CRISPR in Research and Treatment of Multiple Myeloma

CRISPR ve výzkumu a léčbě mnohočetného myelomu

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Summary
In the recent years, there was a remarkable advance in research and clinical implementation of the genome editing technologies. The most remarkable was a discovery of the bacterial adaptive immune system called CRISPR and its rapid transformation into a robust and broadly applicable technology that completely revolutionized both basic and applied biomedical research. Implementation of CRISPR makes genome modification easier, faster and significantly cheaper compare to any other currently available technology. It also offers a tremendous potential for desiging novel research approaches and future treatment options for various genetic diseases including multiple myeloma. The hightroughput use of CRISPR in pooled screen formats promises faster identification and validation of valuable drug targets together with revealing high-confidence biomarkers and unknown resistance mechanisms. This can provide clinicians with new diagnostic and prognostic tolls and ultimately allow more accurate patient stratification for personalised treatment with better eficacy. In this review, we summarize current knowledge about the CRISPR technology and focus especially on its impact in exploring gene functions, screening for novel drug targets, diagnostic markers and genes involved in resistance to commonly used drug in the treatment of multiple myeloma. Finally, we also highlight a potential future use of CRISPR in actual clinical practise.

Key words
multiple myeloma – CRISPR – therapeutics

Souhrn
V posledních letech došlo ke značnému pokroku v oblasti vývoje technik editace genomu a možnosti jejich klinického využití. Především objev adaptivního imunitního systému bakterií známého jako CRISPR a jeho rychlá implementace jako široce využitelné technologie způsobila zásadní převrat jak v základním, tak v aplikovaném biomedicínském výzkumu. Technologie CRISPR umožňuje editovat genom snadněji, rychleji a výrazně levněji než jakákoli jiná v současnosti dostupná technologie. Tímto se nabízí obrovský potenciál pro realizaci nových výzkumných přístupů a budoucí možnosti léčby nejrůznějších genetických onemocnění, vč. mnohočetného myelomu. Robustní využití CRISPR technologie v rámci genetických screeningů slibuje rychlejší identifikaci důležitých terapeutických cílů a současné odhalení biomarkerů s vysokou prediktivní hodnotou a doposud neznámých mechanizmů lékové rezistence. Výsledky takto směřovaného výzkumu tak mohou poskytnout nové diagnostické a prognostické přístupy, které umožňují přesnější stratifikaci pacientů pro personalizovanou léčbu s vyšší účinností. V tomto přehledném článku shrnujeme dosavadní znalosti technologie CRISPR s důrazem na její uplatnění při hledání nových terapeutických cílů, diagnostických markerů a genů zapojených do mechanizmů resistence na běžně používanou léčbu u mnohočetného myelomu. Závěrem prezentujeme potenciální budoucí využití technologie CRISPR v klinické praxi.

Klíčová slova
mnohočetný myelom – CRISPR – léčiva

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Introduction

In the last decade we have seen tremendous improvements in analysing human genome. Genomic methods such as next generation sequencing (NGS) and gene expression profiling (GEP) are gradually moving from the research laboratories to rutine use in everyday clinical practise. Although powerfull, these techniques are not used in the actuall treatment processes and serve rather as important dianostic and prognostic tools. The real currative potential for genetic and oncological diseases including mutliple myeloma (MM) comes with the gene editing technologies.

Genome editing technologies

Adenoviral vector-based approach was the first gene therapy tool used in childrens the ADA-SCID syndrom. It was also considered as potential treatment option for MM patients [1], but due to the early clinical failures – imprecise virus insertion resulting in the activation of oncogenes and development of leukemia – this approach was withdrawn from the clinic [2]. To overcome these issues novel gene editing techniques implemented additional step – generation of double stranded brakes in precisely defined positions in genomic DNA. This increased both specificity and efficacy of the gene targeting.

Initial, highly specific gene modifying techniques were based on engineered zinc finger nucleases (ZFN) or transcriptional activator-like nucleases (TALEN) that are designed to cleave in predefined sites in genomic DNA. Both ZFN and TALEN are modular proteins, where one module is set to bind to a desired DNA sequence and the other generates double-strand breaks near the binding site (Fig. 1A, B) [3,4]. Even through they achieve higher specificity and efficacy than adenoviral vectors, these techniques are still too complex and very difficult to use laboratory research, and it would be even more complicated to transfer them into actual clinical practise.

The recently described method called clustered regularly interspersed short palindromic repeats (CRISPR) is the most powerfull and versatile gene editing tool that ever existed. It was originally described in bacteria, where it functions as an adaptive immune system fighting againts invaiding bacteriophages and other DNA or RNA-based infection elements [5]. Since the 2012, CRISPR reached a spectacular level of interest both in academia, biotech companies and even several clinical trials using this technique will be initiated soon (see below). All that came with masive investments into further optimalisations for transfer of CRISPR-based technologies into clinical practise together with a remarkable engagement of public media making CRISPR well know technology with a huge potential [6].

CRISPR basic

CRISPR is composed of two components – the protein Cas9 and the short non-conding small guide RNA (sgRNA). Cas9 is an ATP-independent endonuclease, originially found in *Streptococcus pyogenes*, that cleaves DNA in precisely definded positions generating double

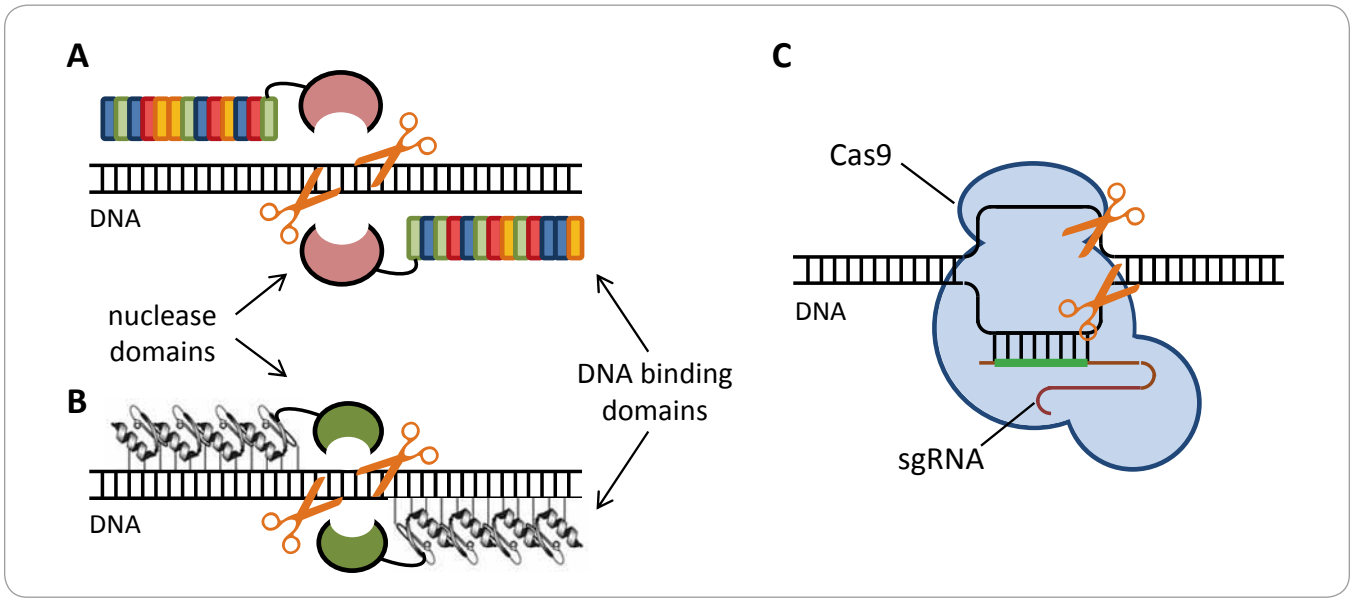

Fig. 1. Genome editing tools.
A. TALEN – Each TALE domain (coloured boxes) binds to one of the four DNA nucleotides. Combining these domain creates protein binding to defined DNAsequences. TALE domains are further fused to the FOK1 nuclease that acts only as a dimer, therefore tandem binding of TALENs is required for DNA cleavage.
B. ZFN – Each zinc finger domain binds to three nucleotides. There are multiple tri-nucleotide specific zinc fingers that can be combined and fused to FOK1.
C. CRISPR – RNA-guided DNA cleavage by the *S. pyogenes* Cas9 endonuclease – Cas9-associated sgRNA contains unique ~20nucleotide "seed" sequence (green) defining binding site in target DNA. Once boud, monomeric Cas9 cleaves simultaneously both DNA strands. The sgRNA seed sequence can be engineered to target Cas9 to any site in human genome.

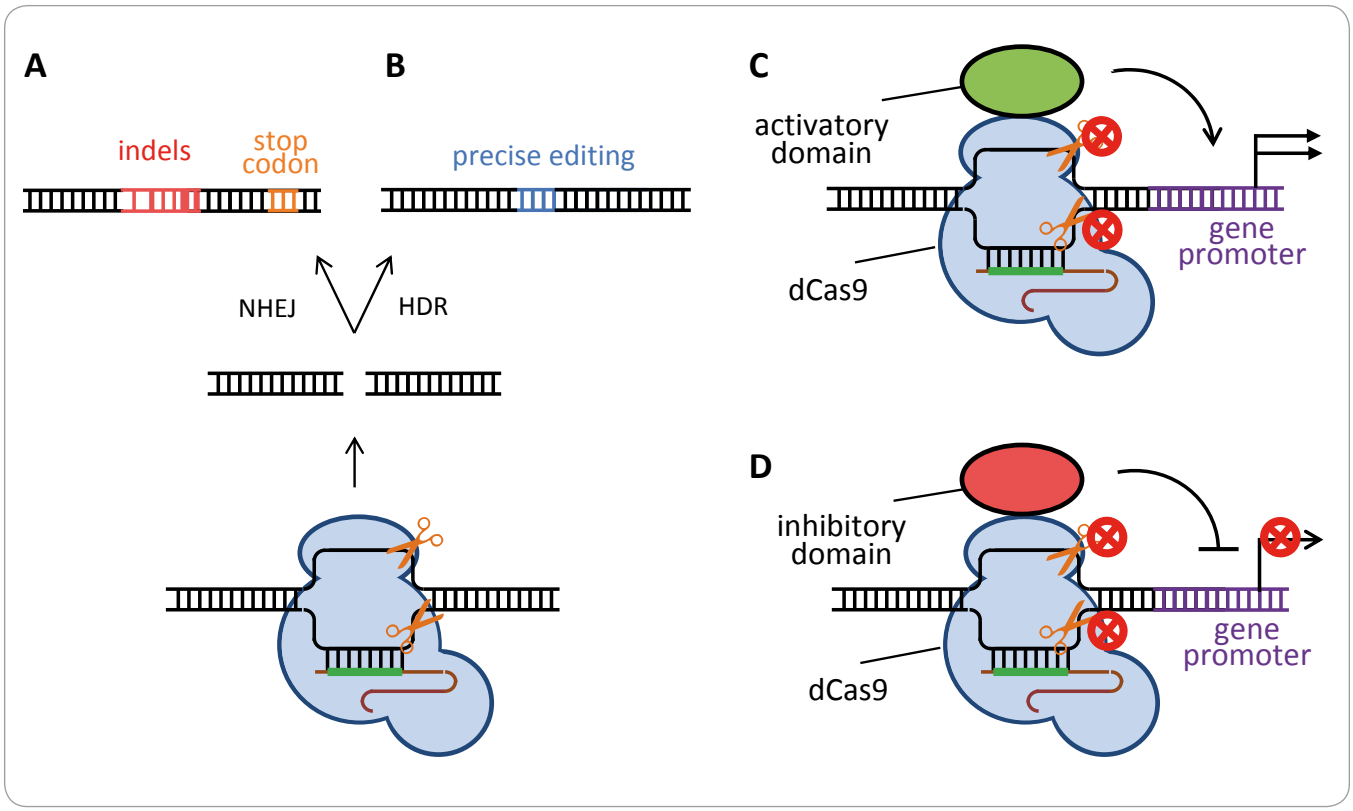

Fig. 2. Gene targeting by CRISPR.
A. Gene knock out – NHEJ repair leads to generation of indels (insertion/deletion) resulting in a premature stop codon abolishing the gene expression.
B. Gene editing – HDR repair can be used to intentionally modify desired genomic sequence (e.g. removing disease/oncogenic mutantions).
C. Activation of gene expression – catalyticaly dead Cas9 (dCas9) fused to transcription activatory domain promotes expression of targeted gene.
D. Inhibition gene expression – dCas9 fused to transcription inhibitory domain downregulates expression of targeted gene.

stranded brakes. To achieve site-specific recognition and cleavage, Cas9 must be complexed with the sequence-specific sgRNA that guides Cas9 to unique DNA motifs on basis of nucleotide complementarity between the 20 nucleotide long sgRNA seed sequence and the target site in genomic DNA (Fig. 1C) [7]. The binding to and cleaving in a particular genomic locus by CRISPR is extremelly specific with either very low or often no detectable off targets [8].

Once, the double stranded brake is made, cells have several ways to repair the damage. The predominant repair mechanism to fix these cytotoxic DNA lesions is the error prone non-homologous end joining (NHEJ). This type of repair is usually inaccurate and frequently leads to insertions and/or deletions (indels) of multiple nucleotides in the vicinity of the cleavage site. If this occurs in the gene coding sequence, the consequences are often frameshift mutations leading to the creation of a premature stop codon, which then results in inactivation of the targeted gene (gene knock out – KO) (Fig. 2A). This can be used in many different applications from switching off a single oncogene in cancer cell to choping off large chomosomal regions generated during aberrant translocation events.

By contrast, the alternative repair mechanism, homology directed repair (HDR), is using the second allele or externally provided homologous DNA as a repair template. Thus, HDR can be applied to intentionally generate precise and specific alterations to desired genomic locus, e.g. mutating the oncogenic variant to the wild type version or inserting a new DNA sequence (Fig. 2B). This phenomenon could be used in treatment of heritable or somatic genetic diseases. Pilot experiments with mouse models using CRISPR to repair the CFTR gene mutated in cystic fibrosis [9] or inactivating the NRL gene involved in retinitis pigmentosa [10] in adult mice are suggesting that succefull and widely applicable gene therapy that was for long time only science fiction might soon becam a reality.

To further extend the genome editing possibilities, CRISPR can be also employed to activate expression of particular genes. When using the nuclease-dead version of Cas9 (dCas9) fused to transcription activator complexes, dCas9 that is no longer active as DNA cleaving enzyme can serve as a hook delivering the transcription activatory domain to the

gene promotor region ultimatively triggering expression of the selected gene. Similarly, this approach can be also used to temporarily and specificaly inactivate transcription (Fig. 2C, D) [5,6]. This offers a possiblitiy of fine tuning the gene expression in a timely dependent manner, especially when inducible version of dCas9 is used. In other words, one can switch on and off expression of particular gene(s) to a certain level only when the cell is exposed to specific stimuli, avoiding unwanted side effects exerted by general transcription modulators such as dexamethasone.

As a research tool, the CRISPR technology enables more efficient modifications of DNA sequences, repression and activation of single or multiple genes at once and it also greatly improves and simplifies creation of complex animal models of many genetic diseases. Therefore, identification of genes, signaling pathways and genetic interactions essential for specific phenotypic changes and disease pathologies is now significantly easier than ever before and it is only a matter of time, when the fundamental findings will be translated into clinically relevant applications.

CRISPR in search for novel drug targets

In a basic research of MM, CRISPR already helped to identify multiple novel players affecting the disease development, progression and offered several novel therapeutic targets. CRISPR-based KO cells are extremelly valuable research tool in revealing the drug specificity. By simple comparison of the wild type cells and the cells with specifically inactivated gene, one can easily distinguish, whether the drug targets given protein or whether the effect is more pleiotropic. In this section, we will describe several recent studies utilizing CRISPR in the identification of new target genes crutial for survial of MM and show how CRISPR can be adopted in testing and validation of the target specificity of novel therapeutic compounds.

It is well established, that expression of MYC oncoprotein is required for survival of MM and many other malignancies. However, very little is known about the regulation of MYC expression in MM cells. Two recent studies used CRISPR to identify and validate novel key drivers of MYC expression. First, knocking out MUC1-C gene by CRISPR or its inactivation by GO-203 inhibitor was associated with a drop in expression of the MYC target genes, including CCND2, hTERT and GCLC [11]. Secondly, CRISPR-engineered cells suggested that let-7, endogenous inhibitor of the translational regulator LIN28, specifically affects MYC expression and cell cycle pathways in MM [12]. Together, these studies provided new pharmacological targets that can be utilised for suppressing MYC in MM.

With the CRISPR technology researchers have already demonstrated the ability to more specifically target genes of the Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3) in MM. IKZF1 and IKZF3 are the primary targets of Cereblon, which activity can be blocked by thalidomide and its derivates including lenalidomide. Analysis of MM cell lines revealed that the loss of IKZF1 and IKZF3 is both necessary and sufficient for lenalidomide's therapeutic effect [13]. This work further inidicated that the anti-tumor and teratogenic activities of thalidomide-like drugs are dissociable providing a completely new perspective of how we look at the Cereblon inhbitors.

MM is charecterised by high genome instability that might result from aberrant function of chromatin regulatory proteins such as histon modifiers. Recent study used CRISPR to delineated the role of histone demethylase KDM6B in MM. Knocking out the KDM6B gene by CRISPR signigicantly abrogated growth and survival of MM cells. The authors also linked KDM6B functions with the NF-κB and MAPK signaling pathway, highligting another drugable target in the treatment of MM [14].

Finally, in a search for the bortezomib-based therapy with a lower toxicity, fewer side effects, higher dose limits and limited resistancy research focused on the identification of compounds that would inhibit proteasome function in MM without blocking the general proteasome activity in the other cell types. CRISPR-based KO of the proteasomal regulatory subunit RPN13 demonstrated that the drug known as RA190 can decrease viability of the MM cell lines and primary cells isolated from MM patients. Importantly, RA190 inhibits proliferation of the MM cells even in the presence of bone marrow stroma and can overcame acquired bortezomib resistance [15].

Identification of the MM resistance genes by CRISPR screens

Although the treatment options of MM have markedly improved especially due to the development of numerous new compounds in the last decade, the recurrent problem in the treatment is disease relaps and fast progression into resistant and refractory stage. The large genomic studies using MM patient samples gave us better insights into the mutation signature in the relapsed MM patients [16–18]. But we still lack precise information, which genes and mutations are the crucial players is development of resistance to certain drugs. Once we will have a solid and validated panel of such genes in MM patients, we might use it as a predictor to stratify patients for a specific type of treatment to enhance its efficacy and avoid resistance. Basically, apply the principles of personalised medicine.

CRISPR technology brings new, exciting possibilities for identification of new genes and their combinations that are responsible for resistance and disease relaps. In so called CRISPR screens, the entire human genome can be profiled to find a particular set of genes that – when altered – might contribute to a loss of sensitivity to a given compound. To systematicaly analyse whole genomes, the CRISPR-based gene profiling (CRISPR screen) is usually done in a high-throughput manner and can be applied on both *in vitro* and *in vivo* systems. In the next section, we will describe the theoretical basis of the CRISPR screens and provide general and simplified protocol how to perform genome-wide screen to identify genes involved in drug resistance.

CRISPR screen methodology

The main part of the CRISPR screen is the CRISPR library, which is a mixture of

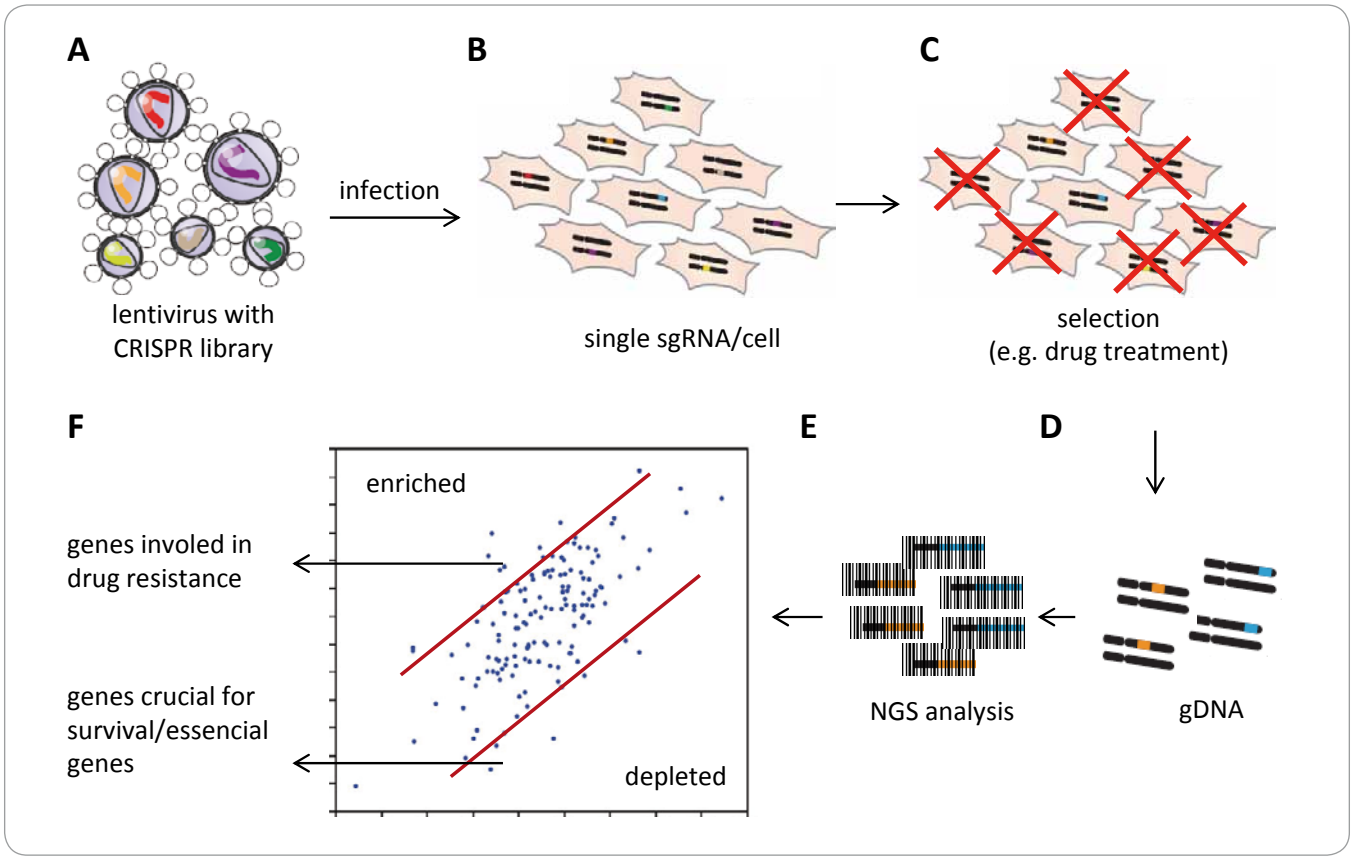

Fig. 3. CRISPR screen – experimental workflow.
A. Lentiviral vectors containe CRISPR components (Cas9 and sgRNA) targeting defined set of genes.
B. Cell line infected with the final rate – 1 cell = 1 sgRNA = 1 targeted gene.
C. Selection pressure is applied on the cell line library (+ control population without selection).
D. Isolation of genomic DNA.
E. PCR amplification and NGS analysis of barcoded sgRNA.
F. Identification of enriched/depleted sgRNA – comparism to control/non-treated population.

plasmids expressing the CRISPR components – sgRNA and Cas9. There are two types of CRISPR libraries – activatory or inhibitory. The first one is used to promote expression of particular genes and the other to knock out (permanently inactivate) selected genes. The library can be either small, focused only on a limited, pre-selected group of specific genes or large, covering every gene in the human genome. The whole genome library contains at least 19,000 different plasmids (small, circular DNA possesing genes to be transfered and expressed in the recipient cells) each containing different sgRNA to cover every coding sequence in the human genome. In most set ups, however, each gene is targted by multiple different sgRNAs to increase the robustness and limit the potential off-target effects.

In most scenarios the plasmids are introduced into the *in vitro* cultured cells usually via viral infection. The screening experiment is designed in a way that every cell will receive only single sgRNA targeting just one specfic gene. Thus, each cell in population will have only one gene activated or knocked out (Fig. 3A, B). After establishing such library of geneticaly modified cells, the selection pressure is applied. Next steps depend on the type of screening and the seeked phenotype.

When performing the resistance studies, cells are treated with a drug of interest. After certain time, only the resistant cells survive and their genomic DNA is collected and analysed by NGS. Abundance (enrichment/depletion) of all sgRNAs is then compared to the control/non-treated population. Because every sgRNA targets only one, unique genomic sequence the hits can be easily assigned to specific genes and their contribution to the cell survival under the tested stress condition is revealed (Fig. 3C–F).

Safety constraints for viral vectors

As already mentioned, in the experimental laboratory settings, the plasmids coding sgRNA and Cas9 are introduced into the cells mostly via infection with retroviral or lentiviral particles. Lentivirus--based systems are more commonly used due to their ability to infect both non-dividing and diving cells. The viral partical is made and delivered in a from of pseudovirus, from which all virulent genes were removed and the virus is unable to replicate in the host cell. To comply with the safety restrictions, production of the

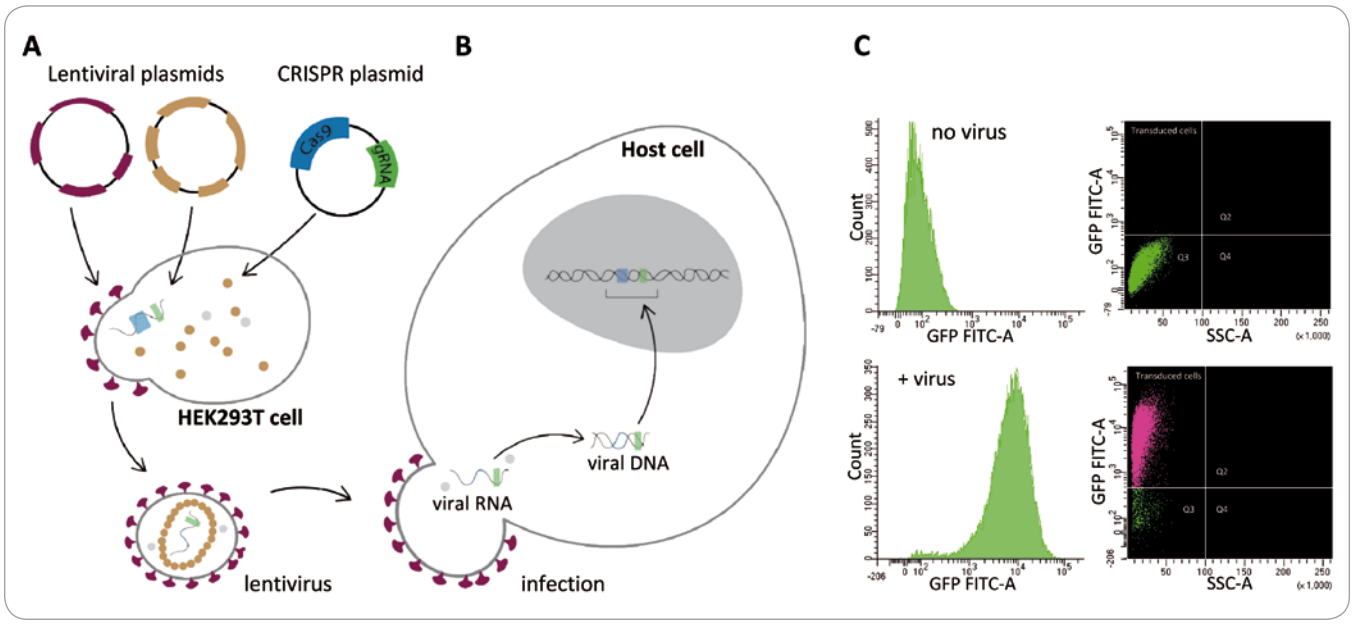

Fig. 4. Generation of pseudo-lentivirus.
A. Virus production – plasmids coding capsid proteins and enzymes required for virus integration are introduced into the virus producing cell line (usually HEK293T).
B. Infection of the target cell – viral RNA is reverted into DNA and intergrated into the host genome.
C. Example experiment – infection with lentivirus coding GFP. The non-infected cells do not show any fluorescence in the GFP/FITC channel analysed by flow cytometry, while > 95% of cells infected with GFP-coding virus are positive.

pseudoviral particles belongs to the biohazard class II and can be done only in the laboratories with dedicated cell culture rooms that are equipped with the appropriate laminar flow hoods containing HEPA filters. Other safety rules including adequate waste managment must be also fulfilled. An official approval for the work with geneticaly modified organisms of class I and II is also needed [19].

To ensure safety of the viral system, genes encoding components for lentivirus are splitted into multiple plasmids. Components of the viral capsid and enzymes required for virus integration in the host cell are usually expressed from two (sometimes even three) plasmids. The third (fourth) plasmid then possess the actuall transgenes – Cas9, sgRNA and selection marker to monitor and enrich the infected cells. To generate the virus all plasmids need to be first transfected into the virus producing cells. In the laboratory settings, the viral particles are made in the specialised HEK293T cells that express the SV40 Large T antigen that is required for the viral genome replication and ensures high virus titers. The finally assembled viral particals are then secreted into the culture media, which can be either directly used to infect the host cells or further concentrated to increase viral titer and improve infection efficacy. Selection of infected cells is mostly based on expression of fluorescent protein (GFP, RFP, etc.) or antibiotic resistance gene (puromycine, blastocidin, etc.) in addition to introduced transgene (Fig. 4A–C).

Successfull CRISPR screens in MM

The first CRISPR screens done with MM cell lines searched for genes responsible for resistance to bortezomib, carfilzomib and lenaliomide [20,21]. The first study identified proteasomal regulatory subunit PSMC6 as the gene confering bortezomib and carfilzomib resistance in the RPMI8266 and KMS11 cell lines. However, the work failed to correlate PSMC6 expression levels in the primary MM samples with the clinical outcome of the bortezomib treated patients. The study currently continues by investigating the PSMC6 mutation rate in the relapsed MM patients after bortezomib treatment. It is likely that also other factors including mutational status of PSMC6 and especially contribution of tumor microenviroment will have an impact on the resistance development.

Another CRISPR whole genome screen in MM cell lines focused on lenalidomide, which primary target is the ubiquitin E3 ligase Cereblon [21]. Lenalidomide is the thalidomide derivate and by inhibiting function of Cereblon it facilitates degradation of the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3). Interestingly, the precise mechanism of action and consequently the therapeutic effects of lenalidomide are different compare to thalidomide even through they both target the same molecule [22]. The study utilised CRISPR screen in the lenalidomide sensitive MM cell line MM1S and highlighted the ubiquitin conjugating enzymes UBE2D3 and UBE2G1 as important players in the lenalidome-mediated regulation of Cereblon function [21]. Further follow-up experiments demonstrated distinct functional roles for both enzymes and provided additional hits for the targeted drug design in the treatment of MM with a potential to avoid development of resistance and lower the side effects exerted by lenalidomide.

A smaller, focused CRISPR screen targeting only 20 critical MM genes was used in an elegant study that examinated clonal evolution of MM cells and development of metastasis in the *in vivo* settings. The study defined specific plasma cell subclones with a high metastatic potential and found unique metastatic gene signature together with two genes acting as potential regulators of metastasis formation in MM [23]. The same group also established a platform for a genome wide *in vivo* CRISPR screening in the xenograft mouse models to investigate genes regulating metastasis dissemination and specific organ colonization in progression of MM. The authors also investigated contribution of multiple genes on several stages of MM development – early primary tumor, late primary tumor, and bone marrow metastasis. As crucial genes for both early and late developmental stages were found genes involved in mTORC1 and DNA repair pathways, many of which are regulated by MYC and cell cycle related targets of E2F transcription factors [24].

The *in vivo* CRISPR screening could be also used to explore essential genes in response to targeted drug therapies and/or immunotherapies. Thus, the CRISPR-based *in vivo* screening offers a very powerful tool for functional genomics and promise many exciting discoveries.

CRISPR in MM therapy

MM takes a reputed privilage as one of the first human diseases for which CRISPR will be used as a method applied directly in the treatment process. The last year (2016), scientists at the University of Pennsylvania announced a launch of a pioneer study using geneticaly modified patient T-cells targeted against cancer cells including MM, melanoma and sarcomas [25,26].

Their strategy is following up on the fingings of high NY-ESO1 expression in plasma cells of MM patients with a poor-prognosis. First, the already established technology of the cell therapy based on the chimeric antigen receptor (CAR) will be used to generate geneticaly modified T-cells (CAR T-cells) specifically targeting cells with increased expression of NY-ESO1 [27]. Secondly, the PD-1 receptor, which is expressed on the surface of antigen-activated and exhausted T-cells, will be deleted by CRISPR. The engagement of PD-1 with its ligands PD-1L and PD-2L, that are highly expressed on cancer cells including MM, leads to temporal down-regulation of T-cells functions and increases their susceptibility to apoptosis [28]. Therefore, removing PD-1 from autologous T-cells is a viable and promising strategy to preserve and activate the tumor specific T-cells (CAR T-cells) that will likely promote specific targeting of patient MM cells.

Conclusion

Application of CRISPR will have a profound effect on our understanding of the development of many hematological malignancies including monoclonal gammapaties and specifically MM. CRISPR-based genome editing offers an unprecedented ability to define novel MM relevant genes and mutations, provide rational targets for new drug development, reveal resistance mechanisms and most importantly it moves the field of genetic manipulation to the next level – a real personalised medicine. In summary, extreme simplicity, broad versatility and aplicability, very high efficacy together with low cost compare to all previous genome modifying methods makes CRISPR the gene eiditing tool of choice in research and clinic with a great potential. Thus, CRISPR technology will undoubtedly transform the way we conduct both medical research and the actual treatment of genetic diseases in the near future.

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