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FACILITATED GENOME EDITING IN HUMAN IPSC TO ADVANCE DISEASE MODELING AND SCREENING

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SUMMARY

Human induced pluripotent stem cells (hiPSCs) have been globally recognized as a multipurpose research tool for modeling human disease and biology, screening and developing potential therapeutic drugs, and implementing cell and gene therapies. The ability to differentiate human iPSCs into any cell type supports the study of biology and disease in these specified cells *in vitro*. The emergence of genome editing tools, including the CRISPR/Cas9 system or TALENs, enable genetic modification of these cells; such as introduction of single base changes or inserting reporters or bio-sensors, which can be used to study the effects of genetic differences or biological functions in the desired cell type.
Key areas of the genome editing workflow that have been addressed to facilitate the genome editing workflow include the genome editing tools themselves, the delivery methods and the maintenance of healthy hiPSC cultures during these stressful manipulations. Using the generation of disease models relevant to cardiac and neuronal disease to explore and identify the best workflow for the genome editing process in hiPSC, we built a reliable approach that reproducibly supports the generation of hiPSC lines carrying small mutations such as SNPs or small deletions. Subsequent studies of the disease relevant cell types then identified cellular phenotypes that corroborated with those previously identified in patient-derived hiPSC-based models. Furthermore, we explored the use of these tools and workflows to insert larger DNA pieces into specific genomic loci to generate fluorescent reporter cell lines for screening for example. Using a small number of loci, we found that introduction of large DNA donors into specific loci was dramatically lower compared to the introduction of SNPs, yet the efficiency obtained was significant enough to allow for the clonal isolation of the edited cells.

INTRODUCTION

Since the availability of feeder-free culture systems for hiPSCs, cell handling has become easier and now allows manipulations of hiPSCs, including at the genomic level via CRISPR/Cas9 or TALEN. In general, the genome editing workflow to generate hiPSC cell lines for disease modeling and screening consists of 4 main steps including (1) the genome editing itself, (2) clonal isolation, (3) characterization of the clones, (4) differentiation of the genome edited hiPSC and running disease relevant assays (**Figure 1**). High editing efficiencies can now be reliably achieved for the majority of genomic targets when using the appropriate tools, yet the downstream process of isolating pure clonal hiPSC lines with the genomic edit of choice remains a labor intensive process. Many clones typically need to be isolated, expanded and screened for the desired genome editing event before any differentiation and biological analyses can be performed.

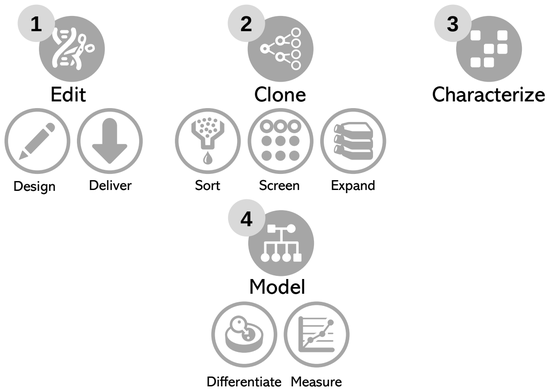

Figure 1. Overview of the common workflow for the generation of a disease model or reporter hiPSC line. *The main steps of the workflow are shown, with sub-activities of each step indicated.*

RESULTS

When the appropriate tools are chosen for genome editing, SNP conversions and tagging with reporters such as GFP can be achieved in most targets (**Figure 1, step 1 and Figures 2-3**). The main pain points of the genome editing workflow we sought to solve here were related to the isolation of clonal lines, which typically is a labor intensive process (**Figure 1, step 2**). Through the implementation of automation approaches, we were aiming to improve the reliability, throughput and timeline of the typical genome editing workflow in hiPSCs. We looked at the deposition of single hiPSCs into 96-well plates (**Figure 4**), clone feeding and clone consolidation into 96-well plates for downstream processing (**Figure 5**).
To allow reliable high-throughput isolation of single cells in 96-well plates on a fluorescence activated cell sorter (FACS) that allows plate seeding, we first implemented stringent gating strategies to ensure that single, viable and pluripotent cells were isolated (**Figure 4**). Factors that may improve survival after sorting were then tested to understand how the most efficient single cell cloning in feeder-free conditions could be achieved. Through extending the RevitaCell™ exposure window from 24h to 72h, using rhLaminin521 as the matrix and StemFlex as the culture medium we were able to dramatically increase clone survival of a few hiPSC seeded into a 96-well (**Figure 4**).

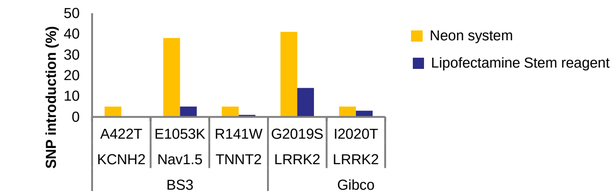

Figure 2: Comparison of electroporation and lipid mediated delivery of genome editing tools. *Cas9 TrueCut v2 protein, gRNA and single stranded oligo donors can be delivered using a Neon Electroporator or Lipofectamine Stem, two approaches that achieve successful genome editing in most targets evaluated. Neon electroporation is the superior method to deliver genome editing tools.*

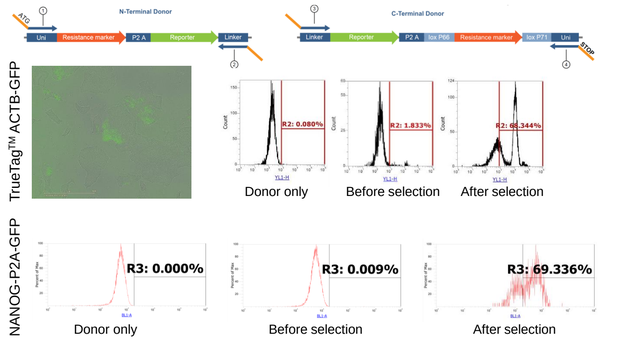

Figure 3: Tagging of proteins expressed in hiPSC. *To generate reporter lines for proteins expressed in hiPSC, the TrueTag system can be used to facilitate insertion of reporter cassettes in a genomic locus of interest via CRISPR/Cas9.*

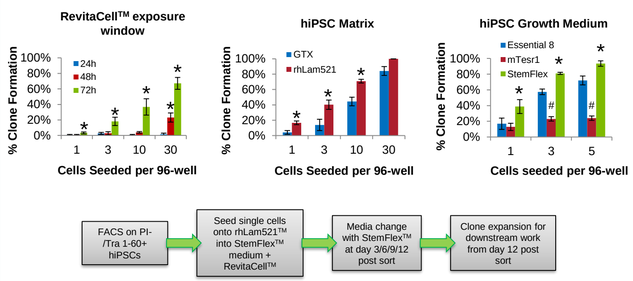

Figure 4: Improvements to increase the recovery of single cell clones isolated through automated single cell seeding via FACS. *A stringent gating strategy was used to identify single, viable and pluripotent hiPSCs, which were seeded using a cell sorter. Effects of RevitaCell™ exposure window, hiPSC matrix protein and hiPSC growth medium were investigated. The improved workflow based on these results is diagrammed.*

Clonal survival after single hiPSC deposition into 96-well plates using a cell sorter can thus be improved dramatically, not only yielding clones in about two weeks, but also facilitating automated workflows downstream. Using whole well-based image analysis, wells with clones can be quickly identified and loaded into plate maps for automated clone consolidation. Using the culture conditions identified for single cell plating, we implemented automated cherry picking to consolidate clones into 96-well plates with a liquid handler that allows single channel manipulations (**Figure 5**). Generally, 2-3 plates containing clones can be consolidated into 96-wells with a post manipulation recovery of ~95%. Consolidated clones recover and proliferate normally, yielding 80% confluency in 3-4 days and have a normal pluripotent morphology (**Figure 5**).

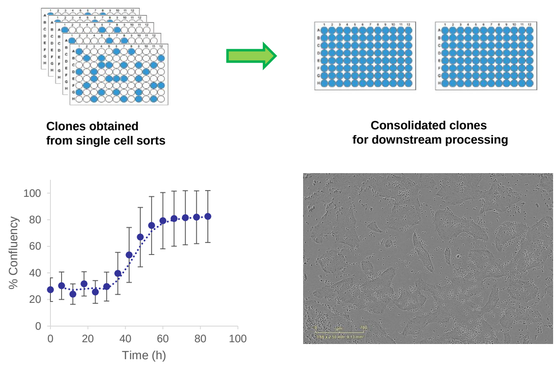

Figure 5: Automated cherry picking of clones to facilitate clone screening. *Using plate maps generated via image based identification of wells containing a clone, cherry picking using a simple liquid handler can be used to consolidate single cell clones into a few 96-well plates. This can be achieved with a ~95% success rate using the culture conditions identified in Figure 4, and hiPSCs expand normally over the next 2-3 days (growth curve of hundreds of clones and representative hiPSCs are shown).*

These tools and methods were tested for the introduction of SNPs into different genomic loci in hiPSCs to validate the tools and to ensure the methods did not affect the biology of the hiPSC. SNPs were chosen based on their relation with a specific disease. After delivery of the editing tools we obtained 11-41% homology driven repair or SNP introduction in the pools (**Figure 6**). Single cell clones were then isolated from each pool using the automated approach and yielded 17%-37% surviving clones, which were screened by Sanger sequencing for the presence of homozygous and heterozygous SNP as well as indel clones (**pie charts Figure 6**). Furthermore, the genome edited hiPSCs generated through the automated workflow had unaffected karyotypes and pluripotency, indicative that the genome editing and clonal isolation methods did not affect the integrity of the cells (**Figure 7**).
To understand whether the introduced mutations had an effect on the biology of the cells, we then differentiated the genome edited hiPSC into specialized cell types to model disease *in vitro* and found that we were able to replicate phenotypes related to the associated disease by simply introducing a single base change (**Figure 8**).

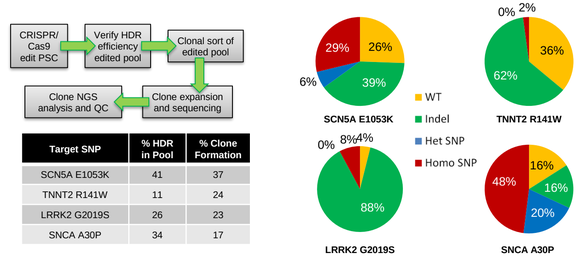

Figure 6. Generation of disease models in Cas9-expressing hiPSCs. *Cas9 hiPSCs were used to introduce SNPs known to be associated with Parkinson's (LRRK2 G2019S, SNCA A30P) or cardiac disease (SCN5A E1053K, TNNT2 R141W). HDR efficiency was target dependent and homozygous SNP clones were identified for all targets.*

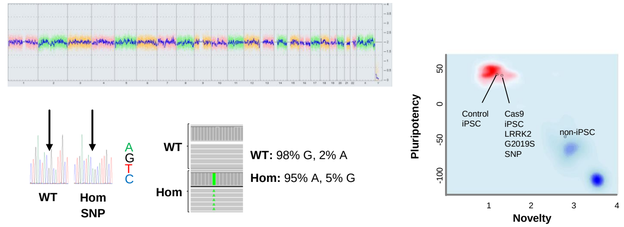

Figure 7: The genome editing workflow does not affect pluripotency or karyotype of hiPSCs. *The genome editing workflow did not affect pluripotency or the karyotype of hiPSCs as assessed by Pluritest and Karyostat respectively. Furthermore, sequencing of the cell line confirmed the introduced mutation and clonality.*

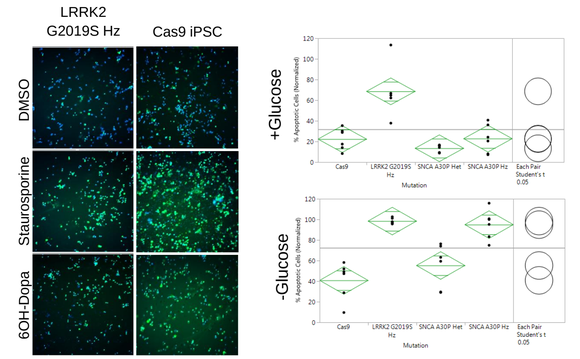

Figure 8: Analysis of an *in vitro* generated model of Parkinson's disease generated through genome editing of hiPSCs. *Genome edited hiPSCs were generated and differentiated into dopaminergic neurons to study the effect of the LRRK2 G2019S mutation, which has been associated with Parkinson's Disease. Diseased hiPSCs formed neurons normally but showed a significant difference in cell survival when exposed to the neurotoxin 6-hydroxy-dopa.*

CONCLUSIONS

With hiPSC-based disease modeling at the forefront of research and drug discovery, the ability to generate disease model cell lines through genome editing has taken an important place in the field. In addition, the generation of reporter cell lines further enhances disease modeling studies by providing the ability to identify different cell types while running assays.
The main challenges around building such models are mostly in the genome editing workflow, for which we have developed a number of solutions. Using the right genome editing tools and methods to deliver them, we demonstrate that desired genome edited cell pools can be generated fairly easily, both for small and larger knock-ins (target dependently). To then facilitate one of the main bottlenecks of the genome editing workflow in hiPSCs, namely single cell isolation, expansion and consolidation, we sought to achieve automation for this step. Relying on reagents that support hiPSC growth under stressful conditions, (rhLaminin-521™, StemFlex™ medium and RevitaCell™), FACS, a simple liquid handler and a whole well scanner, automation of clone isolation was implemented, which not only dramatically reduced hands on time and scalability, but also improved reliability and timelines of genome edited hiPSC line generation.
This achievement illustrates that hiPSC workflows can be easily automated, which allows the scale up of hiPSC cell line generation for a diversity of applications, including *in vitro* disease modeling for basic research and drug discovery.

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