Molecular Pharmacology Fast Forward. Published on August 8, 2022 as DOI: 10.1124/molpharm.122.000553
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Extrachromosomal circular DNAs (eccDNAs), Amplified Oncogenes, and CRISPR–Cas9 System

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Running title: **Extrachromosomal circular DNAs (eccDNAs) and CRISPR–Cas9 System**

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Abstract

Structurally rearranged extrachromosomal circular DNAs (eccDNAs) have been identified in tumor cells, many of which carrying regions related to cancer-driver recurrent oncogenes (e.g. *CCND1*, *EGFR* and *MYC)*. In a tumor cell, eccDNAs are carrying regions associated with oncogene amplification (>10-fold amplified-copy numbers in human tumors) and poor outcome across multiple cancers. Even though dual-delivery of pairs of CRISPR/Cas9 guide RNAs into human normal cells was reported to induce circularization of genes and chromosomes, but in bacteria, CRISPR-Cas9 system primarily targets extrachromosomal rearranged elements. Likewise, in cancer cells, it is expected that a designed CRISPR-Cas9 system is able to target extrachromosomal copy-number amplifications and produces double strand breaks to be detrimental to cellular fitness through dictating gene-independent copy-number loss-of-fitness (LOF) effects and anti-proliferative responses**.** A designed system against amplified amplicons may provide a novel approach for cancer therapy and propose a practical implication for CRISPR-Cas9 as a path in therapeutic strategies of cancer.

Keywords: Extrachromosomal circular DNA (eccDNA), cancerous cell, CRISPR–Cas9, oncogenic amplicons.

Significance statement: Structurally rearranged extrachromosomal circular DNAs (eccDNAs) have been identified in tumor cells. Many of eccDNAs are carrying regions related to cancer-driver recurrent oncogenes (e.g. *CCND1*, *EGFR* and *MYC)*.

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It is expected that a designed CRISPR-Cas9 system is able to target extrachromosomal recurrent oncogenes.

1. Introduction

Extrachromosomal circular DNAs (eccDNAs) that are present in both prokaryotes and eukaryotes, are anticipated to be the major source of somatic rearrangements. Circular DNA elements act as supplements for chromosomal genetic material that deviate from normal rules of Mendelian inheritance with accelerated mutation (Koche et al., 2020; Hull and Houseley, 2020). In human cancers, however, eccDNAs have been reported to be associated with copy-number amplification of proto-oncogenes and transposable elements circularization, invasion of genes and mutagenesis (Koche et al., 2020; Møller et al., 2018). Whole-genome sequencing analysis of cancer patients has demonstrated that eccDNA-based oncogene amplification is common in cancers. EccDNAs and extrachromosomal rearrangements are different from chromosomal amplicons, where deriving multiple copy-number genes across cancer types and are associated with poor outcomes in patients. In compare to chromosomal amplifications, patients with eccDNA-based oncogene amplifications have shown shorter survival. Cancer types contain certain eccDNAs (Table 1) with high oncogene amplification and recurrence in individuals. The most common and recurrent oncogenes or related regulatory sequences are highly amplified on eccDNAs (Table 1). Additionally, in compare to copy number-matched chromosomal oncogenes, eccDNA-related amplicons exhibit enhanced accessibility where can lead to higher levels of oncogene transcription and frequent fusions of the transcripts (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020).

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Circular and chimeric DNA elements are able to reintegrate into the genome, cause chromatin remodeling and oncogenic rearrangements (Koche et al., 2020). Thereby, eccDNAs are able to derive complex chromatin rearrangements and an ongoing mutagenic process through which lead to further cancer lesions and poor outcomes (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020)**.**

Even though, in human somatic cells, eccDNAs are most likely arising by mutational processes, but in their own, they can cause genome-wide, driving-mutagenic processes.

1) EccDNAs become highly amplified and derive extrachromosomal oncogene amplification by carrying a large, copy number of oncogenes and accumulating in soma cells,
2) They contribute to elevated levels of oncogene transcripts through chimeric circularization and enhanced amplicon accessibility, which is coupled with transcript fusions. Even more, in the nuclei, eccDNAs are able to amplify the expression of chromosomal genes through the regulatory regions presented on eccDNAs and associated with gene expression.
3) They represent a major source of oncogenic remodeling and complex DNA rearrangements through circularization and reintegration into linear chromosomes (Koche et al., 2020; Hull and Houseley, 2020; Møller et al., 2018; Kim et al., 2020).

The CRISPR–Cas9-based system is attending as a genetic tool to tractably edit organisms genomes (Molenda et al., 2019; Kovač et al., 2010; Halpin-Healy et al., 2020). By providing a single guide RNA (sgRNA) and Cas9 endonuclease, the CRISPR-Cas9 technology has evolved an ideal tool for gene therapy and is expected to be enabled for efficient gene targeting at predetermined sites in the human genome (Fig. 1). It provides a powerful approach to generate

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genetic models both for fundamental and preclinical research. According to the literature, the CRISPR-Cas9 system would specifically target copy-number amplified regions outside the chromosomes wherein would conferred anti-proliferative/-survival effects on cancerous cells and is detrimental to tumor cellular fitness (Song et al., 2016; Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016).

2. EccDNA and copy number variations (CNVs) in somatic tissues

Accordingly, in a tumor, eccDNAs can be found at the pre-amplification stage, where they are transpososase-sensetive and representing resistance marker to therapy. In an analysis of various tumor types, high copy numbers of eccDNAs were identified that carrying recurrent regions related to cancer-driver genes and amplifications. For example in glioma cells, hundreds of eccDNAs were identified that carrying recurrent regions related to EGFR oncogene (which is located on chr7) (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020)**.**

There are reports from patients with lung cancer that eccDNAs are able to migrate from the malignant tissue into the healthy tissues. EccDNAs have been also detected in healthy muscle and blood samples from normal individuals, where exhibiting nuclei origin and exosome secretion (Møller et al., 2018; Kim et al., 2020**).** It must be noted that eccDNAs may exist in normal cells and tissues such as muscle and blood where carrying genes or gene-related fragments and accounted as a result of mutations or aging process in the cells (with the size of < 25 kb) (Hull and Houseley, 2020; 2018; Kim et al., 2020; Hull et al., 2017). In yeast and in cattle, deletions have been found to produce eccDNAs, however in human aged tissues, eccDNAs were found to include one or several full-length genes and be associated with mutations or aging process (Hull and Houseley, 2020; Møller et al., 2018).

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Three characteristic properties have been leveraged to eccDNAs: (1) eccDNAs are circular elements, (2) lack a centromere, and (3) are highly amplified (Kim et al., 2020). EccDNAs of chromosomal origin are believed to exist in aged tissues to provide accelerated adaptation through stimulated copy number variation (CNV) of driver genes in response to environmental change (Hull and Houseley, 2020; Møller et al., 2018; Hull et al., 2017; Lanciano et al., 2017). Thus, somatic aged cells are rich in environment-driven eccDNAs that may influence phenotypes through amplified gene-copy numbers (copy number variation (CNV)) and transcription of full-length or truncated genes (Møller et al., 2018; Hull et al., 2017)**.** Transcriptional activity of eccDNAs and their contents lead to formalizing this idea of adaptive function in response to particular environmental conditions, whereby simply connecting eccDNA formation to transcriptional induction and amplification of specific genes. By this clever strategy to up-regulate specific genes by accumulating eccDNA, cells could gain the maximum chance of adaptation and thereby stability. Therefore, eccDNAs make benefits for cells by providing gene copy numbers (CNs) and the ability of a high mutation rate, without the associated risk of generating deleterious chromosomal mutations (Hull and Houseley, 2020; Hull et al., 2017)**.**

Data propose a general mechanism for CNV formation and define the key mechanistic elements underlying this selectivity. Quantification of eccDNAs in individual cells revealed remarkable allele selectivity and recurrence of certain eccDNAs in several individuals in the rate at which specific circular amplicons was highly nonrandom (Kim et al., 2020; Hull et al., 2017). The chromosomal origin of the circular amplicons was found to be highly nonrandom. In tumors with amplified oncogenes *CCND1*, *EGFR* and *MYC* for example, chromosomal breakpoints and locations are non-randomly distributed and occur across all eccDNAs (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020)**.** In human somatic tissues, specific chromosomal locations have

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tendency to break and form circular DNAs (recurrence of certain eccDNAs in human somatic tissue exhibits DNA circularization hotspots) (Hull and Houseley, 2020; 2018; Kim et al., 2020; Lanciano et al., 2017)**.**

1) EccDNAs arise mostly from gene-rich chromosomal regions. Actively transcribed locations are more vulnerable to circularization and eccDNA formation per megabase and per gene (Møller et al., 2018; Hull et al., 2017). In human stem cells, DNA circularization events occurs in protein-coding regions or transcriptionally active genes that would explain a large variation and heterogeneity exists in tumors arising from individual stem cell or otherwise genetically identical cells (Møller et al., 2018)**.** CNVs are considered to be a result of inducible transcription at actively transcribed loci that leads to recurrent and environmental-specific genetic changes. CNVs are arisen through transcription interference with replication fork progression and stability that eventually leads to increase mutations at actively transcribed loci (Møller et al., 2018; Hull et al., 2017; Lanciano et al., 2017).

At transcriptionally active DNA, single-stranded DNA is exposing where an R-loop, a DNA:RNA hybrid, naturally forms. R-loop contributes to repeat recombination as well as circular DNA formation through various circularization mechanisms mediated by DNA repair machinery, most likely by non-homologous end-joining. Evidence exhibit that in addition to oncogenes, actively transcribed DNAs such as DNA repeats and 5S ribosomal DNA (rDNA) are also found on circular DNAs. In human cells under certain conditions, circular DNAs can thereby form and make up a fraction of the genome that would be expressed (Møller et al., 2018; Kim et al., 2020; Hull et al., 2017; Lanciano et al., 2017). In budding yeast, certain conditions for example, environmental-copper stress induces eccDNAs that containing the ribosomal DNA

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(ERCs) and copper-resistance gene *CUP1*. Formed eccDNAs highly accumulate in stressed cells where drive premature ageing and shortened lifespan. ERCs and *CUP1* carry active replication origins that are accounted for the massive abundance of eccDNAs (Hull and Houseley, 2020; Hull et al., 2017). In other words, as the cell aged or in stressful conditions, a subset of eccDNAs forms and reaches high copy numbers while the diversity of circular DNA decreases (Hull and Houseley, 2020; Møller et al., 2018)**.**

Herein, in replicating cells, oncogenes are having a plurality for being amplified by circular structures. EccDNA-based amplifications that occur in cancers, are different from chromosomal-oncogene amplifications, and are associated with patient poor prognosis (Møller et al., 2018; Kim et al., 2020).

2) In addition, upon certain conditions, highly repetitive DNAs can contribute to circular DNA formation. For example, epigenetic mechanisms strictly silence retrotransposons, whereby epigenetic erasing during development or upon stressful conditions, leads to retroelements activation and getting the ability to form eccDNAs (Hull et al., 2017; Lanciano et al., 2017)**.** To limit the number of active elements and their insertions into the genome, repeat end-joining mechanisms of non-homologous/homologous recombination by DNA repair processes are involved that form eccDNAs (Møller et al., 2018; Lanciano et al., 2017)**.** Other genomic repeats such as transposon elements, tandemly repeated genes (the ribosomal DNA (rDNA)), and telomeres can contribute to eccDNA formation (Hull and Houseley, 2020; Molenda et al., 2019; Lanciano et al., 2017).

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3) The genome sequencing of pediatric medulloblastoma exhibited catastrophic event of eccDNA formation that was linked with p53 mutation**.** Findings indicate that there is a strong association between somatic p53 mutations and chromothripsis in medulloblastoma and acute myeloid leukemia where massive chromosome rearrangements and eccDNA formation unavoidable (Rausch et al., 2012)**.**

3. *Recurrent oncogenes in highly amplified eccDNAs*

Copy number variation (CNV) or high-copy number amplifications (recurrence of certain eccDNAs in human somatic tissues, originating from certain-DNA circularization hotspots) occur in cancers, whereby associated with tumor progression and resistance to chemotherapy, as well as, with human age-related disorders (Hull and Houseley, 2020; 2018; Kim et al., 2020; Hull et al., 2017). It has been found that the highly amplified and recurrent oncogenes (with CN >10), are present on circular amplicons (Table 1 &Fig. 2). Data demonstrate that eccDNA-based amplifications/CNVs are present and common in cancer types for example glioma, sarcoma and esophageal carcinoma where can drive poor outcomes for patients (Møller et al., 2018; Kim et al., 2020; Hull et al., 2017, Sun et al., 2021). More than 18,000 eccDNAs have been identified in tumor types, in particular, in gliomas and glioblastomas, many of which carrying known cancer driver genes including the well-known EGFR gene amplicon from chr7 (Kim et al., 2020; Kumar et al., 2020; Rausch et al., 2012)**.** Despite providing high-copy number amplifications and diversity, eccDNAs are maintaining a mechanism by which oncogenes are easily accessible and highly transcribed (Fig. 2). EccDNA mechanisms of amplification are evident in various human

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tumor types independent of cancer lineage and negatively affect patient prognosis (Møller et al., 2018; Kim et al., 2020; Aguirre et al., 2016; Munoz et al., 2016).

Oncogene CNVs can enhance cell growth, bestow drug resistance, and complement genetic defects particularly in challenging environments. Oncogenes CNVs drive tumor growth (e.g., of *AKT4, MYC, FGFR2* or *CDK4*) or mediate drug resistance (e.g. *DHFR, KRAS* or *BRAF*) (Table 1) (Munoz et al., 2016; Hull et al., 2017; Lanciano et al., 2017). CNVs or high-copy number amplifications of recurrent oncogenes (e.g. *CCND1*, *EGFR* and *MYC*), are the most frequent types of genomic alterations occurs in cancers where make the main contents of circular amplifications in tumor samples (Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016).

Whole-genome sequencing (WGS) approaches have characterized 85% of amplicons in tumor cells as eccDNAs with a median count of 16.6 eccDNA per cell. Circular amplicons have tendency for high copy number and reintegration into the chromatin where induce worse outcomes in patients whose tumors contain at least one circular amplicon. During cancer development, there is a selection for higher copies of growth-promoting genes which leads to primary eccDNA formation and rapid oncogene amplification. Formed eccDNA has tendency to be amplified and unevenly inherited which results in intratumoral genetic heterogeneity. Onco-eccDNAs are detectable at the primary stages of a tumor that predict patient's resistance to chemotherapy, poor prognosis, and shorter survival. In the nuclei of cells, eccDNAs are able to amplify the expression of chromosomal genes through the regulatory regions presented on eccDNAs and associated with gene expression (Kim et al., 2020).

As mentioned earlier, well-known oncogenes are enriched on the recurrent eccDNAs where are highly amplified and actively transcribed. Besides exhibiting higher levels of onco-

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transcriptional activity, onco-eccDNAs are able contributing chromosomal DNA accessibility and onco-transcript fusions (Møller et al., 2018; Kim et al., 2020).

4. EccDNAs in tumor metastasis and patient outcomes

There is close association between high levels of eccDNAs and poorer cancer prognosis and outcomes. In a study, whole-genome sequencing of about 3,212 cancer patients was recorded. Data analysis demonstrated that oncogenes were highly enriched on amplified eccDNAs, and the most common recurrent oncogene amplifications arose on eccDNAs. Patients whose cancers carried eccDNAs had significantly shorter survival, even when controlled for tissue type, than patients whose cancers were not driven by eccDNA-based oncogene amplification (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020). Another study on the samples from pediatric high-grade glioma and adult glioblastoma patients, showed that there existed some large signals for EGFR, CDK6, and MYC with eccDNAs in the relapse but not in diagnosis (Noer et al., 2022).

In a genome-wide mapping of neuroblastoma, eccDNAs were found associated with oncogene massive amplification and expression. Additionally, cancer-causing lesions and ongoing mutagenic process in neuroblastoma were also found to emerge out of circle-derived rearrangements and associated with adverse clinical outcome (Koche et al., 2020).

Another whole-genome sequencing of pediatric medulloblastoma brain tumor and samples from myeloid leukemia patients revealed highly complex chromosome rearrangements, massive oncogenes amplifications and chromothripsis, all of which could be linked to eccDNAs. The clinical follow-up data for patients also uncovered an association of chromothripsis and complex DNA rearrangements with poor survival and prognosis. From aforementioned evidence, these events could be linked to onco-eccDNAs formation (Rausch et al., 2012).

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In a study, the level of eccDNAs was markedly elevated in patients with metastatic colorectal cancer. The patients who were carrying at least one circular amplicon of eccDNA exhibited poorer survival and prognosis than those patients without eccDNA-associated amplicons (Spindler et al., 2017; Wang et al., 2021).

There was also a genome-wide presence of eccDNAs in samples from esophageal squamous cell carcinoma (ESCC) patients. ESCC is a leading cause of cancer-related mortality with high incidence and poor prognosis. The pathway analysis of genes associated with the massively expressed eccDNAs showed enrichment in cancer-related pathways, all of which have been shown to play key roles in ESCC progression and mortality (Sun et al., 2021).

In respect to poor outcome, recent studies put fundamental roles for cancer-cell exosomes and eccDNAs in the base and origin of tumor growth, metastasis and invasion. Onco-eccDNAs could be in the origins of tumors propagating neoplastic stem-like cells (cancer stem cells, CSCs) in the body and may also explain their relationships to the bulk population of tumor cells. Propagating tumors and primary cancer cells export eccDNAs to the extracellular space by exosomes as a messenger to transmit oncogenic information to other cell types. Messenger eccDNAs cause cancer progenitor cells (CSCs) get the capacity to dedifferentiate and acquire a stem-like phenotype. These findings have linked eccDNAs to cancer metastasis and poor patient outcomes.

In the description of the role of eccDNAs in tumor stem-like cell generation and stemness maintenance, cancer cells use exosomes and eccDNAs to transmit oncogenic information to other cell types. The eccDNAs mediate amplification of oncogenes (e.g. *EGFR, MYC, CDK4*, and *MDM2*) in targets cells as a driving force for the acquisition of the cancer stem cell–like phenotype and signaling of invasiveness and chemoresistance. Results of human studies have linked onco-eccDNAs to increased cancer metastasis and poor patient outcomes. According to

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the findings, eccDNAs can induce dedifferentiation and reprogramming of progenitor cells to acquire a stem-like phenotype in response to genetic manipulation (Wang et al., 2021; Turner et al., 2017; Li and Laterra, 2012; Noer et al., 2022; Xu et al., 2019).

5. *CRISPR-Cas9: As a tool for gene targeting*

There are several protocols developed by different research groups to investigate CRISPR-Cas9 system as a novel technology and ideal tool to edit genomes (Koche et al., 2020; Kovač et al., 2020; Halpin-Healy et al., 2020). This molecular system represents a defense immune mechanism type II in bacteria and is a candidate to become an efficient technology for targeted-gene editing in tractable organisms. A single guide RNA (usually about 20 nucleotides complementary to the target gene or locus), is designed to target a specific sequence site and anchored by a spacer motif to the system (Fig. 1). Cas9 nuclease then cleaves DNA at the specific sequence site where is targeted by the guide RNA and generates double-strand breaks (DSBs), subsequently DNA damage responses are activated and arrest cell growth (Fig. 1) (Song et al., 2016; Gonçalves et al., 2019; Aguirre et al., 2016). Even though dual-delivery of pairs of CRISPR/Cas9 guide RNAs into human normal cells was reported to induce circularization of genes and chromosome, but in bacteria, CRISPR-Cas9 system primarily targets extrachromosomal rearranged elements. Likewise, in cancer cells, it is expected that a designed CRISPR-Cas9 system is able to target extrachromosomal copy-number amplifications and produces double strand breaks that is detrimental to cellular fitness through dictating gene-independent copy-number loss-of-fitness (LOF) effects and anti-proliferative responses**.** A designed system against amplified amplicons may provide a novel approach for cancer therapy and propose a practical implication for CRISPR-Cas9 as a path in therapeutic strategies of cancer

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(Møller et al., 2018). When targeting CNVs or high-copy number amplifications by CRISPR-Cas9 system, the nuclease can introduce multiple strand breaks and deletions in targeted DNAs where DNA-damage response is highly activated and leads to cell cycle arrest and death (Fig. 2) (Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016). By targeting copy-number amplifications on eccDNAs formed mostly from proto-oncogenes and repeats in the human genome, CRISPR-Cas9 mediates loss-of-fitness (LOF) effects on cancerous cells that are associated with genomic instability signals and deleterious responses (Koche et al., 2020; Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016)**.**

6. *Gene-independent copy-number loss-of- fitness (LOF) effects by CRISPR-Cas9*

Data demonstrate that somatic gain-of-function alterations in cell-growth driver genes play a central role in cell transformation and cancer development. Deriver-genes gain-of-function alterations enable transformed cells to evade the checks and balances exist in the cell cycle to keep homeostasis. However, eccDNA-based oncogene amplifications seem to be the most gain-of-function alterations occurred in cancers (Kim et al., 2020; Gonçalves et al., 2019; Aguirre et al., 2016). In normal cells, oncogene copy-number amplifications are actually rare and detrimental, but in tumor cells, they are the most frequent genomic events occurred. In fact, tumor cells show lethal dependencies on oncogene gain-of-function alterations that exhibit great impacts on cellular fitness (Koche et al., 2020; Gonçalves et al., 2019).

Data represent a high association between oncogene CNs and eccDNA amplifications, in all types of cancers (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020). Onco-eccDNA

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amplifications enable a proto-oncogene to reach gain-of-function alterations and high CNs (> 8) of tumor deriving genes. Inheritance of onco-eccDNAs do not obey the chromosomal mechanism and leads to genetic heterogeneity throughout a tumor. Oncogenes amplified by onco-eccDNAs, attain higher CNs than the same genes amplified on noncircular structures and is associated with much more aggressive cancers (Kim et al., 2020; Hull et al., 2017).

Herein, CRISPR-Cas9 as a natural defense system in bacteria targets extrachromosomal elements to protect the host genome from invading mobile elements. This system was reported that primarily targets extrachromosomal circular elements in bacteria species to prevent from gene-clustering or tandem-duplications reintegration (Molenda et al., 2019; Petassi et al., 2020). On the other side, utilizing CRISPR-Cas9 to knockout or target tandem or interspersed-structurally rearranged amplifications exhibited highly detrimental effects on different cancer cell lines (Fig.2). In this case, gene-independent copy-number LOF effects by CRISPR-Cas9 would be robustly expected whereby targeting high copy-number genes commonly found in eccDNAs and amplified in tumors (e.g. *MYC, PI3K, CCND1* and *EGFR*) (Table 1, Fig. 2). LOF effects induced in targeting copy-number amplifications, could also be associated with other structural rearrangements by eccDNAs such as tandem duplications (Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016). ~~However,~~ EccDNAs are amongst the most frequent structural variables found with tandem duplications or gene amplifications and are associated with CRISPR-Cas9-mediated deleterious effects (Gonçalves et al., 2019).

In compare to the heavily rearranged and linear chromatin, the amplicons of eccDNA type is significantly more accessible for CRISPR-Cas9 system (Hull et al., 2017). The most frequent and recurrent genomic alterations occurred in cancers, were found to be on circular amplicons which containing amplified oncogenes such as *PI3K, MYC, CCND1, EGFR, PAX8*, and *CDK4* (onco-

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eccDNAs) (Koche et al., 2020; Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020). Genomic copy numbers on circular amplicons dictate a cell-loss-of fitness response to CRISPR-Cas9 targeting (Gonçalves et al., 2019).

Recently, gene-independent copy-number loss-of- fitness (LOF) effects by CRISPR-Cas9 were observed in blood cancer cell lines. CRISPR-Cas9 was inducing anti-survival effects associated with increased levels of DNA damage markers by targeting amplified regions in the BCR-ABL rearrangement and JAK2 amplification. Targeting amplified oncogenes (targeting > 20 sites) showed also similar deleterious effects on cancer cell lines of differing lineages. These findings may provide researchers great approaches to design guide-RNAs that target amplified onco-eccDNAs and induce cell cycle arrest, LOF, and anti-survival effects ~~eccDNAs~~ (Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016).

By designing single guide RNAs that map to multiple genomic sites, Cas9 nuclease introduces double-strand breaks (DSBs) in genomic DNAs (Fig. 1 & 2), whereby induces disasters effects on the cells; cell cycle arrest (mostly at the G2 checkpoints), and cell deaths ~~eccDNAs~~ (Gonçalves et al., 2019; Aguirre et al., 2016).

Conclusions

The most frequent and recurrent genomic gain-of-function alterations occurred in aggressive cancers, are amplified cell-growth driver genes such as *PI3K, MYC, CCND1, EGFR, PAX8*, and *CDK4* (CNVs >>8), which are associated with circular DNA amplifications. Herein, CRISPR-Cas9-targeted amplicons with CNV > 8 would put cancer cells in a DNA double-stranded break (DSB) shock whereby results in DNA repair responses and cell cycle arrest. There would be a

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strong correlation between extrachromosomal elements/gene copy number amplifications and decreased cell viability after genome targeting by CRISPR-Cas9. The number of target loci and DSBs by CRISPR-Cas9 correlates strongly with regions of copy number gain, both expressed and unexpressed genes, as well as intergenic loci. By designing a single guide RNA which maps to these multiple genomic sites, DSBs in these regions eventually leads to anti-proliferative effects through induction of a G2 cell cycle arrest. Cell response to CRISPR-Cas9 DSBs correlates with the number of target loci. CRISPR-Cas9 targeting of onco-eccDNAs elicits a gene-independent copy-number loss-of- fitness (LOF) effects. Through induction of multiple DSBs in DNA by the Cas9, in a sgRNA-directed sequence-specific manner, DNA damage response is activated and confers anti-proliferative/anti-survival effects.

Abbreviations: Extrachromosomal circular DNAs (eccDNAs); clustered regularly interspaced short palindromic repeats (CRISPR); RNA-guided Cas9 nuclease (Cas9); single guide RNA (sgRNA); copy number variations (CNVs); ribosomal DNA (rDNA); copper-resistance gene1 (CUP1); Copy number (CN); double-strand breaks (DSBs); loss-of- fitness (LOF); ribonucleoprotein (RNP).

Declaration

- Ethical Approval and Consent to participate (NA).
- Consent for publication (NA).
- Availability of supporting data (Yes).
- Competing interests (NO).
- Funding (NO).

- Authors' contributions: Fatemeh Pourrajab: Proposing, supervising and writing article, Mohamad Reza Zare-Khormizi: Performing literature survey, collecting data, and writing the primary article.

Acknowledgements: The authors are grateful to Shahid Sadoughi University of Medical Sciences, Yazd, Iran, and Kerman University of Medical Sciences, Kerman, Iran.

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Authorship Contributions*: Participated in research design:* Pourrajab, F., Zare-Khormizi, M.R.*; Performed literature survey, collected data analysis:* Zare-Khormizi, M.R.*; Wrote or contributed to the writing of the manuscript:* Pourrajab, F., Zare-Khormizi, M.R.

Funding information: We received no external funding for this work.

Conflict of interest statement: No author has an actual or perceived conflict of interest with the contents of this article.

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Molecular Pharmacology Fast Forward. Published on August 8, 2022 as DOI: 10.1124/molpharm.122.000553
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Figure Caption list

Fig 1. A strategy is proposed to design a CRISPR-Cas9 system expressing single guide RNA (sgRNA) and Cas9 endonuclease and targeting sequences on amplified onco-eccDNAs. Three copy numbers (A, B, and C) of a homologous sequence are presented here as representatives for multiple copies of genes or tandem interspersed duplications structurally occurred during eccDNA formation and in genome rearrangements in various tumors. By designing multiplex guide RNA (sgRNAs) targeting predetermined specific sites in the genome, the CRISPR-Cas9 is able to introduce targeted breaks and deletions by the Cas9 endonuclease and can be engineered for multiple-copy number breakages and gene knock-out processes**.** G: copy-number amplified genes or oncogene-related sequences; sgRNA: single guide RNA; consensus sgRNA: single stranded RNA that is designed in CRISPR-Cas9 system to target conserved sequences shared by all copy-number amplified oncogenes; specific sgRNA: single stranded RNA in CRISPR-Cas9 system that distinctly targets a diverged region specifica for each oncogenic amplicone.

Fig 2. Somatic gain-of-function alterations in cell-growth driver genes, CRISPR-Cas9 system against copy-number amplifications and its association with loss of fitness effects on tumor cells. The illustration represents different genomic rearrangements and somatic gain-of-function alterations in cell-growth driver genes by copy-number amplifications that can be a potential target for CRISPR-Cas9. Targeting copy-numbers amplifications (copy-number ratio >>8), by designed CRISPR-Cas9 system, elicits a gene-independent anti-proliferative/DNA damage response, in a sgRNA-directed sequence-specific manner and through the induction of double stranded breaks in DNA by the Cas9. Targeting cell-growth driver genes that are scored as essential in tumor growth such as AKT2, MYC, or

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CDK4, confer also decreased proliferation effects. G: copy-number amplified genes or oncogene-related sequences.

Table 1. Some human cancers and the most known-driver genes amplified in eccDNA/gene duplications*.

Adrenocortical carcinoma	*EGFR, FGFR2*, *KIT*, *FOXA1*, *NFE2L2*, *SOS1, H3F3A*, *PCBP1*, *PMS1*, *SF3B1*,
Breast invasive carcinoma	*EGFR*, *FGFR2*, *KIT*, *FOXA1*, *MYC, WT1, KRAS*, *H3F3A*, *CCND1*, *PIK3CG*, *PIK3R2*, *AKT1*, *MTOR*, *SF1*, *PTPRC*, *PTPN11*, *ERCC2*, *MSH3*, *ERBB2*, *ERBB3*, *SOX17*
Colon adenocarcinoma	*EGFR*, *FGFR2*, *CDK4*, *FOXA1*, *KRAS*, *WT1*, *H3F3A*, *CCND1*, *PIK3CG*, *PIK3CA*, *PIK3R2*, *MTOR*, *SF1*, *PTPRC*, *KLF5*, *ERCC2*, *ERBB2*, *ERBB3*, *SOX17*
Brain Lower Grade Glioma	*WT1*, *KIT*, *PIK3CG*, *MTOR, MACF1*, *PCBP1*
Glioblastoma multiforme	*EGFR*, *MYC, CDK4*, *PIK3R2*, *ERCC2*, *GRIN2D*, *H3F3A*,
Kidney renal papillary cell carcinoma	*EGFR*, *FGFR2*, *MYC*, *FOXA1*, *PIK3CG*, *PIK3CA*, *PTPRC*, *ERCC2*, *ERBB2*, *SOX17*
Liver hepatocellular carcinoma	*WT1*, *KIT*, *CDKN1A*, *CCND1*, *PIK3CG*, *PIK3CA*, *PIK3R2*, *KRAS*, *SF1*, *DHX9*, *PTPRC*, *PTPN11*, *ERCC2*, *SOS1*, *ERBB2*
Kidney renal clear cell carcinoma	*WT1*, *FGFR2*, *KRAS*, *MTOR*, *FOXA1*, *PIK3CG, PIK3R2*, *PTPRC*, *CDK4*, *ERCC2*, *SOS1*, *ERBB2*, *ERBB3*, *SOX17*
Lung adenocarcinoma	*MYC, WT1*, *FGFR2*, *KRAS*, *MTOR*, *PIK3R2*, *SF1*, *H3F3A*, *PTPRC*, *CDK4*, *CCND1*, *ERCC2*, *SOS1*, *ERBB2*, *ERBB3*
Stomach adenocarcinoma	*MYC*, *WT1*, *EGFR*, *KRAS*, *NRAS*, *MTOR*, *PIK3CA*, *PIK3R2*, *H3F3A*, *SF1*, *CDK4*, *CCND1*, *ERBB2*, *ERBB3*, *SOX17*
Skin Cutaneous Melanoma	*EGFR, KRAS*, *FOXA1*, *PIK3R2*, *CCND1*, *ERCC2*
Thyroid carcinoma	*NRAS*, *PIK3CG, KLF5*, *ERCC2, ERBB2*
Uterine Corpus Endometrial Carcinoma	*PIK3CG, PIK3R2*, *PIK3CA*, *KLF5*, *CCND1*, *ERBB2*
Prostate adenocarcinoma	*EGFR*, *MYC*, *KRAS*, *KIT*, *MTOR*, *FOXA1, PIK3CG*, *PIK3R2*, *CDK4*, *ERCC2*, *ERBB2*, *ERBB3*, *SOX17*

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Lung squamous cell carcinoma	*FGFR3, NRAS*, *KRAS*, *PTPN11*, *H3F3A*, *CDK4*, *ERCC2*, *SOS1*, *PCBP1*, *ERBB3*

Note: for more details, refer to the references (Kumar et al., 2020; Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016; Hull et al., 2017; Lanciano et al., 2017).

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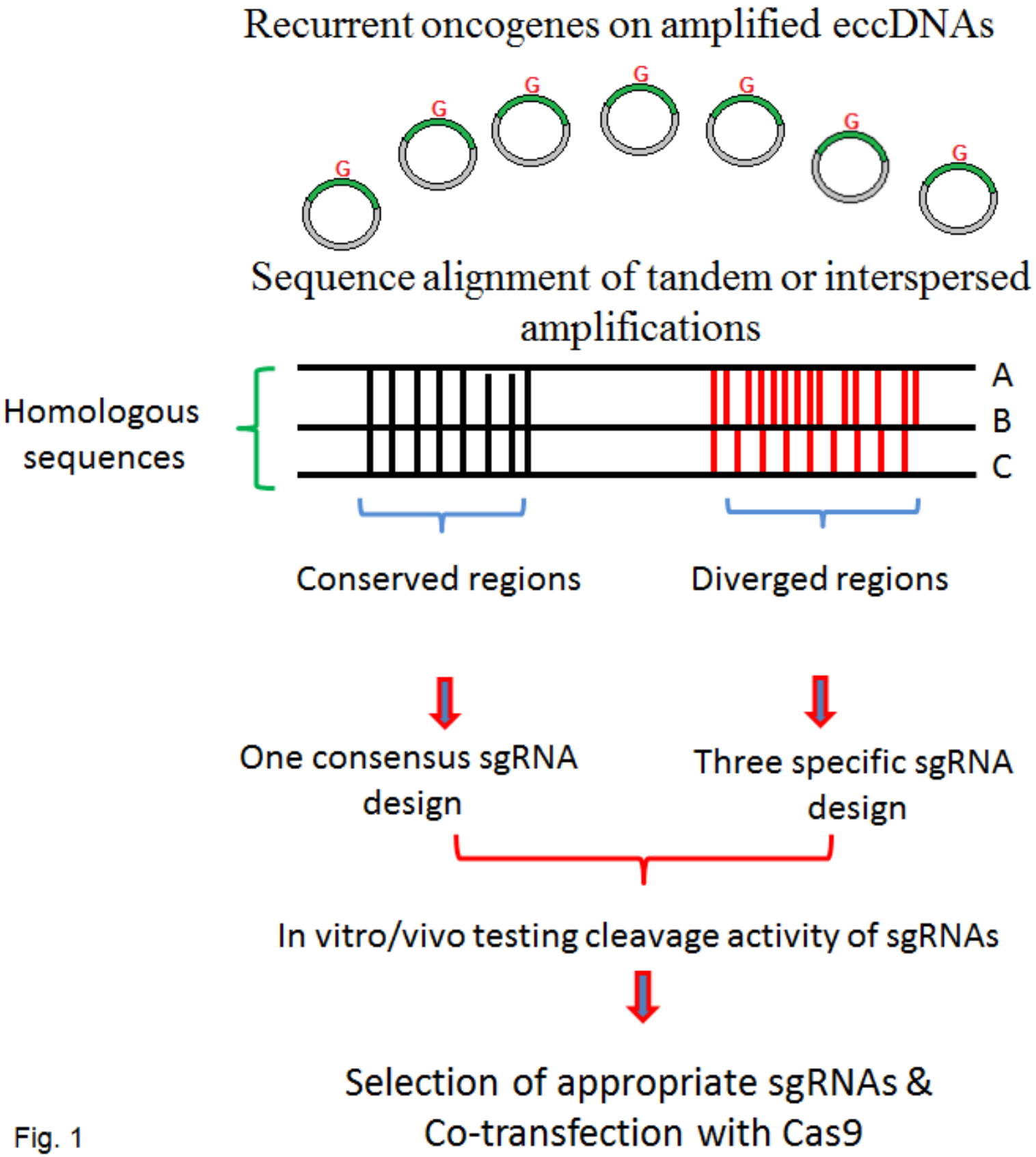

Fig. 1

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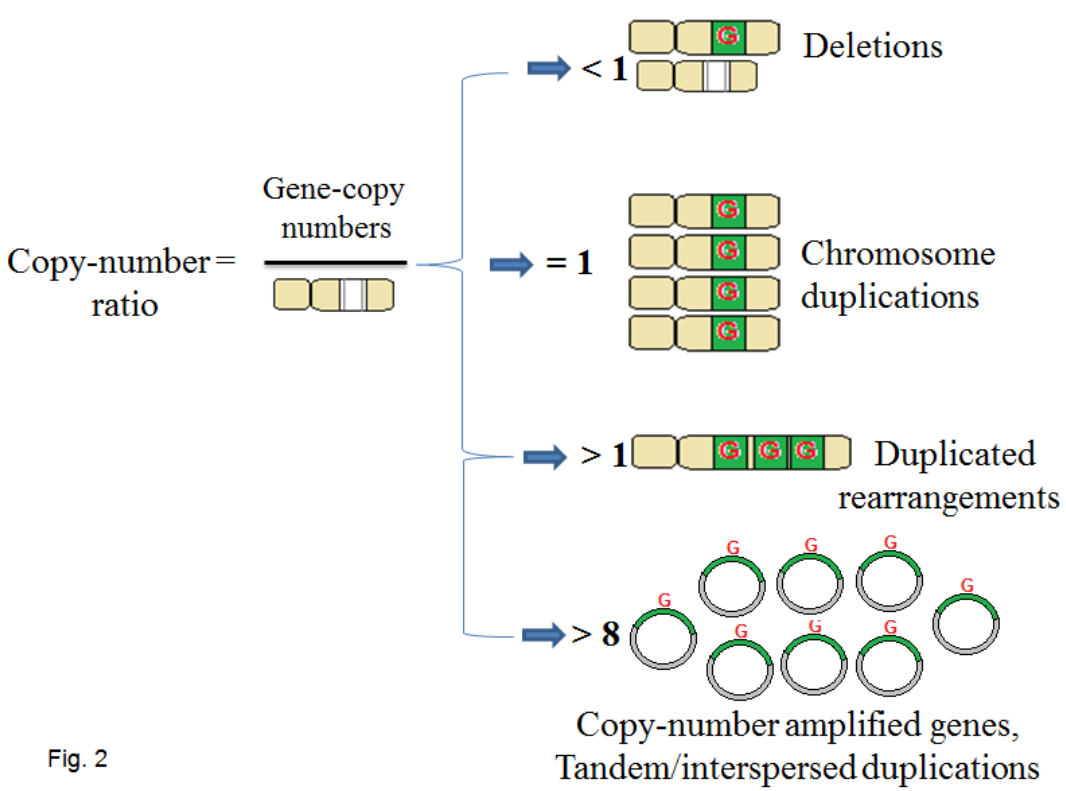

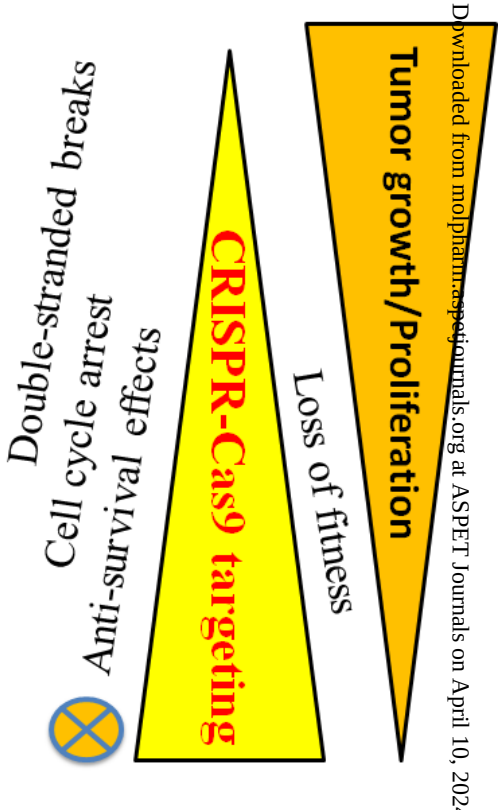

Fig. 2