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Targeted genome engineering using designer nucleases: State of the art and practical guidance for application in human pluripotent stem cells

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ABSTRACT

Within the last years numerous publications successfully applied sequence specific designer nucleases for genome editing in human PSCs. However, despite this abundance of reports together with the rapid development and improvement accomplished with the technology, it is still difficult to choose the optimal methodology for a specific application of interest. With focus on the most suitable approach for specific applications, we present a practical guidance for successful gene editing in human PSCs using designer nucleases. We discuss experimental considerations, limitations and critical aspects which will guide the investigator for successful implementation of this technology.

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Contents

1. Introduction

With their almost unlimited potential for proliferation and differentiation, human pluripotent stem cells (PSCs) and especially patient-specific induced pluripotent stem cells (iPSCs) offer an unprecedented capacity of applications in medical research and clinical therapy. Therefore, the ability to genetically modify PSCs is indispensable for their application in disease modelling, drug screening or cellular therapies. For introduction of transgenes into human PSCs a variety of methods has been established including viral transduction, chemical transfection and electroporation, and different vector types such as standard plasmids, artificial chromosomes, transposon elements or various viral vectors can be applied. Thereby one has to distinguish between "transient" introduction of transgenes that are lost over time, self-replicative elements and approaches that lead to stable chromosomal

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integration. In the latter case, either random integration of transgenic elements or targeted insertion into known genomic sequences is possible.

Conventional non-targeted transgene insertion using viral or non-viral gene transfer technologies results in more or less random integration, which bears the risk of insertional mutagenesis, misregulated expression and transgene silencing (Hacein-Bey-Abina et al., 2003). A more specific but elaborate alternative is the classic technique of gene targeting, which uses the homologous recombination (HR) pathway and a donor plasmid carrying a selectable transgene flanked by homologous DNA stretches of substantial length to specifically target the favoured locus. However, low targeting efficiencies and challenging culture characteristics have prevented classic gene targeting in human PSCs from becoming broadly applicable, and positive and negative selection markers have been indispensable to identify the rare events of targeting among the typically more frequent off-target events.

This issue has been overcome by the discovery of targeted introduction of a double strand break (DSB) strongly stimulating HR at the breakpoint (Choulika et al., 1995; Johnson and Jasin, 2001; Rouet et al., 1994). The recent advancement of customized engineered endonucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeat (CRISPR) RNA-guided nucleases, opens new perspectives for HR-based strategies in human PSCs. These nucleases enable locus specific introduction of DSBs, which can be repaired either by non-homologous end joining (NHEJ) or by HR (Shrivastav et al., 2008), thereby increasing the gene targeting efficiency by 100–10,000 fold (Cho et al., 2013; Durai et al., 2005; Miller et al., 2011; Porteus and Carroll, 2005).

Within the last seven years, since the first reports on designer nucleases in human PSCs (Hockemeyer et al., 2009; Lombardo et al., 2007; Zou et al., 2009), a rapid development and improvement in genome editing has been accomplished with numerous publications successfully applying the technology. However, despite this abundance of reports, it is still difficult to choose the optimal methodology for a specific application of interest. The different types of designer nucleases have diverse characteristics concerning the level of specificity, limitations regarding the choice of target sequences, the complexity of the design and the expenditure of time for manufacturing. Moreover, designer nucleases can be introduced into the target cells via different technologies and can be utilized in different ways by taking advantage of miscellaneous cellular repair mechanisms and different types of donors for homologous recombination. Finally, comparable data concerning off-target specificity, the background of non-targeted transgene integrations and targeting efficiencies are rarely available, because different loci in different cell types or cell lines were targeted. Moreover, a direct comparison of targeting efficiencies is further complicated since in most cases only the resulting numbers of targeted clones after selection instead of primary targeting efficiencies are provided, and levels of transfection-related cell death were typically not included for calculation.

In the following we provide an overview on recent applications of designer nucleases in human PSCs and on the diversity of possible targeting and selection strategies (Supplemental Table 1). Thereby, Fig. 1 highlights the key parameters which have to be considered before starting gene editing and which will be discussed here. Despite the aforementioned limitations concerning a quantitative comparison of the published approaches, we will outline the most suitable approaches for specific applications. In particular, the purpose of this review is to present a practical guide, in terms of experimental considerations, limitations and other critical aspects, for successful gene editing in human PSCs using designer nucleases for various applications.

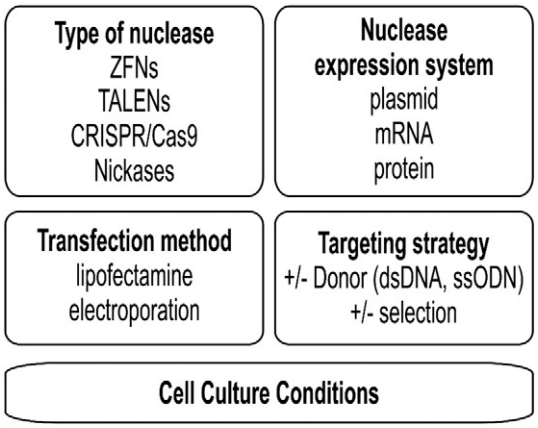

Fig. 1. Selectable key parameters for genome engineering in human PSCs. dsDNA, double stranded DNA; ssODN, single strand oligonucleotide.

2. Choice and design of the nucleases

Fig. 2 briefly illustrates the structure and characteristics of three types of designer nucleases. In summary, ZFNs and TALENs consist of target-specific DNA-binding domains fused to an unspecific nuclease domain, whereby in the CRISPR/Cas9 system a chimeric RNA containing the target sequence guides the Cas9 nuclease to cleave the DNA (Gaj et al., 2013). The development and improvement of customized engineered endonucleases is continuously progressing but efficiencies and grade of specificity of the different nuclease systems are still controversial. TALENs and the CRISPR/Cas9 system have already replaced ZFNs as it is still technically challenging and time-consuming to engineer active ZFNs and only a few academic labs have established routine production (Maeder et al., 2008). The generation of TALENs is much less labour-intensive and time-consuming once the system has been established in the lab, although a typical TALEN requires ~1800 bp to be assembled for each new target site. The CRISPR/Cas9 system is the easiest to use, time-saving and relatively cheap, as the synthesis of an only 20 bp guide RNA is required to program the nuclease. Undoubtedly, the production of several CRISPR guide RNAs for one genomic locus and the validation of the most effective one is less time-consuming than doing the same for TALENs. On the other hand, it has to be emphasized that in both cases design, construction and preparation of the respective plasmid vectors is usually the minor part of the entire targeting approach compared to the establishment of correctly targeted single cell clones. In general, once the respective systems are established in a lab, the construction can be accomplished in about two weeks for TALENs and in one week for CRISPR/Cas9. Technical guidelines and different TALEN assembly kits (https://www.addgene.org/talen/) as well as different CRISPR cloning systems (https://www.addgene.org/crispr/) are available via Addgene or commercial sources. Online tools that facilitate optimal design of such nucleases and their evaluation concerning efficiency and potential off-target activity can be found on https://tale-nt.cac.cornell.edu/, https://bao.rice.edu/research/, http://www.rgenome.net/cas-offinder/, http://www.genome-engineering.org/ or https://chopchop.rc.fas.harvard.edu/index.php. However, previously validated nucleases are already available from academic and commercial sources for many genomic targets and the effort of designing new ones has to be weighed.

After defining the genomic target region, the respective individual DNA sequence should be confirmed to exclude single-nucleotide polymorphisms (SNPs) or other variations in the recognition site of the nuclease. Due to context-dependent interactions between neighbouring zinc-fingers, not all genomic sites can be targeted by ZFNs. In general, one ZFN site can be found every 125–500 bp of a random genomic sequence, depending on the assembly method (Kim et al., 2009; Sander et al., 2011; Sander et al., 2010). In contrast, TALENs can be designed

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