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CRISPR/Cas9: A tool for immunological research

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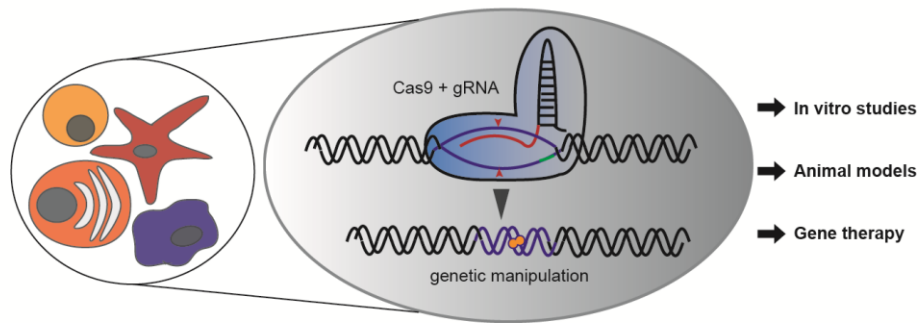
Abstract

The CRISPR/Cas9-system was originally identified as part of the adaptive immune system in bacteria and has since been adapted for the genetic manipulation of eukaryotic cells. The technique is of particular value for biomedical sciences, as it enables the genetic manipulation of cell lines and primary cells as well as whole organisms with unprecedented ease and efficiency. Furthermore, the CRISPR/Cas9-technology has the potential for future therapeutic applications in the clinic. Here, we discuss the use of CRISPR/Cas9 for the genetic modification of haematopoietic cells and the generation of mouse models for immunological research. Additionally, we explain how the technique can be applied as a screening-tool to identify genes involved in different immunological processes. Moreover, we will talk about recent extensions of using the CRISPR/Cas9 technology, such as a transcriptional activator or repressor. Finally, we discuss the first clinical trials that use CRISPR/Cas9 and discuss potential future applications.

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The review article by Hochheiser et al. discusses the use of the CRISPR/Cas9 technology for the genetic manipulation of haematopoietic cells and whole animals, and its potential application in the clinic.



CRISPR/Cas9: A Tool for Immunological Research

Introduction

The genetic manipulation of cells as well as whole organisms is an important technique used to decipher the function of genes and their products in biological processes. Although the techniques used to over-express genes are well established, the inhibition or abrogation of gene expression has been comparatively challenging, particularly before the development of designer nucleases such as zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN). However, while ZFN and TALEN have initially been used to delete genes, their laborious design and production have prevented the wide-spread use of these techniques. Since the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)-systems as part of the adaptive immune system in bacteria and archaea[1], those systems have been quickly adapted to genetically modify eukaryotic cells and organisms[2]. There are different types of CRISPR/Cas-systems that utilise different Cas proteins and RNA molecules. These systems can be divided into two main classes according to their use of multiple-protein complexes (class 1) or a single nuclease protein (class 2). These classes of CRISPR/Cas systems can further be divided into different types according to the specific proteins they utilize (e.g. class 2, type V uses the nuclease Cpf I and type II uses the nuclease Cas9). The type II CRISPR/Cas9-system is the best characterized and most widely used technique amongst the different systems. It requires only one protein and one RNA component to function as a DNA endonuclease[3]. The only requirement for Cas9 to cut the target DNA is the presence of a short nucleotide motif, referred to as protospacer adjacent motive (PAM). Sequence-specific guide (g)RNAs (single engineered chimeric RNA molecules consisting of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) mediate sequence specific DNA targeting) direct Cas9 to a specific genomic region or DNA sequence. The interaction between Cas9/gRNA complexes with target DNA sequences mediates conformational changes and thus activation of Cas9 endonuclease activity, which subsequently induces DNA double strand breaks[4]. These DNA double strand breaks are in turn repaired by distinct cell-intrinsic DNA-repair pathways, either by homologous recombination (in the presence of a donor DNA repair template with homologous sequence flanking the DNA cut site), by micro homology mediated end joining (MMEJ or alt-NHEJ) or by non-homologous end-joining (NHEJ or c-NHEJ). While MMEJ leads to deletions of various length at the DNA breaks, NHEJ leads to random base pair insertions or deletions (InDels) (Fig. 1A). Notably, both pathways lead to functional inactivation of targeted genes with high efficiency [5, 6]. Due to its high efficiency, simplicity of designing and producing gRNAs, as well as high specificity and minimal off-target effects (if high scoring PAM sites can be used), the CRISPR/Cas9-methodology has rapidly become the state-of-the-art technique for genetic manipulation of mammalian cells and genetically modified mice, and has the potential to be used in a diverse range of gene therapy approaches in the future (summarized in Fig. 1A). Furthermore, modifications of the Cas9-enzyme, e.g. abrogation of the endonuclease activity and fusion to transcriptional activators or repressors or genetic markers, make the CRISPR/Cas9 system suitable for specific activation or repression of endogenous gene expression or for marking of specific genomic regions, directed by sequence specific gRNAs (Figure 1B) (a more detailed discussion can be found below)[7]. Different applications of the CRISPR/Cas9-technology are summarised in Fig. 1B.

Manipulation of haematopoietic cells

A detailed understanding of the molecular pathways underlying immunological processes requires the systematic genetic manipulation of cells involved in the respective responses. Since the adaptation of the bacterial CRISPR/Cas9 system for the genetic manipulation of eukaryotic cells, the technique has

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been used in numerous scientific fields. Due to its high efficiency and precision, CRISPR/Cas9 technology can not only be used to modify immortalised cell lines, which can (theoretically) be cultured and duplicated indefinitely, but will also be particularly useful for manipulation of primary haematopoietic stem and immune cells (Fig. 1A) that have a limited lifespan and cannot be subjected to time-intensive selection processes. The recent development of Cas9 transgenic mice[8, 9] facilitates the more efficient usage of the CRISPR/Cas9-technology in primary cells, which until recently has been hampered by difficulties in introducing all components (namely Cas9 protein and gRNAs and in some cases a DNA template for homologous recombination) into the same cell. Recently a few groups have reported efficient manipulation of primary haematopoietic stem cells and lymphocytes by using ribonucleoprotein (RNP) complexes, which involve the complexing of gRNA and Cas9 protein *in vitro* prior to delivery by transfection [10-13]. The advantage of this system is its application in versatile cell types without the need of transgenic Cas9 expression. Furthermore, Cas9 is only present for a short amount of time, reducing the likelihood of introducing unwanted off-target mutations in other genes. However, these systems, in which Cas9 is only present for a short amount of time, do not allow the conditional CRISPR/Cas9-mediated deletion of genes, e.g. via inducible guideRNAs, which is particularly important for studying genes essential for cell survival[14]. Furthermore, CRISPR/Cas9-mediated manipulation of haematopoietic precursor cells and their transfer into irradiated mice constitutes a fast and efficient method to generate animals with genetic modifications in the haematopoietic system[14]. This method will be particularly useful for immunological and cancer research, especially due to the fact that multiple genes can be efficiently manipulated at the same time, as outlined in the next section of this article. As CRISPR/Cas9-mediated genetic manipulation of human haematopoietic precursor cells does not seem to influence their differentiation potential [15], ex-vivo genome modified cells could also form the basis for cell-based therapies. In line with this, several studies have applied the CRISPR/Cas9 technology to edit primary human T cells [12, 16] and to generate chimeric antigen receptor (CAR)-T cells, which show efficient anti-tumour activity in vitro and in vivo [13, 17-19].

We discuss potential future therapeutic applications and their implications later on in this article.

Animal Models

The generation of knockout mice via traditional ES cell technology is very time consuming, laborious and often rather inefficient. Targeting of embryonic stem (ES) cells by homologous recombination, breeding of chimeric mice and crossing heterozygous mice to obtain homozygous offspring takes many months to years. To obtain mice with more than one genetic alteration, complex crosses are required. However, the generation of mice using CRISPR/Cas9 technology in ES cells or by direct microinjection of CRISPR/Cas9 components into the single cell zygote has resolved many of the problems present when using the traditional ES cell approach. For instance, the introduction of modifications is often bi-allelic and is not limited to one gene. Recently, it was shown that bi-allelic mutations can be introduced in up to five genes at once using CRISPR/Cas9 technology in ES cells[20]. To this end, ES cells were simultaneously transfected with Cas9 mRNA and five gene-specific gRNAs. Although these mutations might disperse when breeding the founder lines to establish quintuple knockout mice, it still demonstrates the potential and efficiency of this technique. Interestingly, the same study also showed that ES cells were no longer required to produce gene modified mice. Instead, one-cell stage embryos were used to inject Cas9 mRNA and gRNAs to delete specific gene products. This led to the generation of knockout founder lines, which in theory could already be used to address the effect of the deleted gene in mice.

While the CRISPR/Cas9 technology facilitates and accelerates the generation of animal models, a few concerns remain: (1) At this stage, it is still difficult to predict how many off-target mutations are introduced by the Cas9 endonuclease, i.e. how many non-targeted genomic spots might be affected by the activity of the Cas9 protein. Although off-target effects have also been observed when generating mice with traditional ES cell technologies, breeding of the founder lines to a wildtype background decreased potential side-effects [21]. While we have seen relatively little off-target phenotypes, backcrossing of CRISPR/Cas9 generated mutant mice is recommended. (2) When genome modifications are introduced in the one cell stage embryo, analysis by next generation sequencing often reveals more than two different sequence types in the founder lines (own observation). In addition, we have frequently detected wildtype and mutant alleles being transmitted to offspring that were previously not noted in founder mice. This is probably due to the fact that targeting can occur between the 1 – 8 cell-stage during embryonic development, resulting in mosaicism. These observations of high mosaicism make it difficult to solely rely on phenotype analysis performed on the founder lines.

Nevertheless, despite these caveats, the ease, low cost and high speed of producing genome-modified mice with the CRISPR/Cas9 technology is unprecedented. While traditional ES cell techniques required the assembly of large targeting vectors for a classical knockout, the CRISPR/Cas9 technology only requires the design of small gRNAs for each gene, making the production inexpensive. Compared to obtaining an ES cell derived Knockout model, the CRISPR technology is approximately 5 to 10-fold cheaper and at least 6 months faster.

So far, we have only discussed the generation of constitutive knockout mice using CRISPR/Cas9 technology. However, deleting genes conditionally (e.g. tissue specific) or engineering reporter mouse lines is indispensable for many research questions in the field of immunology. While early on the group of Rudolf Jaenisch described a supposedly easy approach of generating conditional or reporter alleles in mice using CRISPR/Cas9[22], it soon became evident that the generation of such complex alleles was rather difficult[23]. However, we have recently made great advances in generating these complex models[24]. Contrary to previous reports, which used short oligonucleotides as DNA repair templates, we have reverted to using larger targeting vectors to engineer conditional or large knock-in alleles using CRISPR/Cas9 methodology. The targeting vectors used contain ~ 2kb homology arms upstream and downstream of the specific target site and are injected in circular form into the zygote [24]. Although not as fast and easy, it results in reliable targeting in a relatively short period of time (6-9 month) and at reduced costs (3-4 times cheaper) compared to traditional techniques.

Since the generation of genetically modified animal models does not rely on ES cell techniques anymore, generation of pre-clinical models in species other than mice is possible. Of particular interest are genome modified rats, as those represent one of the prime models used in multiple sclerosis research. Indeed, it has been shown that the CRISPR/Cas9 technology can be used to generate total as well as conditional knockout alleles in the rat[25, 26]. Furthermore, other species such as non-human primates, can be modified via CRISPR/Cas9 [27]. Table 1 gives an overview of advantages and caveats of CRISPR/Cas9 based approaches and traditional ES cell targeting for the generation of genetically modifies animals.

Overall the CRISPR/Cas9 technology represents a fast and easy way to generate gene modified mice as well as other animals, an advancement which will greatly accelerate immunological findings, a branch of research, that highly depends on sophisticated animal models.

Screening

Functional whole genome screening approaches are a useful tool to identify genes involved in certain biological processes. Until recently, such screens were performed using RNA interference (RNAi). However, RNAi only partially reduces but does not completely abrogate gene expression. Additionally, RNAi can have many off-target effects, which can potentially lead to false positive hits[28]. Recent studies have shown that CRISPR/Cas9 based screens using whole genome gRNA-libraries can be used to quickly and efficiently identify genes involved in different biological processes [29, 30]. (gRNA-libraries: a mixture of thousands of guide RNAs, which are capable of targeting every gene in the genome). Importantly, this technology can be used to fully understand complex processes in primary immune cells. In a recent study, Parnas et al.[31] generated bone marrow derived dendritic cells from Cas9 transgenic mice and lentivirally transduced these cells with a genome wide gRNA-library. Subsequently, these cells were stimulated with LPS and the production of TNF α was measured by intracellular cytokine staining. Cells were FACS sorted according to their TNF α levels and their genomic DNA was isolated and sequenced by next generation sequencing for the stably integrated gRNAs. This approach identified genes known to be involved in TNF α production, which served as a positive control, as well as novel genes involved in the production of TNF α in dendritic cells. Furthermore, more target specific CRISPR/Cas9 screens in primary immune cells have recently been reported. In a study by Chu et al.[32] the authors isolated primary B cells from Cas9 transgenic mice and transduced the cells with retrovirally expressed guide RNAs targeting a set of 83 genes upregulated during plasma cell differentiation. 22 genes were identified to be important for plasma cell survival and proliferation and eight genes proved to be important for differentiation. Additionally, the authors showed that single gRNA transduction in primary T cells and bone marrow-derived macrophages led to efficient gene deletions, making it very likely that small scale, but also whole genome screenings will be a possible application in different immune cells in the future.

Excitingly, Jaitin et al.[33] recently combined CRISPR/Cas9 based screening with single cell RNA sequencing (CRISPR-seq) *in vitro* and *in vivo*. These experiments form the basis for unravelling complex immunological signalling networks. In this study, the authors lentivirally transduced bone marrow cells with gRNAs targeting different transcriptional regulators known to affect the differentiation of myeloid cells. The gRNA vectors expressed additional fluorescence and genetic markers (so called unique guide identifier sequences) to allow the identification of individual cells. Through RNAseq based phenotypic analysis and simultaneous correlation with different gRNAs, Jaitin et al. identified new signalling cascades and transcriptional regulators important for myeloid differentiation.

Modified Cas9 proteins and their applications

By mutating two residues within the Cas9 nuclease domains, the endonuclease activity can be inactivated (dead-Cas9 (dCas9)) and thus converted into a gRNA-programmable DNA-binding protein that can be used for various applications: by sterical interference or fusion to transcriptional activators or repressors, modified dCas9 can function to either activate (CRISPRa) or repress (CRISPRi) endogenous gene transcription[34, 35] (Fig. 1B). Those modified dCas9-proteins can not only be used for loss-of-function but also for gain-of-function screens by modifying endogenous gene

expression via targeting extensive genomic promotor regions with tiled gRNA libraries[36]. Furthermore, CRISPRa can be used to identify unknown enhancers in non-coding regions around genes of interest. This is particularly interesting, as many disease-associated genetic variants have been mapped to non-coding regions of the genome. A recent study used an CRISPRa screen (using dCas9 fused to the transcriptional activator VP64) to identify transcriptional enhancers in the non-coding regions around the genomic areas encoding for CD69 and IL2R α , two key autoimmunity risk loci [37]. The authors first validated their method by targeting an enhancer at the CD69 locus. This enhancer was readily identified by sorting transduced Jurkat T cells according to their CD69 expression and sequencing for enriched gRNAs. With the same approach, the authors identified previously unknown enhancers around the IL2R α locus. The identified enhancer regions showed chromatin features of physiologic enhancers and one of those regions harboured a single nucleotide polymorphism (SNP), which has previously been implicated as a risk factor for autoimmune disease in humans. Therefore, the authors tested the impact of this SNP on IL2R α in cell lines and a mouse model harbouring the genetic variant. *In vivo* experiments showed a dysregulated IL2R α expression which skewed the polarisation of naïve T cells towards autoimmunity promoting Th17 cells. This study impressively demonstrates the ability of CRISPRa to identify relevant regulatory elements in complex transcriptional networks.

Besides acting as transcriptional regulators, modified Cas9 proteins can be applied for visualising genomic elements as shown in a recent study by Chen et al. [38]. In this study EGFP-tagged dCas9 in combination with specific gRNAs was used to visualise chromosome dynamics in living human cells. The authors demonstrated that this technique enabled the imaging of repetitive elements as well as non-repetitive genomic sequences by tiling gRNAs specific for the target locus. This technique complements the use of DNA-binding proteins with limited target sequences and Fluorescence in situ hybridisation (FISH), which can label any genomic region through base pairing of nucleic acid probes but is incompatible with live imaging because samples need to be fixed and denatured.

Other applications of modified Cas9 proteins are discussed in more detail elsewhere[2].

Clinical studies and potential applications

Therapies in which haematopoietic stem cells derived from patients are genetically modified via ectopic expression of a functional copy of a defective gene and transplanted back into the patient have already been used in the clinic for a while[39]. However, delivering genes via viral vectors results in random genomic integration of the transgene. This bares risks such as insertional mutagenesis or dysregulated expression and can thus lead to immunological rejection of the modified cells or to the activation of oncogenes[40]. Gene-therapy associated risks can be minimized by either transient expression systems or sequence-specific mutagenesis, using non-integrating viral vectors, physical (e.g. electroporation or microinjection) or chemical (e.g. lipofection) transfection of non-viral vectors for template delivery. In a recent study by Genovese et al. [41], the gene defect underlying X-linked Severe Combined Immunodeficiency was corrected in a sequence-specific manner in patient-derived stem cells, leading to restoration of its gene function. While in this study zinc finger nucleases (ZFNs) were used to target a corrective DNA template into the stem cell genome, the CRISPR/Cas9 technology has recently been shown to be suitable for inducing sequence-specific alterations in human haematopoietic stem cells[15]. Similar to ZNFs and TALENs, the CRISPR/Cas9 methodology can be

used for both, correction but also deletion of defective genes. Compared to ZFNs and TALENs, the CRISPR/Cas9 system is less complex, less costly and less time consuming and thus more suitable for individual patient-based therapies. This feature promises to be useful for immune-therapies and could soon find its way into the clinic.

A number of clinical trials using cells which have been genetically modified by CRISPR/Cas9-technology are already underway. The first clinical trial involving CRISPR/Cas9 mediated gene modification started in October 2016 at the West China Hospital in Chengdu [42]. To render the immune system more aggressive towards cancer cells, T cells from lung cancer patients were isolated, genetically modified to lack PD-1 expression and re-transferred into the same individuals. Immunotherapies using blocking antibodies for PD-1 have proven successful, setting the ground for this approach. However, critics argue that a successful treatment with blocking antibodies makes a gene therapy approach unnecessary[42]. Nevertheless, numerous clinical trials targeting PD-1 in T cells from patients with various cancers are planned. Additionally, several CRISPR/Cas9-based clinical trials, in which T cells will not only be deprived of PD-1 expression but additionally equipped with tumour-specific T cell receptors (chimeric antigen receptor (CAR)-T cells), might commence in the near future[43].

CRISPR/Cas9-mediated gene editing could not only be applied for the treatment of cancer but might also be beneficial in other contexts. Thus, CRISPR/Cas9 could be used in anti-viral therapy against infections with the human immunodeficiency virus (HIV) as well as other persistent viruses. To this end, cells can be rendered resistant towards viral infections, e.g. by deleting the gene for the chemokine receptor 5 (CCR5), an important co-receptor for HIV. Furthermore, studies have shown that the viral genome could be eliminated from the infected cells using CRISPR/Cas9 technology[44, 45]. Along these lines, a potentially ground breaking clinical trial aims to edit human papillomavirus (HPV) infected cells via CRISPR/Cas9 and TALENs *in vivo* as a therapy for HPV-associated cervical cancer[46].

Furthermore, CRISPR/Cas9-based therapies of several diseases caused by mono-genetic mutations, such as beta-thalassemia, haemophilia, and Duchenne muscle dystrophy have shown promising results in patient derived cells and in animal models. Thus, the mutation causing beta-thalassemia can be corrected in induced pluripotent stem cells (iPSC) derived from patients. Excitingly it was shown that these corrected iPS cells could give rise to a functional haematopoietic system when transplanted into mice[47]. Haemophilia- and Duchenne-causing mutations could even be successfully edited *in vivo* by using the CRISPR/Cas9 technology in mice[48-51].

The number of diseases of haematopoietic, but also non-haematopoietic origin that might be treatable by the CRISPR/Cas9 technology is very large. Nevertheless, the therapeutic application of this technology remains highly controversial. First of all, it is still unclear how safe this technique is, especially with regards to potential off-target effects. Secondly, there are a number of ethical questions associated with CRISPR/Cas9 mediated gene therapy: Where will the line be drawn between genetic defects that should be corrected and genetic “anomalies”, that remain untreated? Which consequences and long-term effects will the correction of pathogenic mutations have in the context of the patients’ individual genetic backgrounds? Should modifications of the genome be allowed in human zygotes or embryos, which will not only effect somatic cells but also transmit to the germ line?

Concluding remarks

The CRISPR/Cas9 technique has revolutionised genomic engineering and is probably one of the biggest breakthroughs for scientific research of the current century. The technique is particularly valuable for immunological research as genetic manipulation of single cell types as well as the generation of a complete gene-edited immune system can be achieved in a very short time frame. Although other genome modifying technologies existed before the discovery of the CRISPR/Cas9 system, the simplicity, precision and versatility are of unprecedented quality. Furthermore, the CRISPR/Cas9 system can not only be applied to manipulate the genome, but genetically engineered Cas9 proteins have been used to modulate transcription of endogenous genes or to label certain genomic regions. Importantly, engineered variants such as *Streptococcus pyogenes* Cas9 High Fidelity, modified to display even more precision, i.e. show less off-target effects than wildtype Cas9 [52]. This might further facilitate research as well as clinical applications. Interestingly, the CRISPR/Cas9 system is not the only genome modifying technique that exists in bacteria: There are a number of CRISPR-systems that use different enzymes and recognise unique PAM sites. The recently discovered Cpf1 nucleases might even be more specific than Cas9 [53]. Thus, CRISPR systems might only be the beginning and there might be different, even better and more efficient gene-editing systems in prokaryotes that could open up new avenues for immunological research.

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Table 1: Comparison of traditional ES cell targeting and CRISPR/Cas9 based generation of animal models

	Traditional ES cell approach	CRISPR/Cas9 approach
Pre-clinical models and species that can be targeted	Only accessible for species, where ES cells are available (e.g. certain strains of mice, rats)	Can be used for the genetic engineering of a wide variety of species (e.g. mice, rats and non-human primates)
Efficiency	Extensive validation possible before generating live mice. Caveat: Time consuming breeding of chimeric mice and crossing of heterozygous mice to obtain homozygous offspring; germline transmission variable.	Often bi-allelic mutations and efficient generation of genetically altered founder lines with almost 100% germline transmission. Caveat: Targeting between the 1 and 8 cell-stage in the embryo can lead to mosaicism with mutant and wildtype alleles that can be transmitted to offspring
Multiple genomic modifications	Complex crosses required to obtain mice with multiple genomic alterations	Multiple mutations can be introduced at once
Unwanted Off-target effects	Off-target mutations are expected, but reduced through breeding of the founder lines to a wildtype background	Off-target effects are still difficult to predict; hence backcrossing is recommended
Complexity	Multiplex targeting and very large genomic replacements possible Caveat: Large targeting vectors required even for classical knockout	Simple mutation of a gene requires the design of a small gRNA only Caveat: Conditional alleles and large knock-in alleles require larger targeting vectors
Costs and time	More time consuming and expensive compared to CRISPR/Cas9	5 to 10-fold cheaper and are generally faster than conventional techniques

Figure legends

Figure 1. Applications of the CRISPR/Cas9 technology. (A) Cas9 is directed to a specific genomic region or DNA sequence adjacent to a protospacer adjacent motive (PAM) by sequence-specific guide (g)RNAs and induces double strand breaks, which are in turn repaired by cell-intrinsic DNA-repair pathways, such as homologous recombination, micro homology mediated end joining (MMEJ) or non-homologous end-joining (NHEJ), leading to sequence-specific alteration or random insertions and deletions (InDels). The CRISPR/Cas9-technology can be used for genetic manipulation of primary hematopoietic and immune cells, enabling *in vitro* and *in vivo* studies and screenings, as well as facilitating the generation of genetically modified animals and individualized therapy of various human diseases. (B) Endonuclease activity-inactivated Cas9 (dead-Cas9 (dCas9)) proteins can be applied for regulating endogenous gene expression or visualizing genomic elements, when fused to transcriptional activators/repressors or fluorescent probes, respectively.

