Molecular Analysis Tools for Epigenomic Characterization of Pluripotent Stem Cells

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ABSTRACT

Induced pluripotent stem cells (iPSCs) offer an ideal platform to generate patient-derived cell models to dissect basic biology and for therapeutic use, either in drug screening or cell replacement. As iPSCs are generated from various genetic backgrounds using different derivation and culture conditions, it is essential to carryout comprehensive characterization to ensure their identity, functionality, and genetic stability. Molecular analysis platforms offer a quantitative, accurate, and fast alternative to current cellular methods of confirming pluripotency.

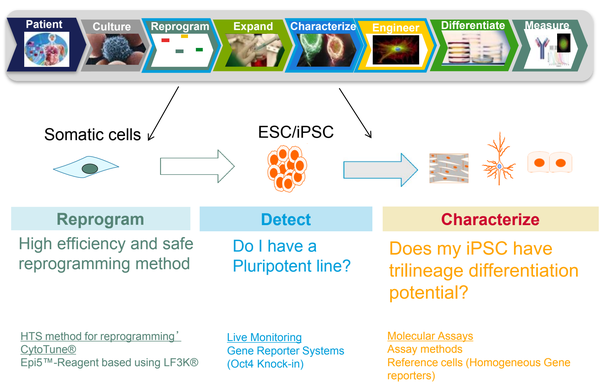
We had reported earlier on the TaqMan® hPSC Scorecard™, a comprehensive gene expression panel for rapid characterization of pluripotent stem cells. In this study, a similar PCR-based approach was utilized to quantitatively detect methylation status of specific genomic loci in pluripotent stem cells. Methylation specific primers were designed to 6 different loci that are known to be either methylated or unmethylated in the pluripotent state; specificity was confirmed in undifferentiated and differentiating H9 ESCs. In order to extend the study, iPSCs were derived from several donor phenotypes using a simplified workflow utilizing the Epi5™ iPSC Reprogramming kit in combination with the Lipofectamine®3000 Reagent transfection system, as well as our CytoTune® IPS 2.0 Sendai Reprogramming Kit. H9 ESCs and the generated iPSC clones will be used to generate Oct4-GFP reporter lines using targeted insertion and GeneArt ® Precision TALs technology, thus enabling the creation of reference standards from high quality pluripotent cells. Creation of pluripotent reporter ESC and iPSC from diverse sources provides a valuable tool to generate homogeneous population of cells for the creation of reference standards and comparison of molecular expression signature with alternate enrichment methods based on surface marker expression. Generation of standardized tools and technologies enables thorough characterization of pluripotent stem cells, and is critical for their downstream application in regenerative medicine.

INTRODUCTION

Advances in the generation of iPSC have led to different methods and culture conditions to successfully establish pluripotent clones. A key component prior to their downstream application is thorough characterization and standardization. Traditional cellular staining and functional behavior, such as differentiation, is now being complemented with thorough molecular characterization (1,2). Transcriptome and methylation analyses have been used to not only confirm pluripotency but also ensure complete reprogramming (2) and determine differentiation bias of specific clones (1).

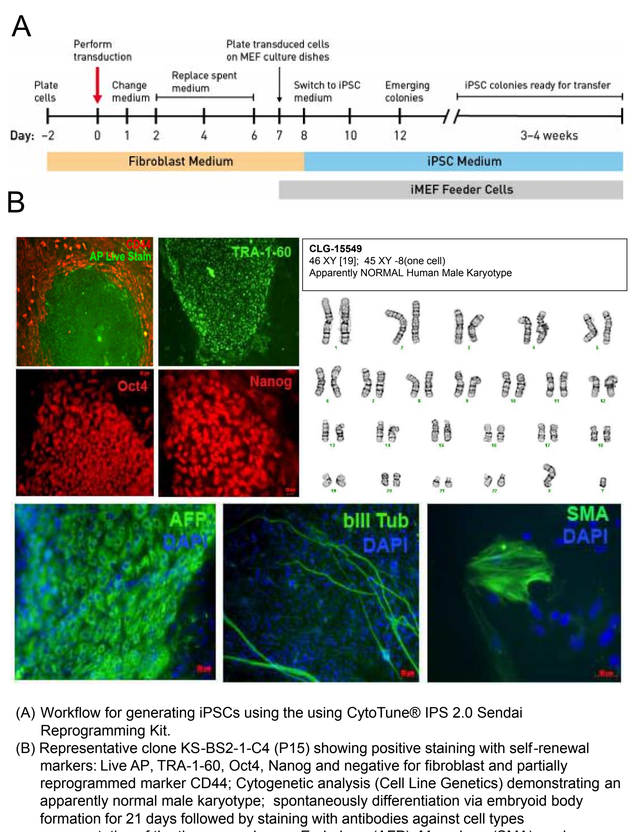
The goal of this study was to develop tools that assist in rapid screening and development of molecular assays for the characterization and standardization of pluripotent stem cell. Foot-print free iPSCs were generated from human fibroblasts using CytoTune® IPS 2.0 Sendai Reprogramming Kit. In addition, reprogramming based on Epi5™ iPSC Reprogramming kit was developed with Lipofectamine®3000 transfection reagent thus enabling a high throughput method. The resulting iPSCs confirmed for pluripotency and trilineage differentiation, was further used in the development of TaqMan® methylation assay to 6 specific genomic loci that are associated with hypermethylated or hypomethylated state in the pluripotent cells. The human ESC line, BGO1v/hOG expressing EmGFP driven by the human Oct 4 promoter integrated in a safe harbor site of the genome (3) was further shown to be an efficient reporter system for secondary fibroblast reprogramming for monitoring the onset of GFP expression during the process of reprogramming. In addition, Oct4-GFP driven expression also serves as a sensitive measure to determine homogeneous population of pluripotent cells. Generation of edited iPSCs with GFP/Neo knocked-in to the endogenous Oct4 promoter using the GeneArt® Precision TALEN™ is currently in progress. The resulting Oct-GFP/Neo iPSCs will provide a reference standard for the development and validation of molecular assays and their confirmation.

METHODS



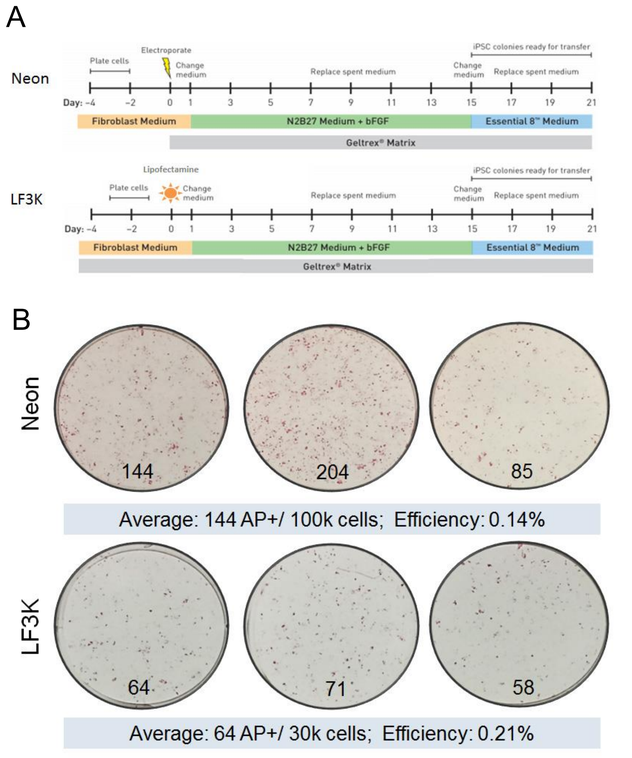
RESULTS

Figure 1. Generation of foot-print free iPSC for Downstream Molecular Analysis



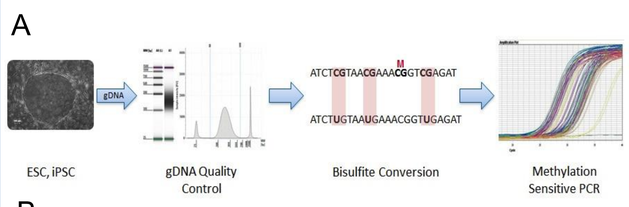
(A) Workflow for generating iPSCs using the using CytoTune® IPS 2.0 Sendai Reprogramming Kit.
(B) Representative clone KS-BS2-1-C4 (P15) showing positive staining with self-renewal markers: Live AP, TRA-1-60, Oct4, Nanog and negative for fibroblast and partially reprogrammed marker CD44; Cytogenetic analysis (Cell Line Genetics) demonstrating an apparently normal male karyotype; spontaneously differentiation via embryoid body formation for 21 days followed by staining with antibodies against cell types representative of the three germ layers: Endoderm (AFP), Mesoderm (SMA), and Ectoderm (βIII-Tubulin).

Figure 2. Development of Lipofectamine®3000 transfection -based reprogramming with Epi5™ iPSC Reprogramming kit



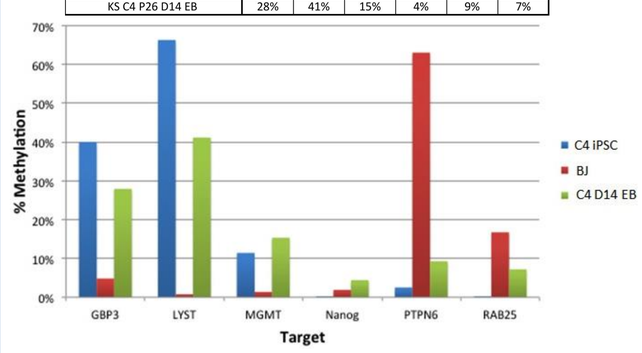
(A) Reprogramming workflow with the Neon® transfection and Lipofectamine®3000. Experiment were run in triplicate under free-free conditions.
(B) Vector® Red Alkaline Phosphatase (AP) colonies obtained with BJ Fibroblast reprogramming with Epi5™ iPSC Reprogramming kit using Lipofectamine®3000 Reagent Transfection or the traditional Neon® Electroporation Transfection System. At day 21, the number of AP+ colonies were counted and the average number of colonies from the three replicates per condition used to determine the efficiency of reprogramming

Figure 3. TaqMan® Methylation-specific PCR Analysis of iPSCs



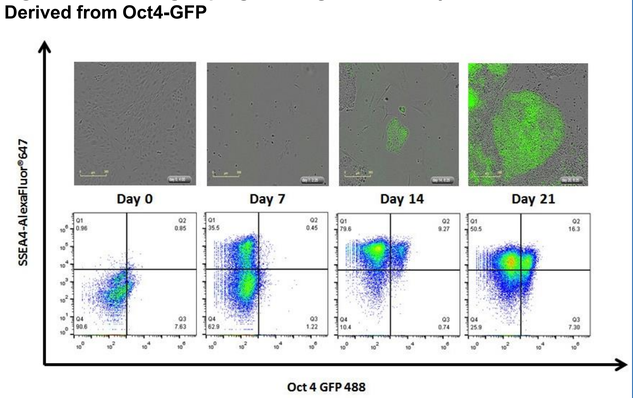
B

	% Methylation in Sample by Target					
Target	GBP3	LYST	MGMT	Nanog	PTPN6	RAB25
KS-BS2-1-C4 iPSC	40%	66%	11%	0%	3%	0%
BJ P4	5%	1%	1%	2%	63%	17%
KS C4 P26 D14 EB	28%	41%	15%	4%	9%	7%



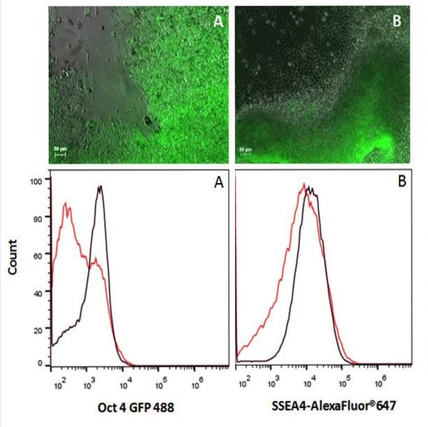
(A) Workflow for TaqMan Methylation-sensitive PCR analysis. Parental BJ fibroblasts, undifferentiated iPSC, and iPSC spontaneously differentiated for 14 days were harvested and genomic DNA (gDNA) isolated for subsequent Bisulfite Conversion. Methylation specific PCR to six gene regions were analyzed using custom made TaqMan® probes and primers targeting methylation and non-methylation specific CpG on each locus. RAB25, NANOG, and PTPN6 are genes that maintain low levels of DNA methylation compared to differentiated cell types; while MGMT, GBP3, and LYST genes maintain relatively high levels of DNA methylation in pluripotent cells compared to differentiated cell types (4).
(B) Percentage of methylation for each sample at the six target gene loci.

Figure 4. Monitoring Reprogramming of Secondary Fibroblasts Derived from Oct4-GFP



Reprogramming of BGO1v/hOG Secondary Fibroblasts using CytoTune® IPS 2.0 Sendai Reprogramming Kit under feeder free conditions. FACS analysis was performed at four different time points and cells were stained with SSEA4-AlexaFluor®647 Antibody. SSEA4 expression is plotted on the Y-axis and Oct4 promoter-GFP fluorescence on the X-axis. The results show that the SSEA4 antigen can be targeted sooner in reprogramming shown at day 7, than the expression of the exogenous human Oct 4 promoter-EmGFP, shown at day 14.

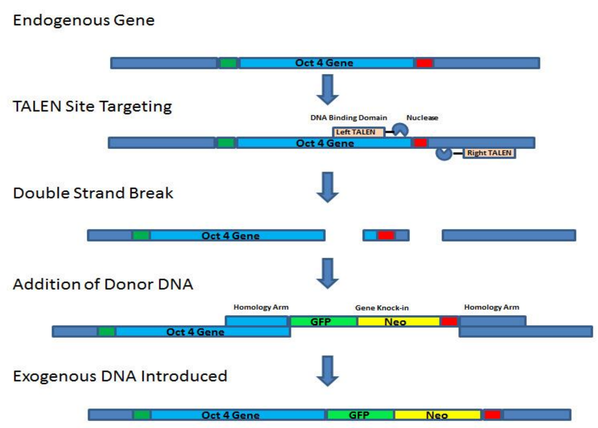
Figure 5. Comparison of SSEA4 Antibody and Oct 4 GFP during Differentiation



Top panels: BG01V/hOG hESCs cultured in feeder free conditions either under (A) regular hESC conditions with drug selection or,
(B) in differentiation EB media without drug selection. Representative images were captured at 5X magnification and shows overlay of phase and GFP fluorescence

Bottom Panels: Following 7 days of culture, cells were stained with SSEA4-AlexaFluor®647 Antibody and analyzed using Flow Cytometry for
(A) Oct4 GFP expression
(B) SSEA4 staining.
In both cases, the black line represents cells in hESC media and the red line represents cells in differentiation media.

Figure 6. Generation of Oct 4 Knock-in using GeneArt® Precision TALEN™



A schematic of GFP-Neomycin knock-in at the endogenous Oct4 Gene Loci using GeneArt® Precision TALEN™.

CONCLUSIONS

CytoTune® IPS 2.0 Sendai Reprogramming Kit was used to generate iPSC clones from BJ human fibroblasts, characterized using cellular methods and used for downstream molecular assays.

Lipofectamine®3000 mediated Epi5™ reprogramming method was developed which offers an alternative to traditional transfection methods and is amenable for high throughput and automated workflows.

TaqMan® methylation specific PCR assay successfully distinguished methylation versus unmethylated regions. The 6 genomic loci chosen showed greater sensitivity in distinguishing parental cells versus iPSC and was less sensitive and more variable in distinguishing Undifferentiated versus differentiated cells.

GFP expression in the human Oct4-GFP gene reporter line BGO1v hESC is a more sensitive and accurate indicator of pluripotency both during reprogramming of secondary fibroblasts and spontaneous differentiation of pluripotent cells, compared to currently used pluripotent specific surface markers such as SSEA4.

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ACKNOWLEDGEMENTS

Thanks to Shoulian Dong and Carmen Gjerstad at Thermo Fisher Scientific for methylation TaqMan® primer and probe design and help with the statistical data analysis of the methylation data. Thanks to Chad, Maha, Joanna and other extended members of the Carlsbad Stem Cell team for their support and suggestions. This study was supported by the CIRM Bridges Program at CSUSM.

TRADEMARKS/LICENSING

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