A ROBUST METHOD FOR TAGGING ENDOGENOUS GENES THROUGH PROMOTER TRAPPING AND SHORT HOMOLOGY ARMS

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ABSTRACT

Precise genome editing via homology-directed repair (HDR) pathway holds great promise for gene and stem cell therapy. However, the efficiency of integrating large DNA molecules into mammalian genome via HDR is inherently low. Recently, we showed that the use of short homology arms (~35nt) was sufficient to introduce small changes in mammalian genome. Now, we take a step further and develop a novel method for tagging endogenous genes through promoter trapping and short homology arms, which dramatically increases the efficiency and specificity of integration. The efficiency of tagging endogenous genes with a 1.4 kb promoterless GFP reporter ranges from 50% to 100% upon antibiotic selection with higher level of specificity occuring at the C-terminus than at the N-terminus. The method has been validated using multiple targets in many different cell lines, including human induced pluripotent stem cells and hematopoietic stem cells. The basal expression levels of various fluorescent fusion proteins and their subcellular locations could be visualized by fluorescence microscopy or detected by western blotting. This method has broad applications in general genome engineering, DNA cloning, protein production and immune cell therapy.

INTRODUCTION

The recent advances in CRISPR-mediated genome engineering enable researchers to efficiently introduce double-strand breaks (DSBs) in genomic DNA. The DSBs are then mostly repaired by either the non-homologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway. In mammalian cells, the NHEJ pathway is predominant and error-prone, which results in disruptive insertions or deletions (indels) at targeted loci allowing for the efficient creation of gene knockouts. Alternatively, the cells may utilize sister chromatids or an exogenous DNA template to repair the DNA damage via HDR, but the efficiency is relatively low. In this study, we developed a robust method for tagging endogenous genes through promoter trapping and short homology arms.

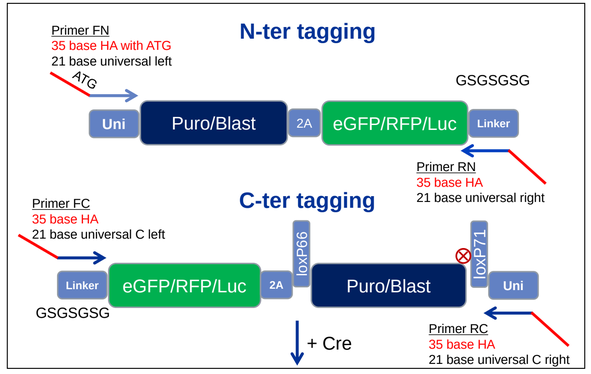
MATERIALS AND METHODS

The gRNAs were designed using GeneArt™ CRISPR gRNA Design Tool from Thermo Fisher Scientific and then synthesized using the GeneArt™ Precision gRNA Synthesis Kit. The concentration of gRNA was determined by Qubit® RNA BR Assay Kit. The genome modification efficiency was determined by GeneArt® Genomic Cleavage Detection kit.

The GeneArt Truetag donor DNA design tool will soon be available from Thermo Fisher Scientific. The donor DNA was prepared by TrueTag™ Donor DNA Kit. The Truecut Cas9V2 protein (Thermo Fisher), gRNA and donor DNA were co-delivered into the cells. At 48 hrs post transfection, the cells were selected with puromycin and then imaged using the Evos Fluorescence microscopy. Alternatively, the cells were subjected to flow cytometry analysis to determine the percentage of EmGFP positive cells using the Attune Nxt flow cytometer.

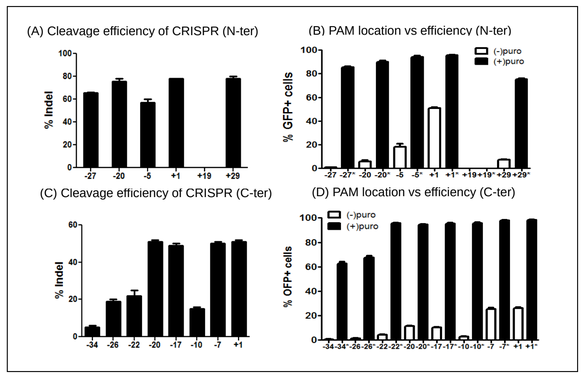
RESULTS

Figure 1. Donor Design for Gene Tagging



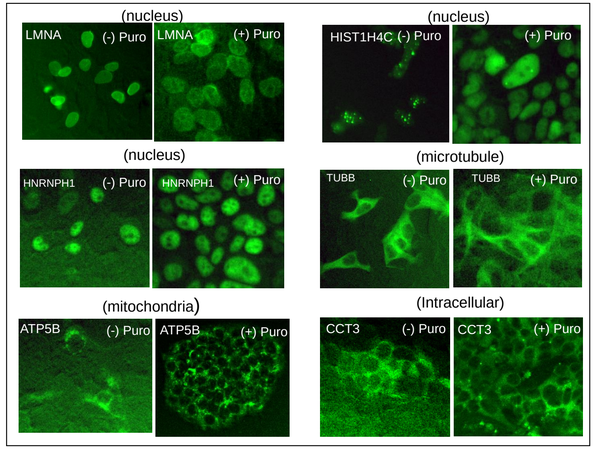
Donor design for either N-terminal or C-terminal gene tagging. Endogenous promoter is used to drive the expression of selection markers and short homology arms are utilized to minimize off-target integration.

Figure 2. DSB in close proximity to insertion site enhances HDR



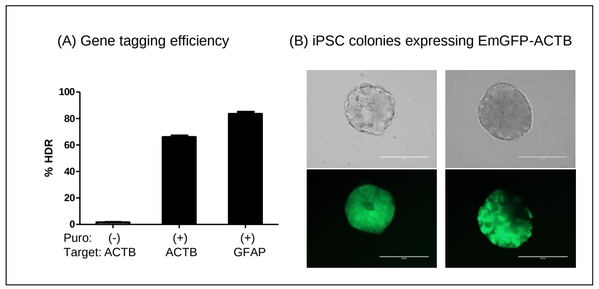
A series of gRNAs were designed and synthesized flanking either N-ter ATG start codon or C-ter stop codon at the ACTB loci. The ± signs indicate the position of DSB upstream (-) or downstream (+) of the ATG or Stop site (0). The various gRNAs were associated with Cas9 nuclease separately and the resulting Cas9 RNPs were transfected into 293FT cells along with various donor DNA. The percentages of Indel were evaluated at 48 hours post transfection (A, C). Meanwhile, the transfected cells were subjected to flow cytometry analysis to determine the percentages of GFP-positive cells (B, D).

Figure 3. Visualization of the subcellular location of endogenous tagged proteins



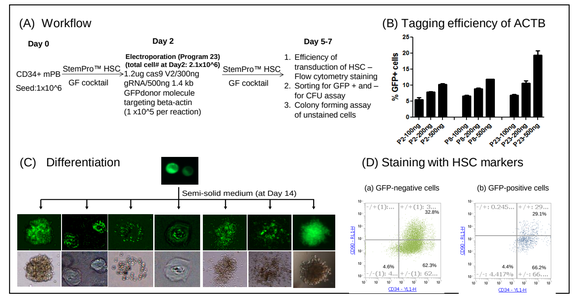
Endogenous proteins localized at different subcellular compartments were tagged with EmGFP by co-delivering Truecut Cas9V2 protein, the corresponding gRNA and donor DNA into 293FT cells. At 48 hrs post transfection, the cells were treated with puromycin for 5-7 days and then visualized using the Evos Fluorescence microscopy. EmGFP+ cells/of % Indel).

Figure 4. Gene tagging in iPSC



Cas9 RNP and Truetag donor DNA targeting either ACTB or GFAP loci were co-delivered into iPSC using electroporation. At 48 hrs post transfection, the cells were selected with puromycin for 7 days, the resulting colonies were randomly picked and expanded for GFAP target whereas the colones targeting ACTB loci were examined by fluorescence microscope (B). The HDR efficiency of ACTB was determined by flow cytometry whereas the efficiency of GFAP was determined by junction PCR and sequencing (A).

Figure 5. HSC genome editing and differentiation



(A) HSC editing workflow. (B) Tagging efficiency of ACTB with EmGFP under different electroporation conditions. (C, D) The transfected cells were were sorted and stained with CD90 and CD34 antibodies (D), and the GFP-positive cells were plated onto semi-solid medium for 14 days and colony formation was visualized under fluorescence microscope (C).

CONCLUSIONS

We developed a simple method for tagging endogenous gene efficiently without the need for preparation of donor plasmid. The tagging efficiency could reach nearly 100% upon antibiotic selection. The method has been validated with different targets in different cell lines.

REFERENCES

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TRADEMARKS/LICENSING