Published in final edited form as:

Cancer Lett. 2016 April 1; 373(1): 109–118. doi:10.1016/j.canlet.2016.01.030.

Development and Potential Applications of CRISPR-Cas9 Genome Editing Technology in Sarcoma

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Abstract

Sarcomas include some of the most aggressive tumors and typically respond poorly to chemotherapy. In recent years, specific gene fusion/mutations and gene over-expression/activation have been shown to drive sarcoma pathogenesis and development. These emerging genomic alterations may provide targets for novel therapeutic strategies and have the potential to transform sarcoma patient care. The RNA-guided nuclease CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein-9 nuclease) is a convenient and versatile platform for site-specific genome editing and epigenome targeted modulation. Given that sarcoma is believed to develop as a result of genetic alterations in mesenchymal progenitor/stem cells, CRISPR-Cas9 genome editing technologies hold extensive application potentials in sarcoma models and therapies. We review the development and mechanisms of the CRISPR-Cas9 system in genome editing and introduce its application in sarcoma research and potential therapy in clinic. Additionally, we propose future directions and discuss the challenges faced with these applications, providing concise and enlightening information for readers interested in this area.

Keywords

CRISPR-Cas9; Sarcoma; Cancer modeling; Gene therapy

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Author contributions

All authors met the International Committee for Medical Journal Editors criteria for authorship, were fully involved in manuscript development, and assume responsibility for the direction and content. All authors have approved the manuscript for submission.

Conflict of interest

None.

1. Introduction

Sarcoma is a rare and heterogeneous group of tumors with 50 different subtypes that exhibit a wide range of differing behaviors and underlying molecular mechanisms [6, 28]. Sarcoma is believed to arise in mesenchymal tissues and often displays highly aggressive behavior with proclivity towards early hematogenous metastasis [15, 62]. Surgery and chemotherapy can significantly improve survival and can be curative when applied early in some types of sarcoma [15, 62]. However, a tendency towards relapse is high and the prognosis of chemo-resistant and disseminated sarcomas remain poor despite multimodal therapeutic approaches [62]. Therefore, further improvements and new approaches for the treatment of sarcoma are essential.

Sarcoma can be classified based on the genetic alterations involved in its development: oncogenic somatic mutations (e.g. *KIT* and/or platelet-derived growth factor receptor- α (*PDGFR- α*) mutations in gastrointestinal stromal tumors (*GIST*)), DNA copy number alterations (e.g. *JUN* gene amplification in dedifferentiated liposarcoma and myocardin (*MYOCD*) gene amplification in leiomyosarcoma), and recurrent chromosomal translocations resulting in abnormal fusion proteins (e.g. *SYT-SSX* gene fusion in synovial sarcoma and Ewing sarcoma breakpoint region 1 and Friend leukemia virus integration 1 (*EWSR-FLI1*) gene fusion gene in Ewing sarcoma) [15, 52, 72]. More commonly, sarcoma pathogenesis is the result of complex chromosomal abnormalities, as in the case of osteosarcomas and high grade undifferentiated pleomorphic sarcomas (neurofibromin 1 (*NF1*) gene deletions, point mutations and indels such as *P53* and *RB*) [15, 52, 72]. In addition, a variety of protein kinases including receptor tyrosine kinases (*RTKs*) are overexpressed or constitutively activated in sarcoma, both in translocation associated sarcomas, such as *GIST* discussed above, and in karyotypically complex tumors, for instance osteosarcoma [17, 85]. Because *RTKs*, *c-KIT*, *ATK*, *EGFR*, *mTOR*, and *IGF-1R*, for example, and their ligand growth factors are so frequently overexpressed/activated in sarcomas, they represent some of the most attractive therapeutic targets in sarcoma [17, 85].

Although the development of adequate transgenic models has been elusive, sarcoma modeling via genetic engineering remains an important approach for sarcoma research. Conventional transgenic techniques often involve the sophisticated and time-consuming processes of germline manipulation and labor intensive animal cross-breeding, and can model the complexity and multistep nature of sarcoma mutations[45]. RNA interference (RNAi) provides an alternative method for genetic engineering, however, it can usually achieve only temporary and partial knockdown [1]. RNAi is also restricted to expressed genes and has pervasive off-target effects [1, 45]. Cre (cyclization recombinase)-loxP (locus of recombination in P1) recombination consists of a single enzyme, Cre recombinase. Cre recognizes a 34-bp site on the P1 genome called loxP and catalyzes reciprocal conservative DNA recombination between pairs of loxP sites [79]. Much of the power of the Cre-loxP system derives from the potential to generate conditional mutants [47, 79]. However, conditional deletion of every gene in every cell type may not be realistic [47, 79]. Conditional deletions are only as good as the promoters that regulate Cre expression, and transgenic Cre driver lines have proven to be somewhat problematic, exhibiting expression outside the target tissue as well as inefficient cleavage leading to mosaicism [47, 79].

Recently, clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas9 protein, a novel genome editing tool has been implemented in a multitude of model organisms and cell types [4, 13]. Compared with RNAi and Cre-LoxP, CRISPR-Cas9 is an exogenous system that does not compete with endogenous processes and functions at the DNA level targeting transcripts, which results in knockdown or complete elimination of gene function. Furthermore, CRISPR-Cas9 provides a larger targetable sequence space in which promoters of the gene may also be targeted. CRISPR-Cas9 genome editing technology holds many advantages and it has already started to supplant incumbent genome editing technologies, such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) (Table 1) [4]. We review the development and potential applications of the CRISPR-Cas9 system in genome editing and introduce these applications in sarcoma modeling and therapy. We also discuss the challenges of CRISPR-Cas9 and future avenues for innovation.

2. Mechanisms of CRISPR-Cas9 in genome editing

The CRISPR-Cas9 system is currently used for RNA-guided endonuclease gene editing. The core components of this system are a nuclease Cas9 and a single guided RNA (sgRNA) [4, 7, 13, 45, 48, 59, 64, 77, 80, 84]. The nuclease Cas9 consists of two catalytic active domains: HNH and RuvC [4, 7, 13, 45, 48, 59, 64, 77, 80, 84]. The HNH domain is a single nuclease domain, whereas the RuvC domain contains three subdomains across the linear protein sequence [4, 7, 13, 45, 48, 59, 64, 77, 80, 84]. RuvC I near the N-terminal region of Cas9 and RuvC II/III flank the HNH domain is near the middle of the protein. The HNH and RuvC nuclease domains are properly positioned for cleavage of the complementary and non-complementary strands of the target DNA, respectively [4, 7, 13, 45, 48, 59, 64, 77, 80, 84]. The sgRNA, which has an invariant scaffold region and a spacer region, is derived from CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The sgRNA binds to Cas9 and directs it to the locus of interest by a 20-nt guide sequence via base pairing to the genomic target [4, 7, 13, 45, 48, 59, 64, 77, 80, 84] (Fig. 1).

The target sequence in the genomic DNA paired to the sgRNA sequence is immediately followed by a NGG sequence called the protospacer adjacent motif (PAM). The PAM sequence is located on the immediate 3' end of the sgRNA recognition sequence, but it is not a part of the 20-nt guide sequence within the sgRNA [4, 7, 13, 45, 48, 59, 64, 77, 80, 84] (Fig. 1). The CRISPR-Cas9 system uses Cas9, which complexes with the sgRNA, to cleave DNA 3–4 base pairs upstream of PAM and generates double-strand breaks (DSBs) in a sequence-specific manner [4, 7, 13, 45, 48, 59, 64, 77, 80, 84]. The DSBs are then repaired either by non-homologous end joining (NHEJ)-mediated error-prone DNA repair or homologous directed repair (HDR)-mediated error-free DNA repair (Fig. 2). The former repair can rapidly ligate the DSB, but generates small insertion and deletion mutations at the target sites [20, 55, 64, 65]. These mutations could disrupt and abolish the function of target genes or genomic elements [20, 55, 64, 65]. For instance, an sgRNA that targets a protein-coding region can produce loss of function frame-shifting indels through NHEJ-mediated DNA repair. HDR-mediated error-free DNA repair requires a homology-containing donor DNA sequence as repair template. Through co-transfection with Cas9, KRAS sgRNA and an

oligonucleotide template into wild-type human intestinal organoids, the *KRAS*^{G12D} (GGT>GAT) mutation was successfully achieved [20, 55, 64, 65].

3. Potential applications of CRISPR-Cas9 in sarcoma

3.1 Modeling sarcoma processes with CRISPR-Cas9

Sarcoma pathogenesis is a multistep process that involves many genetic alterations and epigenetic changes. Genome sequencing studies have identified a large collection of genetic and epigenetic alterations that occur in different types of human sarcomas [22, 65]. Experimental strategies to manipulate the genomes of either normal cells or tumor cells are crucial for understanding the pathogenesis of sarcoma and for discovering potential therapeutic targets. Although several genetic engineering technologies, such as Cre-loxP, ZFNs, and TALENs, have been applied to different tumor model systems, they are generally labor intensive, time consuming, and have low efficiency for gene targeting [26, 47]. Thus, the need for easier, rapid, and high efficiency genetic modeling approaches remains. The CRISPR-Cas9 genome editing technology has revolutionized the field of genetic engineering and may overcome many of the limitations of earlier techniques including Cre-loxP, ZFNs, and TALENs, on carrying out deletions, insertions, translocations, and inversions at specific sites in the DNA of cells [4, 7, 13, 45, 48, 59, 64, 77, 80, 84]. Although it is capable of generating transgenic mice via embryo manipulations similar to those in Cre-lox techniques, CRISPR-Cas9 has been shown to be easy to design and use, and its multiplexing nature streamlines the generation of animal and cellular cancer models enabling rapid functional interrogation of cancer-associated genes (Table 2) [4, 7, 13, 45, 48, 59, 64, 77, 80, 84].

3.1.1 Loss of function mutations and gain of function mutations using CRISPR-Cas9—Genome sequencing studies have identified multiple driver gene mutations in human sarcomas. CRISPR-Cas9 is capable of inducing loss of function (LOF), and gain of function (GOF) mutations *in vitro* and *in vivo*. So far, CRISPR-Cas9 has been used in some malignant tumor models, including lung cancer, colorectal cancer, and myeloid leukemia [22, 27, 28, 30, 31]. By delivering the combination of sgRNA and Cas9 with a lentiviral vector, five genes (*TET2*, *DNMT3A*, *RUNX1*, *NF1*, and *EZH2*) demonstrated LOF in a single hematopoietic stem cell, leading to clonal outgrowth and myeloid malignancy [30]. Hematopoietic stem cells were also derived from mice with knock-in of the *FLT3* internal tandem duplication (*FLT3-ITD*) mutation [30]. Using cooperating LOF mutations in genes encoding epigenetic modifiers, transcription factors, and mediators of cytokine signaling (such as *TET2*, *DNMT3A*, *RUNX1*, *NF1*, *SMC3*, *P53*, *ASXL1*, and *EZH2*), researchers were able to generate models of acute myeloid leukemia (AML). These models recapitulated the combinations of mutations observed in patients [30]. A recent study has identified that deregulation of *APC*, *P53*, *KRAS*, and *SMAD4* is sufficient for transformation of cultured mouse colon cells into colorectal cancer [20]. CRISPR-Cas9 was utilized to induce LOF mutations in *APC*, *P53*, and *SMAD4*, and GOF mutation in *KRAS* in cultured human intestinal stem cells [20]. To introduce the GOF mutation, an oligonucleotide with the *KRAS* mutation and two silent mutations was designed to serve as a template for HDR. Mutant organoids (*KRAS*^{G12D}/*APC*^{KO}, *KRAS*^{G12D}/*APC*^{KO}/*P53*^{KO}, and *KRAS*^{G12D}/

APC^{KO}/P53^{KO}/SMAD^{KO}) were selected by removing individual growth factors from the culture medium. Upon xenotransplantation into mice, quadruple mutants grew as tumors with features of invasive carcinoma [20]. Another colorectal cancer model using CRISPR-Cas9-mediated engineering of human intestinal organoids has been established by Matano et al. [50]. By modulating the culture conditions to mimic the intestinal environment, LOF mutations of *APC*, *P53*, *SMAD4*, and GOF mutations of *KRAS* and *PIK3CA* were introduced into organoids derived from normal human intestinal epithelium [50]. To knock in the *KRAS^{G12V}* mutation, researchers electroporated an sgRNA targeting a sequence adjacent to *KRAS* exon 2 together with a donor vector containing homologous arms but encoding the *KRAS^{G12V}* mutation. They then selected the isogenic organoids that carried mutations in the tumor suppressor genes *APC*, *P53*, and *SMAD4*, and the oncogenes *KRAS* and/or *PIK3CA* [50]. Organoids engineered to express all five mutations grew independently of niche factors *in vitro*, and an adenocarcinoma sequence model was formed after implantation under the kidney subcapsule in mice [50].

Hydrodynamic injection is an established method of delivering plasmids selectively to the liver in animal models [78]. Tail vein injection is the most common procedure in rats and mice [78]. DNA plasmids encoding Cas9 and sgRNAs can be delivered to the liver through hydrodynamic tail vein injection to target the tumor suppressor genes *PTEN* and *P53* alone or in combination [82]. As a result, when *PTEN* was mutated by CRISPR, elevated *AKT* phosphorylation and lipid accumulation in hepatocytes were observed, while simultaneous mutation of *PTEN* and *P53* induced liver tumors [82]. Furthermore, the feasibility of inducing GOF mutations by CRISPR-Cas9 in the liver was also determined by co-injection of Cas9-sgRNA plasmids targeting β -catenin gene and a single-stranded DNA oligonucleotide donor carrying activating point mutations, which led to the generation of hepatocytes with nuclear localization of β -catenin [82]. To broadly enable the application of Cas9 *in vivo*, a Cre-dependent Rosa26 Cas9 knock in mouse was established [57]. Using this type of mouse, the top three significantly mutated genes in lung adenocarcinoma (*KRAS*, *P53*, and *LKB1*) were simultaneously modeled, leading to macroscopic tumor of lung cancer pathology [57].

Recently, CRISPR-Cas9 has been used to knock out tumor suppressor genes *cyclin-dependent kinase inhibitor 2A (CDKN2A)*, *P53*, and *PTEN* in mice to establish three murine sarcoma models [2]. First, scientists developed a multiple lentiviral expression (MuLE) viral system which allows multiple sgRNAs to be expressed together with *hCas9* from a single viral construct expression [2]. To model human sarcoma, a series of MuLE vectors were designed to systematically investigate the single and combinatorial effects of gain of *H-RAS* function and loss of *CDKN2A*, *P53*, and *PTEN* functions [2]. To investigate the single effects of the driven gene, researchers generated tricistronic MuLE vectors expressing sgRNAs targeting exon 7 or exon 8 of the *P53* locus or exon 2 of the *CDKN2A* locus together with expression of *hCas9* and puromycin resistance [2]. To demonstrate that cooperative genetic tumor modeling can be achieved using CRISPR-Cas9, they generated tetracistronic MuLE vectors designed to express either scrambled sgRNA or sgRNA targeting *P53* exon 7 or exon 8, as well as *H-RAS^{G12V}*, *hCas9*, and puromycin resistance [2]. Strikingly, several types of soft tissue sarcomas were developed in mice when their muscles

were injected with *CDKN2A+H-RAS*, *P53+H-RAS*, and *P53+PTEN+H-RAS* combinatorial viruses. Histological analysis of these sarcomas revealed that they were undifferentiated sarcoma with pleomorphic and rhabdoid features [2]. These studies demonstrate that CRISPR-Cas9 is a powerful genome editing technique that is able to simultaneously target genetic mutations to multiple loci and allows for the rapid and systematic generation of genetically complex [2].

3.1 2 Generation of chromosomal rearrangements using CRISPR-Cas9—

Abnormalities in chromosome number and structure are frequently observed in sarcoma cells but have been difficult to generate in a highly specific manner for function analysis [15]. CRISPR-Cas9 may be able to mediate complex manipulations of gene structure, such as chromosomal rearrangements, which give rise to oncogenic fusion genes, oncogene amplifications, or oncosuppressor deletions [24, 45]. In lung cancer, three types of chromosomal rearrangements were successfully generated by CRISPR-Cas9, including the *CD74-ROS1* translocation event and the *MLL4-ALK* and *KIF5B-RET* inversion events [5, 12, 46]. Authors designed sgRNAs targeting intron 6 of *CD74* and intron 33 of *ROS1*, which were then co-expressed with Cas9 in HEK293T cells. Translocations were detected in cells expressing both *CD74* and *ROS1* sgRNAs [5, 12, 46]. Similarly, for both *MLL4-ALK* and *KIF5B-RET*, the expected inversions were only detected in cells expression Cas9 along with the appropriate pair of sgRNAs [5, 12, 46]. In addition to modeling chromosomal rearrangements in cell lines, mouse models of *MLL4-ALK* gene rearrangement by CRISPR-Cas9 in non-small-cell lung cancers (NSCLCs) were generated [5]. First, sgRNAs targeting intron 14 of *MLL4* and intron 19 of *ALK* were cloned into the Cas9-expressing plasmid PX330, and the resulting constructs were co-transfected into NIH/3T3 cells [5]. The desired *MLL4-ALK* inversion was detected in cells. Next, to deliver Cas9 and sgRNAs to the lungs of adult mice, the dual sgRNA-Cas9 cassette was transferred into an adenoviral shuttle vector and recombinant adenoviruses (Ad-EA) were produced [5]. Then, a cohort of adult mice was infected by intratracheal instillation of Ad-EA. The *MLL4-ALK* rearranged NSCLCs developed in mice two months after inoculation [5]. While various types of sarcomas show characteristic translocations, gene fusions generated from these translocations are the initiating events of many sarcomas and are likely essential in some subtypes of these tumors [15].

Alveolar rhabdomyosarcoma (A-RMS), the third most common soft-tissue sarcoma in children, is typified by a translocation that fuses the *PAX3* gene on chromosome 2 to the *FOXO1* gene on chromosome 13 [15, 62, 75]. It is complicated to mimic this translocation in a mouse, because the *PAX3* and *FOXO1* genes in the mice are in an opposite orientation on their respective chromosomes [42]. To circumvent this limitation, Lagutina and colleagues took a two-step approach [42]. First, they created a mouse model by chromosomal engineering via Cre recombinase-mediated genetic alterations [42]. In this model, the orientation of a 4.9Mb syntenic fragment on chromosome 3, encompassing *FOXO1*, is inverted. Then, they used Cas9-sgRNAs targeting chromosome 1 in intron 7 of *PAX3* and chromosome 3 in intron 1 of *FOXO1* to induce DNA DSBs in *PAX3* and *FOXO1* [42]. Using the above method, a CRISPR-Cas9-mediated *PAX3-FOXO1* fusion gene was successfully generated in mice [42]. The study showed that myoblasts isolated from fore and

hind limbs kept their *PAX3*-expressing identity and co-localization of *PAX3-FOXO1* had a higher frequency in fore limb myoblasts than hind limb myoblasts [42]. This mouse model will be a valuable tool for studying mechanisms underlying the initial stage of the A-RMS implicated chromosome translocations.

To generate human chromosomal translocations using CRISPR-Cas9, Ewing sarcoma (ES) was chosen as the test model as it is defined by the occurrence of a chromosomal translocation. ES is a rare aggressive malignant neoplasm that occurs primarily in teenagers and young adults and that typically arises in tissues of mesenchymal origin. It is characterized by the t(11;22)(q24;q12) chromosomal translocation, which leads to the generation of the *EWSR1-FLI1* fusion gene [15]. To target the fusion gene, Torres et al. designed four sgRNAs to target intron 4 (F1, F2) of *FLI1* and intron 7 (E1, E2) of *EWSR1* [73]. First, the ES hallmark t(11;22)/*EWSR1-FLI1* chromosomal translocation was induced and characterized in HEK293 cells and in human primary mesenchymal stem cells (hMSCs) [73]. Then, DNA DSBs in the target loci were generated by transfection of HEK293 cells with a plasmid expressing Cas9 and sgRNAs E1, E2, F1, and F2 [73]. Quantification in metaphase spreads and interphase nuclei revealed a reciprocal translocation rate of $1.76 \pm 0.2\%$ or the E1F2 pair-wise sgRNA combination, whereas none of the three other combinations scored positive for illegitimate events in 280–290 nuclei examined [73]. Next, the group addressed whether the chromosomal translocation driven by CRISPR-Cas9 would be able to replicate the synthesis of the functional *EWSR1-FLI1* fusion gene [73]. The functionality of the fusion protein in the pool of hygromycin-selected t(11;22) positive populations was examined. qRT-PCR confirmed the upregulation of six well-known target genes (*EZH2*, *HMGA2*, *NKX2.2*, *ID2*, *NR0B1*, and *SOX2*) of the *EWSR1-FLI1* fusion protein, which suggested that the fusion protein expressed after induction of a chromosomal translocation by the CRISPR-Cas9 system has a similar activity to that expressed in primary ES cells [73].

3.2 Investigating gene function in sarcoma with CRISPR-Cas9

Genome sequencing studies demonstrate that human sarcoma is a complex process that involves a large collection of genetic alterations; the determination of which mutations are casually related to tumorigenesis remains a major challenge [75]. CRISPR-Cas9 has been used for rapid functional investigation of candidate genes in well-established models of cancer. Using a *KRAS*^{G12D}-driven lung cancer model, CRISPR-Cas9 was adopted to perform functional characterization on a panel of tumor suppressor genes (*NKX2-1*, *PTEN*, and *APC*), with known LOF alterations in human lung cancer [65]. Cre-dependent somatic activation of oncogenic *Kras*^{G12D} combined with CRISPR-Cas9-mediated genome editing of *NKX2-1*, *PTEN*, and *APC* genes resulted in lung cancer with distinct histopathological and molecular features. Through the introduction of constitutive Cas9 and inducible *P53* sgRNAs into hematopoietic stem/progenitor cells (HSPCs), which were then transplanted into myelo-ablated recipient mice, a hematopoietic-cell-restricted *P53*-knockout mouse model was developed [3]. Interestingly, this study generated mutations that not only caused loss of *P53* protein but also novel mutant *P53* proteins that could promote lymphoma development [3]. In addition, a genome-wide Cas9 knockout screen was used to develop a mouse model of tumor evolution [9]. In the study, the initial lung cancer cell line had little

capacity to form metastases; however, after being mutagenized with mouse genome-scale CRISPR knockout library A (mGeCKOa), the cell population formed highly metastatic tumors [9]. This study provides a roadmap for *in vivo* Cas9 screens and makes genome-scale CRISPR screening feasible using a transplant model with virtually any cell line or genetic background (e.g., mutations in *KRAS*, *CDKN2A*, *P53*, *PTEN*, etc.).

Recently, mouse models of osteosarcoma, ES, A-RMS, and synovial sarcoma have shown that inactivation of the *P53* pathway is present in the vast majority of human sarcomas (Table 3) [8, 14, 15, 18, 19, 27, 29, 34, 37, 38, 41, 43, 44, 54, 58, 61–63, 68, 70, 75, 76, 81]. For example, mutant *KRAS* expression and *P53* loss cooperate in the development of undifferentiated pleomorphic sarcomas [15, 75]. Therefore, the CRISPR-Cas9 system could be useful for functional genomic studies in these established sarcoma models.

3.3 Gene therapy with CRISPR-Cas9 in sarcoma

3.3.1 Editing sarcoma genome for anti-sarcoma—CRISPR-Cas9 can not only build various sarcoma models, but can also be used to explore drug treatment and resistance. In the NSCLC mouse model mentioned above, CRISPR-Cas9 mediated *EML4-ALK* rearrangement. The resulting tumors harbored the *EML4-ALK* inversion, expressed the fusion *EML4-ALK* gene, and responded to treatment with the *ALK* inhibitor crizotinib [46, 51]. In a new mouse model of *ARF*^{-/-} *Eμ-myc* B-cell lymphoma, CRISPR-Cas9-mediated disruption of *p53* conferred cells with resistance to doxorubicin treatment [49, 51]. These mouse models provide powerful tools for studying the mechanisms of drug resistance and testing novel therapies [49, 51].

Several studies have revealed that *CDK11* is essential in cancer, including in sarcoma cell growth and survival [21, 23, 33, 71, 86]. Our previous study identified that *CDK11* is critical for the growth and proliferation of liposarcoma cells, suggesting that *CDK11* may be a promising therapeutic target for treatment of liposarcoma patients [33]. Recently, CRISPR-Cas9 was applied to determine the effect of targeting endogenous *CDK11* at the DNA level in osteosarcoma cell lines [23]. Furthermore, the migration and invasion activity was markedly reduced by *CDK11* knockout, indicating that *CDK11* maybe a novel therapeutic target for osteosarcoma [23].

The development of multidrug resistance (MDR) is one of the major obstacles in the chemotherapy treatment of osteosarcoma [69]. Multidrug resistance gene 1 (*MDR1*), which encodes the membrane efflux pump P-glycoprotein (*P-gp*), plays an important role in the drug resistance process [69]. Overexpression of *MDR1* results in an active efflux of anti-cancer agents from cells, thus lowering intracellular drug concentrations and inducing cancer cell resistance to chemotherapeutic drugs, such as doxorubicin and paclitaxel [69]. Similar to targeting *CDK11* as described above, the CRISPR-Cas9 system can also be applied to knockout *MDR1* in drug resistant sarcoma cells to reverse drug resistance. Our group has designed an sgRNA targeting exon 5 of *MDR1* gene (Fig. 3). When plasmids with Cas9 and sgRNA were transfected into osteosarcoma MDR cell lines (KHOSR2 and U2OSR2), *MDR1* was efficiently knocked out (Fig. 4).

3.3.2 Genome-wide screens for anti-sarcoma drugs—The validation of a drug target is an essential step in drug discovery and development [35, 77]. While identification of drug resistance mutations is considered as the gold standard for target confirmation, further validation of drug-target interaction requires the introduction of a resistance mutation into the wild-type background [35, 77]. However, such an approach has not been straight forward in mammalian cells until the advent of the CRISPR-Cas9 technique. Recently, a proof of concept for drug validation in human cells was achieved based on resistance selection and genome sequencing in combination with CRISPR-Cas9 genome editing [35, 77].

Human exportin-1 (XPO1), also known as chromosome region maintenance 1 protein (CRM1), is considered an anticancer target [53]. In recent years, over expression of XPO1 has been observed in osteosarcoma and has been correlated with poor prognosis and resistance to therapy [83]. Selinexor (KPT-330), an inhibitor of XPO1, is currently undergoing phase 2 clinical trials and has demonstrated high response rates as a therapeutic agent in phase 1 trials for heavily pretreated, relapsed, and refractory hematological and solid tumor malignancies in humans [16, 36, 53]. Selinexor could inhibit the function and formation of XPO1, possibly through binding to the cysteine 528 residue of XPO1, which leads to an accumulation of tumor suppressor proteins in the nucleus of treated cells, and thus cell cycle arrest and apoptosis [16, 36, 53]. To validate this drug-target interaction, CRISPR-Cas9 was adopted to introduce a single XPO1 C528S mutation in acute T cell leukemia Jurkat cells. As expected, this mutation prevented Selinexor-mediated functional inhibition of XPO1 by blocking XPO1-Selinexor binding [53, 77]. The study confirmed that XPO1 served as the prime target of Selinexor in cancer cells [53, 77].

Ispinesib, an inhibitor of kinetin spindle protein (*KSP/HsEg5*), has entered clinical trials as an anticancer drug [60]. The drug demonstrated a high level of *in vivo* anti-tumor activity against ES [31] and has been applied to patients with rhabdomyosarcoma and osteosarcoma in a clinical trials [67]. Sequencing studies and bioinformatics analysis suggest that mutations in the Ala133 residue of KSP may be responsible for ispinesib resistance [35, 77]. To confirm this, vectors harboring the Cas9 ‘nickase’ and sgRNAs along with template DNA bearing the desired mutation were transfected into HeLa cells [35]. In the mutant transfectants, mutagenesis of Ala133 was confirmed by using the Surveyor mutation-detection and Sanger sequencing of the genomic locus. The results showed that the A133P substitution conferred >150-fold resistance to ispinesib, which validated previous results from sequencing and bioinformatics studies [35].

In addition to specific drug-target validation, CRISPR-Cas9 systems can also be used to conduct genome-scale screens for mutations that confer drug resistance [77]. The *Braf* V600E mutation has been recently detected in a subset of histiocytic tumors, particularly in histiocytic sarcoma and Langerhans cell histiocytosis [10, 32, 56]. Vemurafenib, a therapeutic *Braf* inhibitor, demonstrated efficacy in some tumors carrying the *Braf* V600E mutation [40]. A genome-scale CRISPR-Cas9 knockout (GeCKO) library targeting 18,080 genes with 64,751 unique guide sequences was used to identify genes whose loss is involved in resistance to vemurafenib [66]. The identified candidates included previously validated genes (*NF1* and *MED12*) and novel genes such as *NF2*, *CUL3*, *TADA2b*, and *TADA1* [66].

Similarly, a library consisting of 70,290 guides targeting all human RefSeq (the Reference Sequence of NCBI) coding isoforms was synthesized to screen for genes that confer resistance to vemurafenib [39]. In this study, a new activation system called synergistic activation mediator (SAM) was used [39]. SAM is an engineered protein complex for the transcriptional activation of endogenous genes [39]. The results demonstrated that the SAM system is robust and specific, and can facilitate genome-scale GOF screening when combined with a compact pooled sgRNA library. The SAM-mediated screens' results confirmed genes previously shown to yield drug resistance, and novel candidates were validated using individual sgRNA and complementary DNA overexpression [39]. In comparison with RNAi screens using a short hairpin RNA (shRNA) library, genome-scale screening with CRISPR-Cas9 showed a higher reagent consistency and stronger phenotypic effects of individual sgRNAs [66, 77]. In addition, these studies exemplified the significant advantages of the CRISPR-Cas9 system in genome-wide LOF and GOF screens for anticancer drug development [77].

4. Challenges and further improvement of CRISPR-Cas9 technology in sarcoma

CRISPR-Cas9 technology has evolved to create a simple, RNA-programmable method to precisely mediate genome editing in mammalian cells, although some shortcomings still exist. One potential limitation of CRISPR-Cas9 technology is that the approach may create off-target effects, though relatively uncommon as compared with other genome editing tools, such as RNAi, TALEN, or ZFN [11, 74]. The efficiency of editing by CRISPR-Cas9 systems can also be further improved [11, 74]. There are several reports demonstrating that the efficiency of CRISPR-Cas9, albeit sufficient to induce tumors, is relatively low [45, 46, 57, 65, 82]. Various strategies have been reported to reduce off-target effects [11, 45, 74, 87–89]. The choice of unique target sequences is important for avoiding off-target effects [11]. The target sequences should differ from any other sites in the genome by at least two or three nucleotides in a 20-nt sequence [11]. In addition, paired Cas9 nickases are highly specific in human cells, and can generate two single-strand breaks or nicks on different DNA strands [11]. Additionally, more efficient deliveries, more powerful sgRNAs, and more potent Cas9 systems are in development [11, 45, 74, 87, 88]. The SAM system, which has been developed by engineering the sgRNA through appending a minimal hairpin aptamer to the tetraloop and stem loop 2 of sgRNA, is specific with minimal off-target activity when SAM-mediated gene activation [39, 87]. *Streptococcus pyogenes* Cas9-HF1 (SpCas9-HF1) was described to be a high-fidelity CRISPR-Cas9 nuclease [88]. It retains on-target activities comparable to wild-type SpCas9 with >85% of sgRNAs tested in human cells [88]. Recently, “enhanced specificity” SpCas9 (eSpCas9) was named. It was able to dramatically reduce “off-target editing” to undetectable levels in the specific cases examined by changing three of the approximately 1,400 amino acids that make up SpCas9 [89].

CRISPR-Cas9 systems have been used in sarcoma modeling and therapy explorations. However, the CRISPR-Cas9 system has an even wider potential for application in sarcoma. For example, CRISPR interference (CRISPRi), which refers to transcriptional suppression of target genes by Cas9 binding, can be used to knock down cancer-associated genes for

functional interrogations [25, 45]. Catalytically inactivated Cas9 (deadCas, dCas9) can be repurposed as a RNA-guided DNA-binding domain fused to various effectors, such as green fluorescent proteins, that enable live imaging of genome loci of interest [25, 45]. When the effector is an epigenetic modifier, such as a DNA methyltransferase or histone acetyltransferase, it reaches the target sites directed by sgRNA and regulates epigenetic alterations, a process termed epi-genome editing [45]. In sarcoma, epigenetic abnormalities and genomic mutations are two sides of one coin. They interact with each other and cooperate to drive carcinogenesis. Given this, dCas9-mediated epigenome editing may become a convenient tool in the future for modeling sarcoma-related epigenetic abnormalities and a promising avenue for sarcoma therapy [45].

In conclusion, CRISPR-Cas9 systems can mediate genome editing, epigenetic regulations, and transcriptome modulations [77]. Though some challenges remain ahead, the application of this technology to several aspects of sarcoma biology, ranging from basic research to clinical and translational applications, offers numerous exciting opportunities for a better understanding and potential treatment of these devastating diseases.

Acknowledgments

This work was supported, in part, by the Gattegno and Wechsler funds, the Kenneth Stanton Fund, and the Jennifer Hunter Yates Foundation. Dr. Duan is supported, in part, through a grant from Sarcoma Foundation of America (SFA), a grant from National Cancer Institute (NCI)/National Institutes of Health (NIH), UO1, CA 151452-01, a pilot grant from Sarcoma SPORE/NIH, and a grant from an Academic Enrichment Fund of MGH Orthopaedics. Dr. Liu is supported by a scholarship from the Chinese Scholarship Council.

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Highlight

1. CRISPR-Cas9 systems can mediate genome editing, epigenetic regulations, and transcriptome modulations.
2. CRISPR-Cas9 genome editing technologies hold extensive application potentials in sarcoma models and gene therapies.
3. CRISPR-Cas9 could be used to explore drug treatment and resistance of sarcoma.

Fig. 1. Overview of the CRISPR-Cas9 system

The core components of the CRISPR-Cas9 system are a nuclease Cas9 and a single guided RNA (sgRNA). The nuclease Cas9 consists of two catalytic active domains: HNH and RuvC. The sgRNA binds to Cas9 and directs it to the locus of interest by a 20-nt guide sequence via base pairing to the genomic target. The black font is the commonly used sequence for sgRNA, not including the 20-nt guide sequence. When Cas9 binds to the target DNA, the target sequence in the genomic DNA is paired to the sgRNA sequence and is immediately followed by a NGG sequence called the protospacer adjacent motif (PAM). The CRISPR-Cas9 system uses Cas9, which complexes with sgRNA to cleave DNA and generate double-strand breaks (DSBs) in a sequence-specific manner about 3–4 base pairs upstream of PAM.

Fig. 2. Genome editing technologies exploit endogenous DNA repair machinery

Double-strand breaks (DSBs) induced by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous directed repair (HDR). Repair by NHEJ usually results in the insertion or deletion of random base pairs (A), or completely remove the exon (B), causing gene knockout by disruption. HDR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions (C) or to achieve gene insertion (D).

AAAGC GATAA GTTTA ACCGA ACTGT TCAAC ATATA CCACC ACCCT TGAAA CCGAC GGTAG
 TAGGT ACCCC GACCT GAA **GG AGAGT ACTAC GACCA CAAAC** CTCTT TACTG TCTAT AGAAA
 CGTTT ACGTC CTTTA AATCT TCTAG ACTAC AGTTT GTAGT GATTA TCTT

Fig. 3. Schematic of U6 ABCB1 sgRNA-CMV Cas9-GFP expression cassette in the single plasmid system

GFP is co-expressed from the same mRNA as the Cas9 protein via a 2A peptide linkage, which enables tracking of transfection efficiency. The exon of ABCB1 selected for guide RNA design is located on the 5th coding exon. The human U6 promoter is used to drive gRNA expression, while the CMV promoter drives expression of Cas9 and GFP proteins. The position of the frame shift that CRISPR-Cas9 knocks out is located at exon 5 within the ABCB1 gene (NM_000927.4MDR1). The red font is the 20bp for gRNA, and AGG (yellow font) is the sequence of PAM.

Fig. 4. Lipofectamine transfection of ABCB1 sgRNA + Cas9+GFP work flow

When plasmids with Cas9 and sgRNA are transfected into osteosarcoma MDR cell lines (KHOSR2 and U2OSR2), *MDR1* is efficiently knocked out.

Table 1

Comparison of the CRISPR-Cas9 system with ZFNs and TALENs

	ZFNs	TALENs	CRISPR-Cas9 system
Target DNA recognition	Zinc fingers (ZFs)	Repeat variable diresidues (RVDs)	crRNA or sgRNA
DNA cleavage endonuclease	Fok I nuclease domain	FoK I nuclease domain	Cas9
Construction of endonuclease	3–4 ZFs domains	8–31 RVD repeats	sgRNA
Minimum DNA base of recognized	Triple	Single	Single
Size of recognized DNA sequences	9–18 bp	(8–31bp) × 2	20 bp + NGG
Restriction of target site	/	Binding site should start with a T base	PAM sequences (NGG or NAG)
Cytotoxicity	High	Low	Low
Advantages (Comparison of ZFNs & TALENs with CRISPR-Cas9)	High efficiency; Lower rate of off-target cleavage than CRISPR-Cas9		Very high efficiency and rapid construction and easy delivery; Multiplexing possible <i>in vitro</i> and <i>in vivo</i>
Disadvantages (Comparison of ZFNs & TALENs with CRISPR-Cas9)	More difficult to assemble than CRISPR-Cas9 and remaining possibility of off-target cleavage		Off-target cleavage possibly more frequent than ZFNs and TALENs

Table 2

Modeling cancer with CRISPR-Cas9

Target Genes	Types of mutation	Cell Lines or cancer Models	Malignancy	Reference(s)
<i>TET2, RUNX1, DNMT3A, EZH2, NFI, SMC3</i>	Loss of function (LOF) mutations	Lineage ⁻ /Sca1 ⁺ /cKit ⁺ (LSK) cell	Myeloid malignancies	27
<i>APC, P53, KRAS, SMAD4</i>	Gain of function (GOF) of <i>Kras</i> and LOF of <i>APC, P53, SMAD4</i>	Human small intestinal stem cell	Colorectal cancer	22
<i>APC, P53, KRAS, SMAD4</i>	GOF of <i>KRAS</i> and LOF of <i>APC, P53, SMAD4</i>	Human intestinal stem cells	Colorectal cancer	28
<i>PTEN, P53</i>	Indels	Mouse 3T3 cells	Liver cancer	30
<i>NKX2-1, PTEN, APC</i>	Loss of function mutations	A <i>KRAS</i> ^{G12D} -driven lung cancer mode	Lung cancer	23
<i>MCL-1</i>	Indels	Human Burkitt lymphoma (BL) cells	Lymphoma	40
<i>CD74, ROS1</i>	Interchromosomal translocation	HEK 293T cells; non-transformed immortalized lung epithelial cells (AALE)	Lung cancer	34
<i>MLL4, ALK</i>	Intrachromosomal inversion within a single arm	HEK 293T cells	Lung cancer	34
<i>KIF5B, RET</i>	Intrachromosomal inversion across both arms	HEK 293T cells	Lung cancer	34
<i>NPM, ALK</i>	Interchromosomal translocation	HCT116 cell line	Lymphoma	33
<i>mGeCKOa</i>	LOF mutations	Non-small-cell lung cancer cell line	Lung cancer	41
<i>KRAS, P53, LKB1</i>	GOF of <i>KRAS</i> and LOF of <i>P53, LKB1</i>	Mouse dendritic cells	Lung cancer	31
<i>MLL4, ALK</i>	Intrachromosomal inversion within a single arm	NIH/3T3 cells	Lung cancer	35
<i>MLL4, ALK</i>	Intrachromosomal inversion within a single arm	Primary fibroblasts cells from mouse tail, or HEK 293T cells and ASB-XIV cell lines	Lung cancer	36
<i>EWSR1, FLI1</i>	Interchromosomal translocation	HEK293A cell line and human mesenchymal stem cells	Ewing sarcoma	39
<i>RUNX1, ETO</i>	Interchromosomal translocation	HEK293A cell line and CD34+ human haemopoietic/progenitor stem cells	Myeloid malignancies	39
<i>PAX3-FOXO1</i>	Chromosome translocation	Myoblast cells from fore and hind limb	Alveolar Rhabdomyosarcoma	38
<i>H-RAS, CDKN2a</i>	<i>H-RAS</i> expression plus knockdown of <i>CDKN2A</i>	Primary mouse embryonic fibroblast (<i>MEF</i>) cells; human melanoma A375 cells; C2C12 cells; NIH3T3 Cells; LLC-1, B16-F10, MC-38 cell lines	Undifferentiated sarcoma with pleomorphic and rhabdoid	36
<i>H-RAS, P53</i>	<i>H-RAS</i> expression plus knockdown of <i>P53</i>	The same as the “ <i>H-RAS, CDKN2A</i> ” line	The same as the “ <i>H-RAS, CDKN2A</i> ” line	36
<i>H-RAS, P53, PTEN</i>	<i>H-RAS</i> expression plus knockdown of <i>P53</i> and <i>PTEN</i>	The same as the “ <i>H-RAS, CDKN2A</i> ” line	The same as the “ <i>H-RAS, CDKN2A</i> ” line	36

Table 3

Xenograft models of human sarcoma

Tumor type	Gene mutation(s)	Significance	Reference(s)
Sporadic/varied	<i>P53</i> —/—	Mice deficient for <i>P53</i> could prone to developing sarcoma.	45
Sporadic/varied	<i>P53</i> mutation	Mice for the <i>P53</i> mutation were most prone to developing sarcoma.	44
OS & Poorly differentiated soft tissue sarcomas (PDSTS)	<i>RB</i> and <i>P53</i> deletion	Deletion of <i>P53</i> in mesenchymal cells that give rise to osteoblasts is a powerful initiator of OS. Deletion of <i>RB</i> does not initiate sarcoma formation in mice, but it accelerates formation of sarcoma.	46
PDSTS	<i>EWS-FLI1</i> or <i>P53</i> deletion	In the setting of <i>P53</i> deletion, <i>EWS-FLI1</i> accelerated the formation of sarcomas.	46
OS	<i>TAX</i> ; <i>P19ARF</i> —/—	The loss of <i>ARF</i> selectively predisposed <i>TAX</i> transgenic mice to development of OS.	47
OS	<i>P53</i> and <i>RB</i> deletions	Osterix-Cre-mediated deletion of <i>P53</i> and <i>RB</i> results in OS.	48
OS/Lymphosarcoma/Leiomyosarcoma	<i>RB</i> and <i>P107</i> deletions	<i>RB</i> haploinsufficiency coupled with <i>P107</i> deletion could results in sarcoma formation.	49
Failed to form sarcoma	<i>PAX3-FKHR</i> fusion	<i>Pax3-FKHR</i> allele causes lethal developmental defects in knock-in mice but might be insufficient to cause sarcoma.	50
Alveolar Rhabdomyosarcoma (RMS)	<i>PAX3-FKHR</i> transgene and <i>P53</i> and <i>RB</i> deletion	Expression of the <i>PAX3-FKHR</i> transgene requires loss of <i>P53</i> and <i>RB</i> for RMS formation.	51, 52
RMS/Various sarcoma	<i>P53</i> and <i>RB1</i> deletion with or without <i>PTCH1</i> haploinsufficiency	Mutant <i>P53</i> , <i>PTCH1</i> , or <i>RB1</i> in satellite cells gives rise to undifferentiated sarcoma; <i>RB1</i> loss acts as an apparent modifier for sarcomas by inducing dedifferentiation	53
Pleomorphic RMS	Mutant <i>KRAS</i> expression and <i>P53</i> deficiency	Cooperation of oncogenic <i>KRAS</i> and <i>P53</i> deficiency resulted in the development of pleomorphic RMS in adult mice	54
Pleomorphic RMS	Mutant <i>KRAS</i> expression and <i>P53</i> mutation or loss	The combination of oncogenic <i>KRAS</i> and loss of <i>P53</i> activity accelerates sarcoma formation; Mutation, but not loss of a single <i>P53</i> allele, is sufficient for sarcoma development.	55
Synovial sarcoma	<i>SYT-SSX</i> fusion	Expression of the <i>SYT-SSX 2</i> fusion gene yields a highly penetrant and representative model of human synovial sarcoma.	56
Liposarcoma	<i>TLS-CHOP</i> fusion with loss of <i>P53</i>	Deletion of <i>P53</i> cooperates in the formation of liposarcomas	57
Neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs)	<i>NF1</i> deletion With or without <i>P53</i> and <i>P19ARF</i> deletions	<i>NF1</i> and <i>P53</i> mutations lead to MPNSTs; <i>INK4a</i> and <i>ARF</i> deficiency cooperate with <i>NF1</i> heterozygosity to yield MPNSTs.	58

Tumor type	Gene mutation(s)	Significance	Reference(s)
Uterine leiomyosarcoma	<i>LMP2</i> deficient	<i>LMP2</i> deficient mice spontaneously develop uterine leiomyosarcoma.	59
Uterine leiomyosarcoma	Overexpression of <i>CRIPTO-1</i> (<i>CR-1</i>)	<i>CR-1</i> play a important role in the formation of uterine leiomyosarcoma.	60
Uterine leiomyosarcoma	Deletion of <i>P53</i> and <i>BRCA1</i>	Conditional deletion of <i>P53</i> in mice results in the development of uterine leiomyosarcoma and that concurrent deletion of <i>P53</i> and <i>BRCA1</i> significantly accelerates the progression of the tumors.	61