In Vitro Application of CRISPR

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Clustered regularly interspaced short palindromic repeats (CRISPR) are widely used in cancer research to edit specific genes and study their functions.

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1. Discovery of CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) are multiple short sequences found in the genome of prokaryotes, hence bacteria. These DNA sequences originate from bacteriophages, which had previously infected the bacteria. Hereby, a memory of phage infections is created in the prokaryote and is seen as an adapted immune system by the integration of a unique DNA sequence from the phages into the bacterial genome [1][2]. During a new phage infection, these DNA sequences will be transcribed together with the CRISPR-associated protein (Cas), and by aligning the specific sequences to the genome of the phage, a DNA break can be induced by the Cas protein [3][4][5]. The discovery of this unique immune defense has changed the modeling of many biological processes and was awarded the Nobel Prize in 2020.

2. The Elements of CRISPR/Cas9

CRISPR has evolved in prokaryotes and resulted in different Cas proteins. Here, Streptococcus pyogenes Cas9 is the most used Cas protein in CRISPR engineering. For genomic DNA to be cleaved by the Cas9 protein, different criteria have to be fulfilled. First, a unique 20-base pair RNA sequence containing the complementary sequence to the genomic target called the guide RNA. This RNA sequence guides the Cas9 protein to the target site and is often fused to a tracrRNA, which binds the Cas9 protein. The whole RNA sequence is called a single guide RNA (sgRNA). Furthermore, a protospacer adjacent motif (PAM) is required for most Cas proteins and, for Cas9 the motive is NGG. The Cas9 protein cleaves the target sequences at position 3+ in the guide sequences and the host organism will repair the break by either homologous or non-homologous end joining (HEJ or NHEJ) [6][7][8]. The homologous repaired genome will maintain the original DNA sequences and can be re-cut by the CRISPR complex. However, after non-homologous end joining, the genomic sequencing is changed by adding or removing base pairs (insertion or deletions (indel)). Hereby, the genomic sequence is changed and can give rise to loss of function mutations in the targeted protein [7][8][9].

The repair through homologous end joining can be used in combination with a “repair template” and hereby a desired genomic sequence can be added to the target of interest. This could be the generation of a driver mutation, such as $Kras^{G12D}$ [10][11]. These are the fundamental principles of CRISPR for genomic editing.

3. In Vitro Application of CRISPR

After the discovery of CRISPR, the method has been applied to study many biological processes in vitro. As CRISPR has made it possible to genetically edit nearly all types of cells, the method allows for addressing biological questions that were previously difficult or impossible to answer. Different methods are now used to deliver CRISPR/Cas9 in vitro but the most common are plasmid, lentivirus, or electroporation of modified sgRNAs with the Cas9 protein. Each method has its advantage, but it will not be further discussed. The strength of CRISPR is to generate cell lines with genetic alterations that are found in human diseases, including cancer, and the chance to study the implications in vitro. Multiple studies have applied CRISPR to investigate gene functions. Chu et al. (2013) showed as some of the first, that in human embryonic kidney cells *CCR5* can be mutated [8]. Later came Mali et al. (2013), which used CRISPR to insert the coding sequence for GFP in the AAVS1 locus by the use of a repaired template in combination with CRISPR/Cas9 [3]. The specific gene editing by CRISPR with the use of a repaired template is currently optimized by prim- and base-editing and will result in easier and higher efficiency, which is crucial from a therapeutic perspective [12]. Targeting the mitochondrial genome is

challenging, as the CRISPR complex is not entering the mitochondrial. Adding a mitochondrial targeting sequence to Cas9 has improved the editing of the mitochondrial DNA and this opens up future treatment options for genetic mitochondrial disorders [13].

The ability of sgRNA and Cas9 to bind specific sequences in the genome has led to the development of CRISPR activation or inhibition (CRISPRa or CRISPRi). Here, a modified Cas9 protein called dead Cas9 (dCas9) is used, as it has lost the ability to cleave the genomic DNA. By fusion of the activating protein VP64 or the repressor protein KRAB to dCas9, these fusion proteins will bind specifically to the promotor region through a sgRNA and alter the expression of the downstream gene [14][15][16][17]. Hereby, it is possible to regulate the expression of a specific gene without the induction of mutations to the genome.

4. Genome Wide CRISPR Screens

The CRISPR/Cas9 ability to induce mutations with high efficiency has allowed for use of the technology for genome wide screening. These screens have been successful to identify genes that are involved in different malignant processes. Special mutations that gain resistance to drug treatment have been identified through CRISPR screens [18]. To perform CRISPR screens, different considerations have to be taken. Here, a library of sgRNAs targeting the genes of interest has to be generated. The mutation frequency is variable among sgRNAs, therefore multiple guides for the same target genes should be included in the library, and normally 3–5 guides are used for each target [6][19]. Lentiviruses are often used for the delivery of the library, as the virus DNA is integrated into the cell genome. The viral genome can then be used as a bar code to identify which sgRNA has been targeting the cell, and hence, which genes have been mutated. It is also of great importance that each sgRNA is represented equally in the library, so no bias is introduced to the screen. Overall, CRISPR screens have contributed to the discovery of new molecular mechanisms in cancer biology, and work on DNA repair is a strong example where the genetic interaction of PARP has been identified [20], which highlights the importance of CRISPR in cancer research.

5. In Vivo Application of CRISPR for Cancer Research

After the discovery of CRISPR, the method was fast introduced to generate in vivo models, especially in mice. Now, many new mouse models have been generated by CRISPR by either genetically engineered ES cells or oocyte injections, which is not further discussed [21][22]. Instead, reserachers focuses on the CRISPR application in somatic cells to generate models for studying cancer. CRISPR technology has been applied to study cancer in vivo by generating loss-of-function or gain-of-function mutations in somatic cells. CRISPRa and CRISPRi have also been used in vivo together with CRISPR screening. The different aspects will be discussed further and their implication for cancer research.

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