binding with ligands, they activate intracellular signaling pathways, promote cell proliferation and inhibit apoptosis. The presence of resistance genes in ecDNA promotes multidrug resistance in tumor cells. B. Oncogenes on ecDNA, such as *MYCN*, promote the invasiveness of tumor cells. C. There is an increase in the amount of extrachromosomal DNA in metastatic tumors.

immunofluorescence, and chromatin immuno-precipitation based on H3K4me1 and H3K27ac was conducted. It was found that the frequency of ecDNA and histone modifications representing active gene transcription was high, while the inhibitory modifications to histone H3K9-me3 and H3K27me3 were few [6, 39] (**Figure 3C**). Studies have been conducted on the histone modification of DMs [83]. Moreover, ChIP-seq has also proved that these modifications are not evenly distributed throughout the whole ecDNA, being found mostly in the regulatory regions of corresponding genes, such as at promoters. Therefore, epigenetic modification is critical for the increase of oncogene expression in ecDNA.

Regulation of mRNA stability

The regulation of the stability of eukaryotic mRNA is one of the main mechanisms for regulating gene expression [88]. The sequence components of mRNA (the 5'-cap structure,

5'-untranslated region, coding area, 3'-untranslated region, polyA tail, and 5'- and 3'-terminal interactions), mRNA-binding protein (5'-cap-binding protein, coding region-binding protein, 3'UTR-binding protein, and polyA-binding protein), translation products of mRNA (autonomic regulation), nuclease, viruses and other factors can regulate the stability of the mRNA. The study of mRNA stability regulation is helpful for understanding the gene expression of ecDNA in more detail. Previous studies on DMs have indicated that DMs are related to the interchromosomal domain (ICD) in the HD-N-16 neuroblastoma cell line, which is believed to contain macromolecular complexes involved in transcription, splicing, DNA replication and repair [89, 90]. Therefore, the preferential localization of DMs on the surface of the chromosome region may be interpreted as a preference that facilitates the entry of the amplified *MYCN* gene into the protein complex for transcription and splicing [90].

Regulation of translation level and protein stability

In the process of ecDNA translation, there is a close correlation between the copy number of the oncogenes in ecDNA and the level of protein expression. The initiation of protein translation in eukaryotes is a complex cellular process that involves a series of proteins called eukaryotic translation initiation factors (eIFs). The initiation of translation in eukaryotes is accomplished by the interaction between these eIFs and other eukaryotic initiation factors and ribosomes, mRNA and initiation tRNA. Eukaryotic initiation factor 5A2 (eIF-5A2) is a member of the eukaryotic initiation factor 5A subfamily [91]. It has been found that eIF-5A2 exists in DMs, and the overexpression of eIF-5A2 is significantly correlated with the deterioration of ovarian cancer and plays an important role in the pathogenesis of ovarian cancer [92]. When the double minute copy number is decreased, the expression level of eIF-5A2 is decreased, and the growth rate of tumor cells is inhibited. Currently, only eIF-5A2 has been found in DM, which does not reflect the unified mechanism of ecDNA translation.

The role of ecDNA in tumorigenesis and the development of multidrug resistance

The role of ecDNA in tumor proliferation

The high expression of oncogenes in ecDNA enables tumor cells to acquire greater proliferation capacity and genetic plasticity. There are a variety of oncogenes in ecDNA, and the genomic variation acquired before and during the whole process of tumorigenesis provides cancer cells with a competitive advantage over neighboring cells, thus improving the survival and proliferation rates and stronger clone formation ability [35, 93]. For examples, *MET* [21, 94], *EGFR* [37], *ERBB2* [35], and *PEGFRA* [35, 38] have receptor tyrosine kinase (RTK) activity induced by ligand binding, and they strictly regulate the stimulation of proliferation and survival through cell signaling pathways, most notably the RAS/RAF/MEK/MAPK and PI3K/Akt pathways, which provide the relevant upstream and downstream proliferative signal, leading to an increase in the adaptive potential of tumor cells [20, 35, 94] (**Figure 4A**). EcDNA carrying *MET*, *EGFR* and *MYC* genes has also

been found in recurrent tumors, maintaining the malignant characteristics of tumors [2]. The oncogene *c-myc* is present on ecDNA, and as a classical nuclear transcription factor [95], its abnormal activation can trigger the initiation of a complex genomic unstable network [96-98]. In nasopharyngeal carcinoma (NPC), abnormal overexpression of *c-myc* alters the expression of target genes, many of which are carcinogenic genes or tumor suppressor genes, thereby significantly promoting cell proliferation, and is significantly associated with a poor prognosis for NPC patients [99]. In addition, studies *in vitro* and *in vivo* have demonstrated that the expression of *c-myc* alone is not sufficient to induce tumorigenesis, and its function combined with that of other oncogenes is needed [100]. In fact, the deregulated expression of the *c-myc* gene can lead to genomic instability, manifested as amplification of the extrachromosomal gene. The extrachromosomal DNA produced upon *c-myc* overexpression is, on average, 10-fold larger than that observed when *c-myc* is not deregulation [7, 39]. In neuroblastoma, most of the extrachromosomal DNA formed is cycled in the region of chromosome 1p36, and because of the growth suppressor genes or tumor suppressor genes in chromosome 1, it is thought that the malignant phenotype of the tumor may be related to the loss of tumor suppressor genes, which enables the tumor to gain an advantage [101-103]. EcDNA is usually derived from DSBs during DNA replication, and DSB repair involves the P53 pathway [104], whether there is an association between ecDNA and cell cycle. The abundant expression and amplification of *MDM2* in neuroblastoma will counteract the transcriptional activity of endogenous *P53*, thus promoting tumor development by inhibiting *P53* [105]. In the process of homologous recombination (HR), the BRCA1 protein plays an important role in the HR pathway and controls the cell cycle by activating checkpoints (such as G2/M). In MTX-resistant cells containing DMs, and the inhibition of HR activity results in a decreased number of copies of the DM amplification gene, increased sensitivity to methotrexate (MTX), and accelerated cell death [17]. Tumor cell proliferation is often associated with poor prognosis and is a cause for resistance to treatment. Clinical staging is an important factor that affects the prognosis of patients with tumors. In patients with neuroblastoma, the prognosis of

stage I and stage II is good, while that of stage III and stage IV is poor. *MYCN* amplification has a strong correlation with stage III and stage IV [101]. The MYCN protein forms a heterodimer with MAX, which acts as a transcriptional activator of target genes and promotes G1/S phase progress in the cell cycle, with a higher total frequency of extrachromosomal amplification [101]. *REL* encodes the nuclear factor-κB (NF-κB) transcription factor, regulates the expression of genes and promotes the proliferation of tumor cells [46]. Therefore, it is of prognostic value to identify the genes and pathways that promote tumor amplification to ensure that they are considered as part of the combination therapy.

The role of ecDNA in tumor invasion and metastasis

EcDNA plays a role in the progression of tumor cells to a more aggressive, malignant phenotype. The discovery of ecDNA in human cancer cells has shed new light on the three-dimensional structure of the tumor genome and epigenome. The study of DMs in tumor invasion and metastasis provides a mechanistic basis for our understanding of the roles of ecDNA. Invasion is one of the ten characteristics of tumors that can turn surrounding normal cells into tumor cells [106]. In the same gene GBM cell line, the invasive phenotype of cells with DMs has been shown, and irradiated STIC cells (containing DM) have paracrine effects on the newly formed STIC cells that were not irradiated, thereby promoting tumor development by promoting cell invasion and angiogenesis [107]. In many neuroblastomas, ecDNA carrying the *MYCN* oncogene is also associated with invasive tumor growth (**Figure 4B**). The cancer is found not only in situ but also metastasizes remotely [106]. There are a large number of oncogenes in ecDNA. Is there a gene level transfer similar to plasmid transfer? *In vivo*, it is difficult for cells to fall off and move to other places, either because of cell adhesion or the killing effect of the immune system; however, DNA transfer is much easier. The presence of DMs can be considered a repeated and overlapping secondary change, which is more frequent in metastatic lesions [108]. DMs appear in the secondary malignant effusion of ovarian cancer [108], metastatic ASML variants [109], malignant fibrous histocytoma in bone [110], and

recurrent GBM tumors, which show many structural and numerical abnormalities compared with those of the primary tumor. The chromosomal morphology of metastatic tumor is poor, and the amplification of oncogenes in ecDNA is increased [109, 110] (**Figure 4C**).

The occurrence and metastasis of tumors are closely related to the internal and external environment of tumor cells. The conditions for tumor survival and development can be changed and maintained by changing the tumor microenvironment. Irradiated cells containing DMs show the ability to change their extracellular microenvironment, promote the invasiveness of surrounding cells, and create a tumor microenvironment that promotes tumor growth [107]. In the case of changes in environmental conditions, the number of DMs can be regulated and even reduced to zero. For example, mutations and amplified genes in the ecDNA in primary GBM tumors are lost during cell culture, accompanied by significant phenotypic and transcriptional differences [9, 35, 111]. Amplification in the form of HSR impossiblely has the same mechanism as ecDNA. This observation suggests that the selection and competition between amplified DNA copies with different genetic backgrounds are different [35]. In summary, ecDNA promotes rapid diversification of the genome and increases the possibility that cells more suitable to changing environments are selected. Differences in ecDNA in tumor cells *in vivo* and *in vitro* constitute a basis for future research, better understanding the molecular mechanisms by which ecDNA drive cancers alter the tumor microenvironment to evade treatment and determining whether changing the relationship between ecDNA and the tumor microenvironment can inhibit tumor progression.

The role of ecDNA in drug resistance

The expression of both oncogenes and drug-resistant genes in ecDNA increases the degree of tumor progression and drug resistance. Oncogenes in specific ecDNA in cells also change with environmental changes; for example, in glioblastoma, they often lead to carcinogenic variation of *EGFRvIII* [112, 113]. Tumor cells can develop drug resistance through the amplification of oncogenes in ecDNA, and as many as 20 DMs containing *DHFR* genes can

be found in some methotrexate-resistant cell lines [41, 70, 114, 115]. DMs are usually detected in cells that are resistant to increasing drug concentrations, and CAD protein is a multifunctional protein that, in the presence of the specific inhibitor PALA, leads to the generation of DMs containing the *CAD* gene [49, 50]. In addition, the random distribution of ecDNA during mitosis leads to the cells that acquire enhanced mutations being more likely to transmit these mutations to their offspring cells, thus promoting tumor progression and therapeutic resistance [116, 117]. Extrachromosomal DNA is also a carrier of drug-resistant genes [29], and the amplification of drug-resistant genes may allow tumor cells to grow in the presence of cytotoxic drugs, which may be related to the overproduction of the corresponding proteins. An extrachromosomal copy of *MDR1* was found during the treatment of multidrug-resistant KB cells. The *MDR1* gene encodes a membrane-binding protein called p-glycoprotein, which is a multidrug transporter that acts as an ATP-dependent efflux pump for chemotherapy drugs, leading to drug resistance in tumor cells [47] (**Figure 4A**). In the process of drug treatment to "eliminate" ecDNA oncogenes, it was found that, once the drug was withdrawn, ecDNA revives and reappears, which was related to drug resistance during tumor treatment and could affect the efficacy of targeted therapy related to the oncogene [36]. Resistance to *EGFR* tyrosine kinase inhibitors (EGFR-TKIs) leads to the reoccurrence of clonal *EGFR* mutations on the extrachromosomal DNA after drug withdrawal. The $MDM2^+$ DM copy number also increased with increasing erlotinib treatment and remained elevated even after drug withdrawal [9, 36]. After the recurrence of the disease, at least one cancer driver of ecDNA is retained in many tumor cells, supporting the idea that ecDNA can prevail after selective pressure is applied to anticancer therapies. This outcome was demonstrated by the significantly reduced time to secondary surgery in patients with at least one ecDNA compared to patients with primary tumors without ecDNA [2]. These results suggest that cancer can evade therapies for target oncogenes maintained on extrachromosomal DNA in a highly specific, dynamic and adaptive way, elucidating the mechanism of drug resistance of cancer cells containing ecDNA, opening up new perspectives on drug treatment regimens, and helping us further explore ways to treat tumors.

EcDNA is expected to be a potential molecular target for tumor diagnosis and treatment

The value of ecDNA in diagnosis

Tissue biopsy detection of ecDNA is used for tumor diagnosis. The morphological characteristics of tumor cells and the expression level of oncogenes can be histologically observed [20]. EcDNA is visible under the microscope. To distinguish the extrachromosomal circular DNA molecules in the normal chromosomes, histological observations together with a measure of the expression level of the coding oncogenes in biopsied tissue samples can be used in combination, and ecDNA can be used in the diagnosis of tumors. However, tissue biopsy requires surgery or other means of acquiring biopsy materials, which may cause some degree of trauma to the subject.

Liquid biopsy can be used to detect ecDNA for tumor diagnosis. Liquid biopsy does not require surgery or puncture sampling, which can reduce the pain for patients. Compared with tissue biopsy, the sample requirements are lower, and it is easy to avoid the deviations caused by heterogeneity. High-throughput sequencing techniques are used with liquid biopsy samples to identify tumor-specific linear DNA fragments present in serum or plasma [118]. Free DNA (CfDNA) has also been found in blood circulation, but its content is very low and limited to only linear DNA [12]. Circular DNA crosses cell membranes relatively easy, making it more likely that cancer patients will have ecDNA in their blood. In tumors, high expression of oncogenes in ecDNA is strongly associated with clinical diagnosis, treatment, and prognosis. EccDNA was recently found in circulation [12, 54, 119]. These circular molecules are released into the blood circulation after cell death. Circle-seq can be used to purify eccDNA [55, 120]; which makes it possible for detecting by liquid biopsy. If the ecDNA detected in liquid biopsy samples can provide ultra early diagnosis of tumor and respond to treatment, then ecDNA can be used as a diagnostic and posttreatment evaluation indicator for tumor patients. For patients after treatment, the number of oncogenes in eCDNA in the tumor can be monitored to help make an accurate judgment on the prognosis of patients. However, currently, there are many eccDNAs in tissues, and we need to identify its source. The differences between eccDNA and ecDNA, as discussed in this paper, include size and other measures. The development of specific meth-

ods to distinguish ecDNA from other extrachromosomal DNA would enable disease progression to be monitored.

The value of ecDNA in tumor therapy

Understanding the underlying molecular mechanisms of tumor evolution can help identify more effective therapies to eradicate tumors. EcDNA is an important mechanism driving the copy number variation of oncogenes, which is closely related to the curative effects and the prognosis of tumors. For example, in patients with non-small-cell lung cancer (NSCLC), there is no significant correlation between the number of mutations in tumor cells and prognosis, but patients with more changes in oncogene copy number have worse tumor efficacy and prognosis [121]. Moreover, ecDNA also plays an important biological function in the development of tumors, which can drive the development, invasion and metastasis of tumors and the formation of multidrug resistance. Therefore, the elimination of ecDNA or oncogenes amplified in ecDNA can effectively reduce the malignancy of tumor cells and can be the basis of tumor therapy. The discovery of ecDNA may lead to a fourth revolution in cancer treatment, according to Boundless Bio, which believes that the replication mechanism of ecDNA is slightly different from that of normal chromosomal DNA, leading to a way to specifically inhibit ecDNA replication directly. Another approach Boundless Bio is exploring involves the inhibition of the metabolic pathways required for ecDNA replication.

The frequency and amplitude of oncogene amplification can be changed with various stimuli, and radiotherapy can destroy all cancer cells as much as possible. Compared with those in unirradiated cells, the gene copy number and expression level of amplified genes in the extrachromosomal DNA of irradiated cells were significantly reduced after exposure to relatively low doses of radiation [47, 122]. Unfortunately, these treatments are limited to the area of exposure, rarely leading to cures, with most tumors locally recrudescing within a few months. In addition, some drugs can effectively reduce the copy number of ecDNA and thus achieve a certain therapeutic effect. In the process of drug-induced differentiation of HL-60 cells into granulocytes, *c-myc* expression was decreased and *c-myc* copies were lost in dou-

ble minutes [26, 31]. Low doses of hydroxyurea and etoposide led to the degradation of extrachromosomal circular DNA molecules into smaller DNA fragments [48], accelerating the loss of oncogene amplification (such as *EGFR* and *c-myc*) in ecDNA [35, 40, 47, 123-127] in a dose- and time-dependent manner [124]. Low concentrations of hydroxyurea showed preclinical activity by eliminating ecDNA, reducing the tumorigenicity of human tumor cells [128], and inducing apoptosis in some cases [23], without including cytotoxic effects or overall toxicity [18, 40, 125, 128]. In addition, hydroxyure (HU) combined with cisplatin has shown enhanced cytotoxicity in tumor cells [129]. Gemcitabine was able to decrease the number of ecDNA in cells at a 7500-fold lower concentration than that used for the common cancer drug hydroxyurea, and it also inhibited the growth, colony formation and invasion of tumor cells [130]. Intricate pathways in cells play different roles in gene amplification and may be new targets to improve the effect of tumor chemotherapy by reduced amplification [32]. CRISPRi can also be used to eliminate ecDNA, but whether the CRISPR technology used in research can be used in the clinic remains to be discerned.

Conclusions and future directions

EcDNA is an important recent discovery in the field of tumor research. Because of its specific presence in tumor tissues and cells and the high copy number and high expression levels of its oncogenes, ecDNA can greatly drive the occurrence and development of tumors and the formation of malignant phenotypes, such as multidrug resistance. Therefore, in-depth study into the formation, role and mechanism of ecDNA in tumor development is expected to lead to important breakthroughs in the study of tumor pathogenesis in the future, and ecDNA is also expected to become an important molecular target for the diagnosis and treatment of tumors in the future. However, at the same time, it must be acknowledged that ecDNA lacks centromeres, and the uneven distribution of ecDNA during mitosis increases the heterogeneity of tumors and the difficulty of tumor diagnosis and treatment. In recent years, although important advances and discoveries have been made in the study of the formation of ecDNA and its role and mechanism in tumors, many problems remain unresolved.

For example, is ecDNA the primary mechanism that drives tumor development? Are there tumor suppressor genes and normal genes in ecDNA, and if they exist, what are their functions? Does ecDNA have a uniform replication mechanism? Is it the same as plasmid or eccDNA rolling ring replication? What is the uniform mechanism of ecDNA elimination? Can the results of research on DMs be applied to into all ecDNA? All of these questions require continuous exploration and research by scientists.

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Disclosure of conflict of interest

None.

Abbreviations

EcDNA, extrachromosomal DNA; 5S rDNA, 5S ribosomal DNA; SpcDNA, small poly-dispersed circular DNA; EccDNA, extrachromosomal circular DNA; EGFR, epidermal growth factor receptor; PDGFA, PDGF receptor α; DHFR, dihydrofolate reductase; TCGA, The cancer genome atlas; FISH, fluorescence in situ hybridization; PDX, Patient-Derived tumor Xenograft; DMs, double minutes; DSBs, DNA double-strand breaks; NHEJ, non-homologous end joining; MMEJ, microhomology-mediated end-joining; MDR1, multi-drug resistance gene-1; TADs, topological associating domains; PLAC-seq, proximity ligation-assisted ChIP-seq; HI-ChIP, In situ Hi-C followed by chromatin immunoprecipitation; ATAC-seq, Assay for Transposase Accessible Chromatin with high-throughput sequencing; MARS, matrix attachment regions; ICD, interchromosomal domain; eIFs, eukaryotic translation initiation factors; eIF-5A2, eukaryotic initiation factor 5A2; RTK, receptor tyrosine kinase; NPC, nasopharyngeal carcinoma; HR, homologous recombination; BRCA1, breast cancer susceptibility gene 1; MTX, methotrexate; GBM, glioblastoma multiforme; STIC, stem-like tumor initiating cells; HSR, homogeneously staining region; PALA, N-phosphonace-

tyl-L-aspartate; CfDNA, cell free DNA; NSCLC, non-small-cell lung cancer; HU, hydroxyurea; CRISPRi, CRISPR interference or inhibition.

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