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New tools for improving the genome editing workflow in human induced pluripotent stem cell applications.

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ABSTRACT

Disease-specific human induced pluripotent stem cells (hiPSCs) can be derived directly from patients with known disease phenotypes or can be mimicked by introducing known mutations into the hiPSC genome through CRISPR/Cas9 or TALEN systems. The genome editing process in hiPSCs - especially homology directed repair (HDR) and single cell cloning - remains inefficient and more optimal workflows are needed to improve the application of genome editing to hiPSC disease modeling. Here, we show that a stably expressing Cas9 hiPSC line and its differentiated progeny can be useful for genome editing-based applications. Stable expression of Cas9 yields up to 75% indels and up to 40% efficiency in HDR-mediated SNP corrections in hiPSCs. Moreover, high indel rates can be achieved when gRNAs are delivered to differentiated cells directly.
Aside from editing efficiency, hiPSC survival after isolation of single cells for derivation of homogenous clones has proven challenging and limits the ability to easily isolate homogeneous clones from genome edited hiPSCs. We demonstrate that, through the combined use of the matrix protein rhLaminin-521™ and the novel culture medium StemFlex™, cell survival is greatly improved during the genome editing workflow. Most importantly, we establish that these reagents are key to supporting single cell isolation by automated cell sorting from genome edited hiPSCs. These tools and methods contribute to improved success in the derivation of homogenous genome edited human hiPSC clones and provide novel alternatives to study disease-causing mutations in vitro.

INTRODUCTION

Reprogramming permits the derivation of hiPSCs from diseased patients, and allow us to model diseases in vitro. Furthermore, with the advent of CRISPR mediated genome editing, we can now mimic disease mutations in control hiPSC lines to study the biological effect of those mutations. hiPSCs can then be differentiated into specified cell types such as cardiomyocytes and neurons which can be used to develop assays for drug safety screening or can be used to model disease phenotypes in a dish to discover new drugs (**Figure 1**)[1].

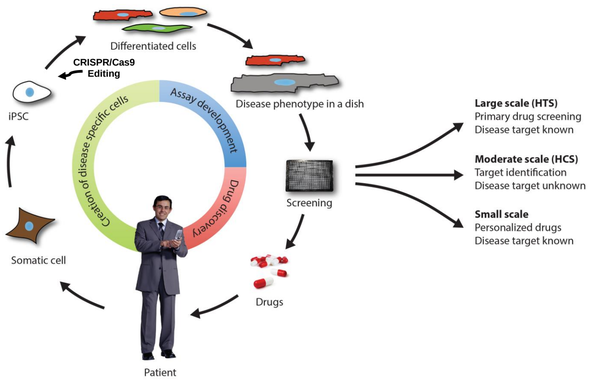


Figure 1. Overview of the applications of disease specific hiPSC (and genome-edited hiPSC) in the drug discovery pipeline. *(Adapted from Mercola et al[1]).*

The implementation of CRISPR/Cas9 in hiPSC disease models has proven challenging due to difficulties with efficient editing tool delivery, survival after delivery and clonal isolation. The more efficient the delivery and clonal isolation is, the higher the success rate in the derivation of the edited hiPSC line of choice. Therefore, we highlight several new tools that can be used to improve the derivation of CRISPR/Cas9-based disease models through use of a hiPSC line stably expressing Cas9 and an improved workflow for clonal isolation of edited hiPSC, which hinges on the use of rhLaminin521™ matrix proteins and StemFlex™ medium.

RESULTS

Most available CRISPR/Cas9 editing methods relying on DNA and mRNA based tools do not allow efficient genome editing in hiPSC and results in the screening of hundreds of colonies to find a correctly edited hiPSC line. Common approaches to facilitate isolation of genome edited hiPSC clones is to introduce a positive selection marker with the desired edit into the genome to enrich for correctly edited hiPSCs, after which hiPSCs are seeded at limited dilutions to isolate potentially single cell derived clones. While the use of Cas9 protein now allows efficient editing in hiPSCs, its reliability is highly target and cell dependent. We thus developed a stably Cas9 expressing hiPSC line as a backbone to easily generate hiPSC-based disease models (**Figure 2**). Through lentiviral delivery we introduced Cas9 into hiPSCs (Cas9 hiPSCs) and after selection a stable cell line was obtained (**Figure 2**). The cell line generation process did not affect the biology of the hiPSCs as the cells maintained their hiPSC morphology, normal karyotype and expression of pluripotent markers (**Figure 2**). We furthermore evaluated the differentiation potential of the Cas9 hiPSCs both through spontaneous differentiation as embryoid bodies and directed differentiation to cardiomyocytes and dopaminergic neurons (**Figure 3**).

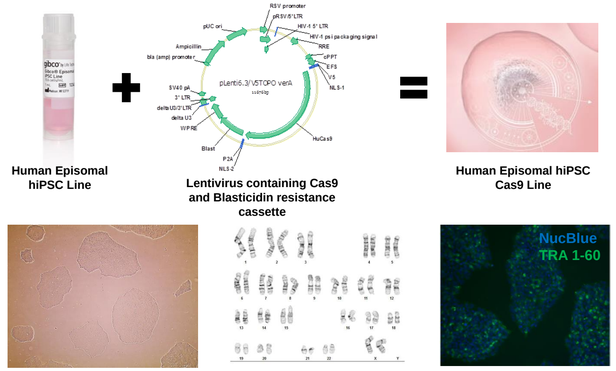


Figure 2. Human Episomal hiPSC Cas9 line (Cas9 hiPSCs). *Cas9 hiPSCs were generated by transducing Human Episomal hiPSCs with a Cas9 expressing lenti-vector after which the hiPSCs were selected for presence of the lenti-vector. The Cas9 hiPSCs have normal morphology and karyotype and are pluripotent.*

Cas9 expressing hiPSCs were able to differentiate to all three germlayers and into cardiomyocytes and dopaminergic neurons (**Figure 3**). To test editing capabilities of the Cas9 hiPSCs, in vitro transcribed gRNA were delivered through electroporation, yielding a high degree of indel induction (**Figure 4**). When single stranded oligos as donor are co-delivered with the gRNA into the Cas9 hiPSCs, homology driven repair was efficient in most cases, although target dependent. Not only Cas9 hiPSCs can be edited, but also Cas9 hiPSC-derived cells through differentiation can be edited and here we show that floor plate progenitors can be edited through delivery of IVT gRNA via RNAiMax™ (**Figure 4**). The ability to edit in Cas9 hiPSC-derived differentiated cell types would allow for functional screening in a variety of cell types and opens up avenues to use gRNA collections such as the LentiArray™ libraries to identify important biological targets.

Sample Name	Self-renewal	Ectoderm	Mesoderm	Endoderm
Cas9 iPSC	0.18	-0.73	-1.16	-1.80
Cas9 EB Day7	-5.45	0.95	2.67	-0.29
Cas9 EB Day21	-7.74	1.36	3.74	1.22

Gene expression relative to the reference standard

Upregulated						Downregulated
x > 1.5	1.0 < x ≤ 1.5	0.5 < x ≤ 1.0	-0.5 ≤ x ≤ 0.5	-1.0 ≤ x < -0.5	-1.5 ≤ x < -1.0	x < -1.5

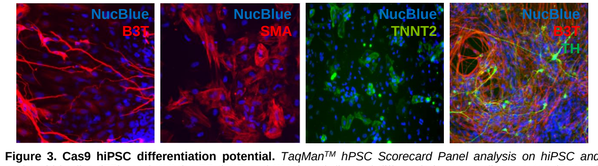
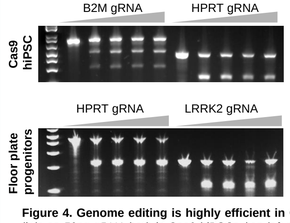


Figure 3. Cas9 hiPSC differentiation potential. *TaqMan™ hPSC Scorecard Panel analysis on hiPSC and embryoid bodies (EB) and ICC for EB (B3T and SMA) and directed differentiation to cardiomyocytes (TNNT2) and DA neurons (B3T and TH) demonstrates that the Human Episomal hiPSC Cas9 Line differentiates into all three germ layers.*



Target	SNCA_1	SNCA_2	SNCA_3	SNCA_4	LRRK2_1	LRRK2_2	TNNT2_1	TNNT2_2	KCNH2	B2M	HPRT
% Indels	38	30	63	23	73	78	42	69	51	62	49
% HDR	37	N/D	34	N/D	26	14	27	19	7	N/D	N/D

Figure 4. Genome editing is highly efficient in Cas9 hiPSCs. *Up to 75% indel induction is observed with as little as 50ng gRNA both in Cas9 hiPSCs (top left panel) and Cas9 hiPSC-derived floor plate progenitors (bottom left panel). Indel induction up to 75% and homology driven repair (HDR) up to ~40% was observed in Cas9 hiPSCs.*

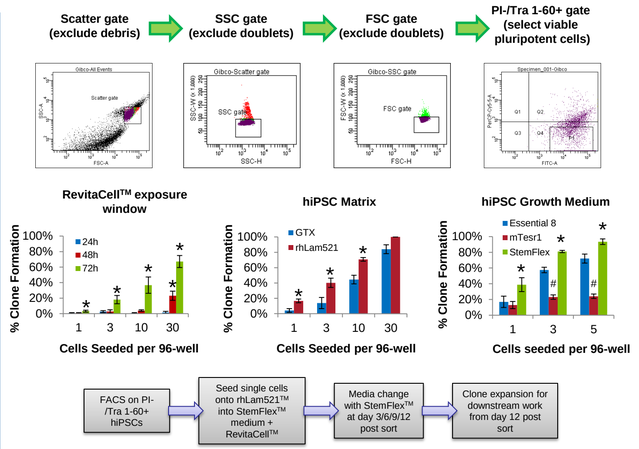


Figure 5: Improvements to increase the recovery of single cell clones isolated through FACS. *A stringent gating strategy was used to identify single, viable and pluripotent hiPSCs which were seeded as indicated. Effects of RevitaCell™ exposure window, hiPSC matrix protein and hiPSC growth medium were investigated. The novel workflow based on these results is diagrammed.*

Use of the Cas9 hiPSCs thus allows robust CRISPR-mediated editing across numerous targets and we next sought to improve clonal isolation of edited hiPSCs. With a FACS-based method to allow high-throughput isolation of single cells, we first implemented stringent gating strategies to ensure that single, viable and pluripotent cells were isolated (**Figure 5**). Factors that may improve survival after sorting were then tested to understand if single cell cloning in feeder-free conditions was at all possible. Through extending the RevitaCell™ exposure from 24h to 72h we were able to dramatically increase clone survival in Essential8™ when seeding limited amounts of cells into a 96-well (**Figure 5, left histogram plot**). Clone survival from seeding one cell was, however, limited and therefore the effect of matrix protein and media system was further investigated. Sorting cells on rhLaminin-521™ further increased clone recovery from single cells, up to 15% and this improvement was enhanced when sorting cells into StemFlex™ medium, yielding up to 40% clone survival from single cell seeds (**Figure 5, middle and right histogram plot respectively**). Clonal survival after single deposition with a cell sorter can thus be improved extensively by following the summarized post sort steps as indicated in the flow chart in **Figure 5**.
This method was then tested on three different hiPSC lines to ensure that the protocol was robust across hiPSC lines from different origins. As shown in **Figure 6**, single cell-derived pluripotent clones were successfully derived from all hiPSC lines tested with different clonal survival rates, but approaching at least 25%. We then further pressure tested the clonal isolation method by isolating single cell clones from CRISPR/Cas9 edited hiPSC pools (**Figure 7**).

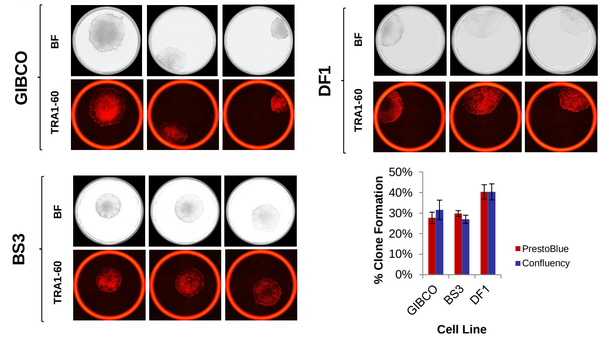
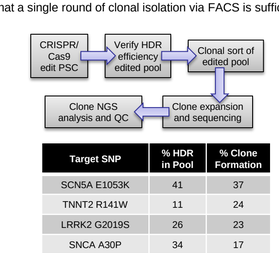
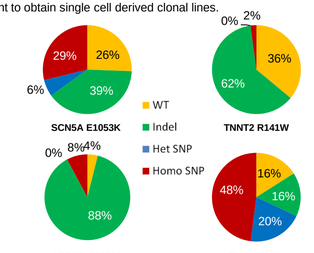


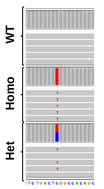
Figure 6. Single cell clone generation from different hiPSC lines. *Individual clones are shown and stained for TRA 1-60 to demonstrate their pluripotent nature. Cloning efficiency measured by confluency or PrestoBlue® for the different lines is also indicated.*

Through delivery of gRNA and single stranded oligos into the Cas9 hiPSCs we introduced SNPs into four different genomic loci. After delivery we obtained 11-41% homology driven repair or SNP introduction (**Figure 7**). Single cell clones were then isolated from each of the pools and yielded 17%-37% surviving clones. These clones were then screened by Sanger sequencing for the presence of homozygous and heterozygous SNPs as well as indels (**pie charts Figure 7**). Selected single cell clones were then further analyzed by next generation sequencing to understand if the isolated clones were really originating from a single cell. Ratios of 100% WT or SNP are expected for unedited or homozygotes, whereas for heterozygotes, both the WT and SNP allele should be represented by equal amounts. Across all for targets and all the derived clonal lines, this is indeed the case (**Figure 7, bottom table**), demonstrating that a single round of clonal isolation via FACS is sufficient to obtain single cell derived clonal lines.



Target SNP	% HDR in Pool	% Clone Formation
SCN5A E1053K	41	37
TNNT2 R141W	11	24
LRRK2 G2019S	26	23
SNCA A30P	34	17





Cell Line	SCN5A E1053K WT	SCN5A E1053K Homo	SCN5A E1053K Het	TNNT2 R141W WT	TNNT2 R141W Homo	TNNT2 R141W Het	LRRK2 G2019S WT	LRRK2 G2019S Homo	LRRK2 G2019S Het	SNCA A30P WT	SNCA A30P Homo	SNCA A30P Het
% WT Alleles	98	2	52	98	1	NA	100	5	NA	100	1	52
% Edited Alleles	2	98	48	2	99	NA	0	95	NA	0	99	48

Figure 7. Generation of disease models in Cas9 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. After expansion clones were analyzed by NGS for allele ratios to demonstrate that clonal lines can be isolated after one round of clonal isolation.*

CONCLUSIONS

With hiPSC-based disease modeling at the forefront of drug discovery, the ability of genome editing has taken an important place in hiPSC-based disease model generation. We therefore sought to develop new tools and methods that improve the efficiency of genome editing and clonal isolation to facilitate generation of isogenic hiPSC lines or hiPSC lines carrying disease causing mutations to facilitate the generation of disease models.
We developed a hiPSC line that stably expresses Cas9, which not only allows efficient genome editing in the hiPSCs themselves, but also allows to study CRISPR/Cas9 mediated perturbations during hiPSC differentiation. To facilitate the clonal isolation of genome edited hiPSCs, we furthermore developed a new method that allows isolation of single cell derived clones via FACS. Key components of post survival are extracellular factors such as the rhLaminin-521™ matrix protein and StemFlex™ medium. The combination of both the Cas9 hiPSC line and the clonal isolation method provide two novel tools that dramatically increase the success of CRISPR/Cas9 edited hiPSC lines, as we demonstrate for four individual targets, and should contribute to the adoption of disease models in research and drug discovery.

REFERENCES

1. Mercola et al. Induced Pluripotent Stem Cells in Cardiovascular Drug Discovery. Circulation Research 2013.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING

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