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Generation and validation of PAX7 reporter lines from human iPS cells using CRISPR/Cas9 technology

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ABSTRACT

Directed differentiation of iPS cells toward various tissue progenitors has been the focus of recent research. Therefore, generation of tissue-specific reporter iPS cell lines provides better understanding of developmental stages in iPS cells. This technical report describes an efficient strategy for generation and validation of knock-in reporter lines in human iPS cells using the Cas9-nickase system.

Here, we have generated a knock-in human iPS cell line for the early myogenic lineage specification gene of PAX7. By introduction of site-specific double-stranded breaks (DSB) in the genomic locus of PAX7 using CRISPR/Cas9 nickase pairs, a 2A-GFP reporter with selection markers has been incorporated before the stop codon of the PAX7 gene at the last exon. After positive and negative selection, single cell-derived human iPS clones have been isolated and sequenced for in-frame positioning of the reporter construct.

Finally, by using a nuclease-dead Cas9 activator (dCas9-VP160) system, the promoter region of PAX7 has been targeted for transient gene induction to validate the GFP reporter activity. This was confirmed by flow cytometry analysis and immunostaining for PAX7 and GFP. This technical report provides a practical guideline for generation and validation of knock-in reporters using CRISPR/Cas9 system.

1. Introduction

Human iPS cells provide an unprecedented opportunity for the science community to model and study different disorders *in vitro* (Inoue et al., 2014). These cells can be used for many *in vitro* applications such as disease modeling, drug screening, genome editing and even *in vivo* regenerative purposes (Tedesco et al., 2012; Wang et al., 2012; Xie et al., 2014; Zou et al., 2011; Soldner et al., 2009; Dimos et al., 2008; Ebert et al., 2009; Moretti et al., 2010; Raya et al., 2009; Maehr et al., 2009). Therefore, generation of different lineage progenitors and tissues from iPS cells has been considered the cornerstone of iPS technology (Mauritz et al., 2008; Zhang et al., 2009; Choi et al., 2009; Grigoriadis et al., 2010). Thus, generation of lineage-specific reporter ES/iPS cell lines enables prospective identification and isolation of various progenitors from differentiating cells and allows optimization of differentiation protocols.

Knock-in reporter cells have been generated through site-specific gene targeting and homologous recombination (HR) mediated inclusion of the reporter cassette in the appropriate genomic locus (Leavitt & Hamlett, 2011; Zwaka & Thomson, 2003). This allowed for in-frame inclusion of the reporter cassette without any undesirable side effects associated with random integration based methods.

However, the low efficiency of HR in human iPS cells makes it hard for gene modification in human iPS cells (Zwaka & Thomson, 2003; Xue et al., 2009; Davis et al., 2008; Ruby & Zheng, 2009). Nevertheless, nuclease-mediated introductions of site-specific DNA double-stranded breaks (DSBs) have significantly increased the efficiency of HR. These include site-specific nucleases such as zinc-finger (ZFN) and transcription activator-like effector nucleases (TALENs), which allowed for precise gene editing (Wang et al., 2012; Zou et al., 2011; Kim et al., 1996; Urnov et al., 2010; Carroll et al., 2006; Deng et al., 2012; Ma et al., 2013; Maetzel et al., 2014; Shin et al., 2014). The only limiting factor with these methods is their complex time-consuming design.

Fortunately, a new class of RNA-guided nucleases has been developed from microbial adaptive immune systems (i.e. clustered regularly

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interspaced short palindromic repeat-CRISPR- and CRISPR associated Cas9-CRISPR/Cas9-systems) (Cong et al., 2013; Mali et al., 2013a). This system provides a very simple and efficient design for site-specific targeting of genomic DNA. A newer version of Cas9 uses a D10A mutant of Cas9 (Cas9n) to introduce a single-strand nick in DNA (Mali et al., 2013b; Ran et al., 2013a). By using as a pair to target a specific locus, Cas9n pair increases site-specificity while reduces the off-target mutations (Shen et al., 2014). Therefore, in this technical report, we used CRISPR/Cas9n system for generation of the human iPS cell reporters. In this case, we decided to generate a PAX7 skeletal muscle reporter iPS cell line.

Pax7 and PAX3 play key roles in specification of neural crest development as well as skeletal muscle specification during embryogenesis (Buckingham & Relaix, 2007; Jostes et al., 1990; Basch et al., 2006). For skeletal myogenesis, PAX3/7 positive cells specify early paraxial mesodermal cells which enter the myogenic program and govern skeletal myogenesis during embryonic and fetal development (Buckingham & Relaix, 2007; Jostes et al., 1990; Lagha et al., 2008; Relaix et al., 2006; Relaix et al., 2004; Relaix et al., 2005). These cells differentiate into myoblasts and move to different parts of body/limb to form mature myofibers or stay quiescent to form muscle adult stem cells/satellite cells. Due to the important role of PAX7 in this process especially for satellite cell formation (Seale et al., 2000), we decided to choose the muscle specific transcriptional isoforms (1 and 2) of PAX7 for generation of the reporter human iPS cell line (Barr et al., 1999; Vorobyov & Horst, 2004).

In this technical report, we designed a Cas9n based strategy to generate the reporter. In addition, by using a nuclease-dead Cas9 (dCas9) gene activation/CRISPR-on system (dCas9-VP160 activator) (Cheng et al., 2013), we induced PAX7 transcription to validate the GFP reporter function.

2. Materials and methods

2.1. sgRNA design and Cas9 nickase (Cas9n) vector assembly

Cas9 target sites were identified using an online CRISPR design tool (crispr.mit.edu) (Ran et al., 2013b). Briefly, 200 bp DNA sequences of human PAX7 gene flanking the stop codon (100 bp before and after the stop codon) were used for designing the sgRNAs. Then, four pairs of sgRNA were selected to generate the optimal 5′-overhang arm length for efficient cutting (Ran et al., 2013a). For each target site, a specific Cas9n vector was made. Briefly, a Cas9 nickase vector (hSpCas9n nickase — pX335, # 42335 from addgene) (Cong et al., 2013) was digested using BbsI and a pair of annealed oligos (20 bp target sequences) was cloned into the guide RNA locus (Ran et al., 2013b). The vectors were then sequenced for confirmation.

2.2. SURVEYOR assay for Cas9n pairs

Assembled Cas9n pairs were tested by transfecting 293T cells. Briefly, 0.5 μg of each plasmid was used for transfection of 293T cells. Three days after transfection, DNA from targeted and non-targeted cells was harvested and amplified by PCR for the targeted region. Then, PCR products were hybridized using a thermocycler to allow heteroduplex formation from targeted and non-targeted DNA. Later on, 400 ng of hybridized DNA was treated with a SURVEYOR nuclease and DNA fragments were analyzed by agarose gel electrophoresis to identify the presence of expected bands. The best pair (5 + 7) with the highest cutting efficiency was chosen for targeting.

2.3. Generation of targeting vector

A BAC clone for the human PAX7 was obtained from CHORI (BAC RP11-164D21). To generate the targeting vector, we used a highly efficient recombination-based cloning method in bacteria (Wu et al., 2008). Briefly, homology arms (5′ — 1.2 kb; 3′ — 4.4 kb) along with target region were captured into a gateway compatible vector (pStart-K, # 20346 from addgene) (Wu et al., 2008) using recombineering. After introduction of a cutting site with a selection sequence (AscI-Cat-AscI) using another recombineering reaction, the reporter with an excisable selection sequence (2A-GFP-loxP-EF1a-RFP-2A-Puro-loxP fragment from HR130PA-1 vector, System Biosciences — SBI) was cloned into the AscI cutting sites using a blunt-end ligation. Finally, through a gateway recombination, the modified genomic fragment was switched into another vector which contained the negative selection (*tk*) as well as unique linearization sites. The final vector was linearized before using for targeting.

2.4. Validation of targeting efficiency in 293T cells

In order to validate the efficiency of the targeting construct and Cas9n vectors, 1×10^7 of 293T cells were electroporated (Gene Pulser Xcell — Bio-Rad, 250 V, 250 μF, ∞ Ω) with the targeting cocktail (10 μg of Cas9n plus 50 μg of linearized targeting vector) and after positive and negative selection (0.75 μg/ml puromycin and 200 nM FIAU from day 3 to day 11), the expanded 293T cell clumps were analyzed for RFP using a fluorescent microscope. Positive individual clones were harvested and PCR-screened for targeting efficiency (PCR primers were designed to span the outside 5′ homology arm and 2A-GFP region to ensure the right target locus).

2.5. Generation of reporter human iPS clones

After validation of Cas9n pairs using SURVEYOR assay and targeting vector in 293T cells, fully characterized integration-free human iPS cells (DF19-9, WiCell) (Yu et al., 2009) have been used for generation of human reporter clones. The human iPS cells were expanded on Matrigel in feeder conditioned medium for a few days and after harvesting, 1×10^7 cells were electroporated (Gene Pulser Xcell — Bio-Rad, 250 V, 250 μF, ∞ Ω) using 10 μg of Cas9 vectors plus 50 μg of linearized targeting vector for PAX7. After electroporation, cells were expanded for a few days and selection was done by addition of positive and negative selection (0.75 μg/ml puromycin and 200 nM FIAU) for 2 weeks. After selection, RFP^+ clones were picked up for expansion and screened by PCR.

2.6. PCR and sequencing validation of reporter clones

PCR primers were designed to span outside the 5′ homology arm up to 2A-GFP region as well as covering the nicking sites of Cas9 nickase pair. PCR products were sequenced to ensure in-frame positioning of the reporter/selection fragment and to rule out the presence of indels at cutting sites of Cas9n pairs. Furthermore, to identify homozygous clones for reporters, PCR primers were designed to identify intact PAX7 as well as the targeted allele.

2.7. Southern blot validation of transgene copy number

In order to prove the correct integration site of the transgene and exclude any random integration of the transgene, a southern blot strategy was designed using an internal probe within the GFP. The genomic DNA from the reporter hiPS cells was extracted and 5 μg of genomic DNA from each cell line was digested with EcoRV, separated in a 0.9% agarose gel and transferred to a Hybond N^+ membrane as described before (Xue et al., 2016). The genomic DNA on the membrane was then hybridized with a Digoxigenin (DIG)-labeled internal probe derived from partial GFP sequence.

2.8. sgRNA design for dCas9-VP160 activator and vector assembly

In order to evaluate reporter activity in targeted iPS clones, we used a nuclease-dead Cas9 activator system (pAC93-pmax-dCas9VP160, # 48225 from addgene) (Cheng et al., 2013). sgRNAs were designed to