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EDITORIAL

Magic wands of CRISPR—lots of choices for gene knock-in

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Introduction

Genome editing technology relies on endogenous repair pathways of DNA double-strand break (DSB), triggered by site-specific nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) (Sakuma and Woltjen 2014). The primary concept of genome editing based on non-homologous end-joining (NHEJ)-dependent gene knockout and homologous recombination (HR)-mediated gene knock-in. NHEJ is an error-prone pathway, resulting in short deletions and/or insertions at the target site without the need for donor DNA. When a donor plasmid containing homology arms is supplied along with the genome editing nucleases, HR reaction between the target chromosome and the donor plasmid is stimulated; thus, HR-mediated sequence additions can be achieved with much higher frequency than in the traditional gene targeting strategy (Capecchi 1989).

Currently, genome editing techniques have become more variable; e.g., gene knockout can be conducted efficiently by utilizing microhomology-mediated end-joining (MMEJ) pathway with or without the overexpression of exonucleases (Mashimo et al. 2013; Bae et al. 2014), and the efficient gene knock-in can be achieved with enhancing or bypassing the HR pathway. Since HR is only active in late S-G2 phases in mammalian cells (Taleei and Nikjoo 2013), HR-dependent knock-in does not always result in efficient gene knock-in even with a powerful genome editing tool. On the other hand, other repair pathways such as NHEJ, MMEJ, and single-strand annealing (SSA) are considerably active during the majority of the cell cycle. In addition, single-strand DNA (ssDNA) can also be incorporated at the DSB site via single-strand template repair (SSTR), which is thought to be independent from HR, because SSTR is not Rad51-dependent (Bothmer et al. 2017; Richardson et al. 2017). In this review, we summarize these HR-independent gene knock-in systems developed recently.

NHEJ-mediated gene knock-in

NHEJ plays a major role in DSB repair in mammalian cells; thus, NHEJ was first harnessed as an alternative pathway to incorporate exogenous double-strand DNA (dsDNA) donor in the manner of simple cut and paste. Since artificial nuclease systems such as ZFNs produce the sticky ends, these overhangs were used as the tabs for sticking, by simply adding the overhangs to the knock-in cassette or the target site of ZFNs to the donor plasmid (Orlando et al. 2010; Cristea et al. 2013). This system was subsequently refined as the ObLiGaRe method, which enabled obligate ligation by switching the left and right target sites of ZFNs and TALENs added in the plasmid donor and utilizing the obligate heterodimeric nucleases

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(Maresca et al. 2013). Furthermore, longer overhangs produced with paired Cas9 nickases can also reportedly be available for the similar knock-in approach (Ran et al. 2013). After the success of sticky ligations, the blunt-end ligation mediated by wild-type Cas9 nucleases were reported by many groups. The successful examples were shown in cultured cells (He et al. 2016; Royba et al. 2017) and in animal embryos such as zebrafish (Auer et al. 2014; Kimura et al. 2014) and frogs (Shi et al. 2015). However, these systems could not control the direction of integration.

Recently, Suzuki and colleagues reported a simple but important improvement in NHEJ-mediated knock-in strategy, referred to as the HITI (homology-independent targeted integration) method (Suzuki et al. 2016a). They used a common single-guide RNA (sgRNA) for the cleavage of genomic target site and donor DNA, but the orientation of the sgRNA target site on the donor DNA was flipped. Based on this design, the integrants with the intended orientation could be concentrated, because the integrants with the reverse orientation would be cut out again by the Cas9/sgRNA. They showed HITI-mediated robust gene knock-in not only in dividing and non-dividing cultured cells but also in somatic cells of living mammalian organisms. Thus, the NHEJ-mediated strategy has become a strong alternative to the conventional HR-mediated knock-in.

MMEJ/SSA-mediated gene knock-in

Although NHEJ-mediated strategy has enabled robust targeted integration, it is unsuitable for seamless and scarless knock-in, because the error-prone NHEJ often results in mutagenic knock-in junctions, and the residual target sequence of genome editing nucleases in the knock-in allele is inevitable even though the improved HITI strategy is used. Therefore, highly efficient, highly accurate, and footprint-free knock-in method has been desired.

In accordance with the current in-depth understanding of DSB repair pathways, end-joining-type pathways are divided into three groups; classical NHEJ (C-NHEJ), alternative end-joining (A-NHEJ)/MMEJ, and SSA (Rodgers and McVey 2016). C-NHEJ does not require homologous sequence, while A-NHEJ/MMEJ and SSA require short and long homologies, respectively. As described earlier in this review, MMEJ has been utilized for the efficient gene knockout. The availability of MMEJ in genome editing applications has also been proven by various studies reporting the frequent observation of the traces of MMEJ repair on the edited alleles (Yasue et al. 2014; Li et al. 2015). The first example of MMEJ-dependent integration of donor plasmid was reported in 2014, where microhomologous ends between the genomic target site and exogenous DNA fragment were generated by simultaneous cleavage of genomic DNA and the donor vector (Nakade et al. 2014). This study showed that MMEJ-directed donor vector, referred to as the PITCh vector, harboring very short (~ 10 bp) microhomologies, can be integrated into the genome in the predefined way, not only in cultured cells but also in silkworms and frogs, which were known as the organisms that HR-mediated knock-in was hardly applicable. Furthermore, CRISPR-mediated PITCh permitted seamless knock-in, which could not be realized by NHEJ-mediated strategies.

The wide applicability of the PITCh system has been shown in various studies, including the creation of antibody-producer cells by engineering CHO-K1 cell line (Sakuma et al. 2015), precise in-frame integration of donor fragment in zebrafish (Hisano et al. 2015), and the production of reporter frogs at the F0 generation (Suzuki et al. 2016b). In addition, the detailed protocol, freely available materials, and online design tool for the CRISPR-mediated PITCh knock-in were provided by the authors (Sakuma et al. 2016; Nakamae et al. 2017). Along with these user-friendly resources, the application examples of CRISPR-mediated PITCh have become more diversified, e.g., high-throughput epitope tagging in cultured cells for chromatin immunoprecipitation (ChIP)-seq (Xiong et al. 2017), the application in mammalian zygotes (Aida et al. 2016; Nakagawa et al. 2017), and in vivo somatic knock-in in mice (Yao et al. 2017a) were reported one after another. Note that recent design of CRISPR-mediated PITCh has employed longer microhomologies (~ 40 bp); thus, it might be based on SSA rather than MMEJ.

In addition to the PITCh system, one recent study showed that longer homology (~ 800 bp) with in situ linearization resulted in higher knock-in efficiency compared to HR-, NHEJ-, and MMEJ-dependent strategies both in vitro and in vivo, excluding some exceptions such as in mouse ES cells and in astrocytes (Yao et al. 2017b). The authors called this strategy HMEJ (homology-mediated end joining); however, the actual mechanism involved in the process of HMEJ knock-in may be SSA, as the authors discussed in the paper. This SSA-mediated knock-in approach is not truly novel, because

previous studies reported similar knock-in enhancing effect by the nuclease-mediated cleavage outside the homology arms in animal embryos such as sea urchins and fruit flies (Beumer et al. 2006; Ochiai et al. 2012). Nevertheless, this is the first report of comprehensive comparison of the efficiency among HR-, NHEJ-, MMEJ-, and SSA-dependent knock-in strategies, and it turned the spotlight again on the utility of the SSA-dependent approach.

SSTR-mediated gene knock-in

The incorporation of ssDNA upon DSB repair was initially shown in mammalian cells using meganucleases and ZFNs (Wang et al. 2008; Radecke et al. 2010). Subsequently, this methodology has widely been applied not only in cultured cells but also in various organisms including zebrafish and mouse (Chen et al. 2011; Bedell et al. 2012; Meyer et al. 2012). Short ssDNA (up to 200 nt) can be chemically synthesized; thus, such single-strand oligodeoxynucleotide (ssODN)-mediated tag integration or replacement of single nucleotide polymorphisms (SNPs) does not require the construction of targeting vector. Moreover, ssDNA is unlikely to cause random integration compared to dsDNA. Because of its simplicity and safety, there is no doubt about the usefulness of ssDNA; however, the size limit of loadable exogenous DNA in chemically synthesized ssODN had been a critical demerit of this method.

Recent developments for the creation of long single-strand DNA (lssDNA) have overcome this problem. One solution is a method referred to as ivTRT (in vitro transcription and reverse transcription) (Miura et al. 2015). In the ivTRT method, the template dsDNA was first transcribed into single-strand RNA with T7 RNA polymerase, and then reverse transcription was performed. After that, the RNA/DNA hybrid was treated with RNaseH to degrade the RNA strand. Using the lssDNA prepared with ivTRT, it was shown that the insertion of knock-in cassette and creation of floxed allele were efficiently achieved in mouse zygotes (Quadros et al. 2017). Another reported method for the lssDNA production was based on the double nicking of plasmid DNA (Yoshimi et al. 2016). The knock-in fragment was first cloned into the plasmid vector, and then a single-strand DNA of the intended region was cut out by the two nicking endonucleases. Yoshimi and colleagues showed the insertion of GFP in rat zygotes using the lssODN created by this method. They also showed in the same paper that whole plasmid or bacterial artificial chromosome (BAC) DNA could be incorporated into the rat genome by bridging with two ssODNs. This approach called 2H2OP (two-hit by gRNA and two oligos with a targeting plasmid) is also an interesting utilization of SSTR in gene knock-in applications.

Conclusions

As introduced in this review, genome editing-assisted gene knock-in technology has become rather diversified and improved from day to day. Since genome editing relies on the endogenous DSB repair machineries, their appropriate utilization is a key to control the editing outcome. The favorable DSB repair pathway for gene knock-in is variable depending on various parameters such as the target cell types and organisms, purpose of gene knock-in, and delivery methods. The adequate system should be chosen by the researchers themselves with a deep and correct knowledge to make the most of genome editing and gene knock-in technologies.

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