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Efficient Recombinase-Mediated Cassette Exchange in hPSCs to Study the Hepatocyte Lineage Reveals *AAVS1* Locus-Mediated Transgene Inhibition

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SUMMARY

Tools for rapid and efficient transgenesis in "safe harbor" loci in an isogenic context remain important to exploit the possibilities of human pluripotent stem cells (hPSCs). We created hPSC master cell lines suitable for FLPe recombinase-mediated cassette exchange (RMCE) in the *AAVS1* locus that allow generation of transgenic lines within 15 days with 100% efficiency and without random integrations. Using RMCE, we successfully incorporated several transgenes useful for lineage identification, cell toxicity studies, and gene overexpression to study the hepatocyte lineage. However, we observed unexpected and variable transgene expression inhibition in vitro, due to DNA methylation and other unknown mechanisms, both in undifferentiated hESC and differentiating hepatocytes. Therefore, the *AAVS1* locus cannot be considered a universally safe harbor locus for reliable transgene expression in vitro, and using it for transgenesis in hPSC will require careful assessment of the function of individual transgenes.

INTRODUCTION

As it has been the case for studies aiming to understand mouse development, transgenesis is an indispensable tool to fully exploit the potential of human pluripotent stem cells (hPSCs). Recent technological advances using site-specific nucleases (Zinc Finger Nucleases [ZFNs], Transcription Activator-Like Effector Nucleases [TALENs], or clustered regularly interspaced short palindromic repeats [CRISPR]/Cas9 system) have allowed to overcome major hurdles hampering genome editing in hPSCs (Li et al., 2014). Gene targeting constitutes the method of choice for transgenesis in hPSCs as it eliminates the drawbacks of random integration methods linked to possible insertional mutagenesis and epigenetic silencing, which lead to variegated transgene expression in subpopulations of cells (Cherry et al., 2000; Yao et al., 2004).

Despite these advances, gene targeting in hPSCs still remains a laborious process, and the development of tools that allow rapid and versatile genetic modification remains of great interest. Site-specific recombinase-mediated homologous recombination with pre-integrated recombination target sequences in "safe harbor" loci, like the *Rosa26* or *Hprt1* loci, has been extensively used in mouse transgenesis. Such safe harbor loci are found in ubiquitously expressed genes with transcriptional competent conformation that allows stable transgene expression with no detrimental effect on the biology of the modified cells. In hPSCs, Cre recombinase systems for recombinase-mediated cassette exchange (RMCE) have been developed either in the adeno-associated virus integration site 1 (*AAVS1* locus) (Ramachandra et al., 2011; Tay et al., 2013; Zhu et al., 2013) or by random integration (Du et al., 2009), though such methods do not constitute a technical

improvement over gene targeting approaches using nucleases.

The *AAVS1* locus, located in the first intron of the *PPP1R12C* gene on chromosome 19 has been described to meet the "safe harbor" requirements in a variety of cell types including hPSCs. Though the function of the *PPP1R12C* gene has not been fully investigated, hPSCs retain pluripotency after targeting. In addition, transgene expression in the *AAVS1* locus appears stable in undifferentiated hPSCs and following differentiation to all three germ layers in vitro and in vivo (DeKelver et al., 2010; Hockemeyer et al., 2009; Lombardo et al., 2011; Qian et al., 2014; Smith et al., 2008).

The goal of this study was to generate an efficient and rapid method of transgenesis in the *AAVS1* locus of hPSCs, based on RMCE using positive and negative selection to allow the generation of non-clonal transgenic lines, to enable stable incorporation of lineage-specific promoters, molecular response sensors, or inducible gene overexpression. We focused on validating the applicability of the RMCE in the *AAVS1* locus during hepatocyte differentiation as only few studies have used transgenesis to characterize this lineage in human (Davis et al., 2008; Duan et al., 2007; Ishii et al., 2008; Umeda et al., 2013; Wang et al., 2011). Using ZFNs to pre-integrate FRT sequences in the *AAVS1*, we generated an RMCE system that allows the generation of cell lines with 100% efficiency and without random integrations in ±15 days. We demonstrate the suitability of the locus to support several applications for the study of the hepatocyte lineage, although we found variable transgene inhibition in the pluripotent and differentiated state of hESC in vitro in a potential lineage-dependent manner, which appears to not be present in teratomas in vivo. Our results suggest that the *AAVS1* locus is not as safe as generally believed.

RESULTS

Generation of an RMCE-Suitable Master Cell Line and RMCE

The master cell line (MCL) was generated as explained in the Supplemental Experimental Procedures (Figure 1A). Amplification of the wild-type allele and Southern blotting was performed to determine whether the integration was mono or biallelic and to rule out random integration events (Figures 1B and 1C). Two heterozygously targeted clones were chosen for further characterization of maintenance of pluripotency (teratoma formation assay was carried out using a protocol approved by the Institutional Ethics Committee at KU Leuven) and a normal karyotype (Figures 1D, 1E, and S1A). In agreement with previous studies, GFP was homogeneously expressed in undifferentiated and differentiated cells from the selected clones, which was stable during passaging and differentiation (Figures 1F and 1G).

RMCE was then performed by nucleofection of the master cell line with the donor vector *pZ:F3-P CAGGS tdTPH-F* and the FLPe-expressing vector (Figure 2A). Control conditions without donor or without FLPe were included to evaluate the rate of random integration events and the specificity of the RMCE mediated by FLPe. The cells were selected with progressively increasing concentrations of puromycin, to select for cells integrating the donor plasmid, combined with negative selection with FIAU, to select for exchange events resulting in loss of the HSV-tk suicide gene and to eliminate possible random integrations (Figure 2B). We detected an average of 12.8 ± 6.8 (n = 6) $Puro^R/FIAU^R$-resistant RMCE colonies, all homogeneous tdT^+/GFP^- demonstrating that efficiency of recombination by cassette exchange was 100% (Figure 2B). No resistant colonies were obtained when cells were transfected with the donor alone, indicating that recombination was mediated by FLPe between the FRT sequences and that the negative selection efficiently selects against random integration events. Following selection, the non-clonal newly generated RMCE lines (all $Puro^R/FIAU^R$ cells) were subjected to PCR and Southern blotting (Figures 2C and 2D) to confirm full RMCE and absence of random integration of either the donor or FLPe plasmids. RMCE lines maintained pluripotency (Figure 2E).

A similar master cell line was generated in an iPSC line, and FLPe-mediated RMCE was validated (Figure 1C; Figures S1A–S1E). Therefore, this constitutes a method allowing highly efficient complete RMCE in ±15 days, without random integrations and without the need for single colony characterization after recombination.

Lineage Tracing Using the *OCT4* Promoter Reveals Inhibition Exerted by the *AAVS1* Locus

To demonstrate the suitability of the *AAVS1* locus for lineage identification and tracing studies, we recombined the *OCT4* promoter-GFP RMCE vector *pZ:F3-P OCT4p-GFP-F* in this locus (Figure 3A, top; Figure S2). Contrary to what was observed when the *OCT4*p was randomly integrated in hESC (Gerrard et al., 2005), we did not detect *OCT4*p promoter activity (Figure 3B). Thus, the *AAVS1* locus might inhibit or silence the *OCT4p* despite the insulator activity ascribed to a DNase hypersensitive region (DHR) region present in the *AAVS1* (Ogata et al., 2003). The epigenetic status of the *AAVS1* was previously described as open chromatin by DNase hypersensitivity and chromatin immunoprecipitation (ChIP) assays, but no study has assessed the DNA methylation in this region. We performed bisulfite sequencing of different fragments of the *AAVS1* locus in wild-type, undifferentiated hESCs and observed DNA hypo-methylation upstream of the DHR (33% methylated CpGs), total absence of

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