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A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of

Philosophy in Molecular and Medical Pharmacology

By

Jason Patrick Awe

2014

ABSTRACT OF THE DISSERTATION

Investigating the Mechanisms of Reprogramming and Optimizing the Generation of Potentially Therapeutically Useful Induced Pluripotent Stem Cell Derivatives

By

Jason Patrick Awe

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2014

Professor James A. Byrne, Chair

Human induced pluripotent stem cells (hiPSCs), derived from easily obtainable skin cells, possess enormous opportunity for autologous cellular treatment therapies, gene correction, and disease modeling without worries of ethical constraints associated with human embryonic stem cells (hESCs). Although lentiviral based reprogramming remains as one of the most popular methods for reprogramming, potentially oncogenic viral integrations in random locations throughout the genome along with non-human antigens associated with the reprogramming process thwart the clinical applications of these hiPSCs. To address these concerns we derived a hiPSC line void of any exogenous reprogramming factors and differentiated these hiPSCs into clinically relevant cell derivatives. In addition, to maintain clinical relevance, we implemented a methodology to clean our hiPSCs from non-human antigens to allow for current good manufacturing practice conditions that could help set the standard for human clinical trials with our factor-free hiPSCs. The field of stem cell reprogramming has rapidly advanced, and a new

technique involving mRNA based reprogramming was introduced that we found to be difficult to reproduce due to an innate immune response based degradation of mRNA when introduced into the cell. To solve this problem, a small chemical compound was utilized that blocked important aspects of the innate immune response to single stranded mRNA that yielded robust and uniform expression of a key reprogramming factor. This stabilization could be important in increasing mRNA based reprogramming efficiency of hiPSC derivation. Another challenge in the hiPSC field is investigating nuanced potential differences manifested in transcriptional, epigenetic, immunological, and differentiation potentials between hESCs and hiPSCs. To help and potentially solve this problem and allow for more complete and faithful reprogramming to a hESC state, global microarray transcriptional analysis of oocyte cytoplasm was utilized to find eight putative novel shared reprogramming factors across multiple species. These factors have identifiable roles in opening up chromatin that can allow reprogramming factors to better access reprogramming loci that could confer the known reprogramming advantage that somatic cell nuclear transfer based reprogramming maintains over current direct reprogramming approaches. To address the recently observed immunogenicity issues of iPSCs, we studied the expression of two normally fetally associated genes implicated in an iPSC-specific immune response. We found high line-to-line variation between both hESC and hiPSC lines across different levels of differentiation and confirmed that current differentiation protocols derive cell types with a fetal phenotype as opposed to the adult phenotype needed for clinical applications as indicated by aberrant expression of specific fetal genes. Taken altogether, we hope these studies allow for more robust, reproducible, and clinically relevant hiPSCs that more closely resemble hESCs and maintain full ability to differentiate into clinically relevant cell types that can be used for potential human clinical trials for disease and cell replacement therapy.

COMMITTEE PAGE

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DEDICATION PAGE

I dedicate this thesis to my amazing wife who has been incredibly supportive of my research, the long hours in the laboratory, and most importantly, of me and all my endeavors. I also dedicate my thesis to my grandmother, Nana, who has supported me unconditionally throughout my whole life in every aspect.

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Publications

1. Xia B, Joubert A, Groves B, Vo K, Ashraf D, Djavaherian D, Awe J, Xiong Y, Cherfils J, Ma D. "Modulation of cell adhesion and migration by the histone methyltransferase subunit mDpy-30 and its interacting proteins." PLoS One. 2010 Jul 23;5(7):e11771. doi: 10.1371/journal.pone.0011771.

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6. Durruthy-Durruthy J., Briggs S., Awe J. et al and Sebastiano V. (2013). "Derivation of clinical-grade human induced pluripotent stem cells using modified mRNAs" (*Under Review at PLOS ONE*)

7. Awe JP, et al and Byrne, J. A. (2013). "Characterizing immunogenicity differences in human embryonic stem cells, induced pluripotent stem cells, and their derivatives" (in preparation).

Molecular & Medical Pharmacology Department, University of California, Los Angeles	***2013***

Molecular & Medical Pharmacology Department Retreat
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CHAPTER 1: INTRODUCTION

Reprogramming methodologies and clinical relevance for hiPSCs

Human embryonic stem cells (hESCs) were first derived in 1998 and were characterized by the ability to both self-renew and differentiate into almost any functional cell type and are commonly derived from the inner cell mass of pre-implantation blastocysts [1]. HESCs quickly ushered in a new era of science research focused on the potential applications in drug discovery, disease biology, and cell replacement therapy [1-4]. However, an ethical concern with the use and destruction of human embryos to derive these cells remains a burden, as well as immunological concerns due to alloantigens on the graft e.g. stem cell derivatives, due to major and minor histocompatibility complex antigens [5, 6]. Mammalian development has been historically thought of as a permanent unidirectional process by which defined epigenetic changes throughout the genome gradually cause a loss of developmental potential during embryonic development [7], whereby the genome is stably set and unable to dedifferentiate again. However, nuclear cloning was developed that provided proof that these previously thought permanent epigenetic marks causing specific lineage specification can in fact be altered and/or removed allowing to reprogram a fully differentiated adult somatic cell back into a pluripotent stem cell that closely resembles ESCs [8]. This reprogramming back to pluripotency has since been extrapolated and fusion of pluripotent stem cells or incubation of pluripotent stem cell extracts with adult somatic cells also can cause this reversal of the differentiated state back into a pluripotent state [9]. Reprogramming with cell extracts from oocyte or embryonic stem cell extracts have also been shown to contain the ability to reprogram differentiated somatic cells, again showing the nuclear plasticity of adult cells [10-12]. These results immediately provided the platform for patient specific cellular therapeutics and disease modeling *in vitro* [3]. Since it is obvious that defined factors in the nucleus of pluripotent stem cells are able to reprogram adult somatic cells, the next step in the evolution of deriving patient specific stem cells was the discovery that forced expression of defined transcription factors, Oct4, Sox2, Klf4, and cMyc,

were able to reprogram adult mouse fibroblasts into ES-like iPSCs when transduced with retroviruses [13]. Almost immediately, however, safety concerns were brought forth concerning insertional mutagenesis which can be caused by retroviral mediated activation of endogenous genes and/or through forced expression of known oncogenes like cMyc [7]. However, multiple retroviruses need to be used to deliver all four transcription factors thereby only allowing a low percentage of cells to have the correct stoichiometric ratio of all four factors properly being transduced and expressed causing very low reprogramming efficiency [14]. This is particularly true as different copy numbers can be found in different clones leading to the hypothesis that precise relative amounts of the individual reprogramming factors are critical for proper reprogramming [7]. In the effort to increase reprogramming efficiency further, the reprogramming field quickly developed better reprogramming viral vectors which culminated in development of a single lentiviral "stem cell cassette" (STEMCCA) that included all four reprogramming factors in a single cassette that was also excisable due to Cre/loxP technology [15]. Part of this motivation to invest in the STEMCCA reprogramming methodology is provided by data showing that the epigenetic and pluripotent state of hiPSCs are influenced by defined stoichiometric ratios of the reprogramming factors, and not as much absolute factor expression levels [16]. This research was further refined with another study showing that the optimal stoichiometric ratio is highly dependent on Oct4 dosage, specifically a 3:1:1:1 ratio of Oct4, Sox2, Klf4, and cMyc [17]. The STEMCCA lentivirus is ideally designed for derivation of factor-free hiPSC lines due to the reprogramming vector expressing the four reprogramming factors at the defined optimized stoichiometric ratios due to "self-cleaving" 2A peptides and having the ability to be excised from the genomic integration site due to Cre/loxP technology incorporated into the vector [15, 18-20]. This led to derivation of a single integrated pluripotent stem cell line that was free from exogenous factors and only a 200bp inactive long terminal repeat (LTR) remaining integrated into the genome, although the concern of insertional mutagenesis is still

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present [21]. Therefore it is important that the genomic site, where the leftover LTR is still integrated into, is sequenced and followed up with gene expression analysis to yield putative factor-free lines that may be clinically applicable [21]. This analysis has become increasingly more relevant at a time when gene targeting by homologous recombination has been shown to be an efficient methodology for site-specific transgene integration [22]. As discussed in Chapter 2, if the US Food and Drug Administration is willing to expand current safe-harbor criteria to include site specific integration into safe areas of the genome, then this would increase the potential quantity of hiPSC lines available for therapeutic needs. At the time of the publication that comprises Chapter 2, other methodologies for reprogramming were becoming available, although they suffered from low efficiencies and hard to reproduce applications to different cell types [23]. Therefore, we decided to bring the more efficient methodology of reprogramming with the excisable STEMCCA vector and reprogram human adult somatic fibroblasts to derive fully characterized transgene-free human iPSCs, as presented in Chapter 2.

Good manufacturing practice quality conditions for future hiPSC derivative based human clinical trials

One of the most important aspects in the development of hiPSC derivative based cell therapeutics is the production of clinical-grade factor-free pluripotent stem cell lines that adhere to current good manufacturing practice (cGMP) guidelines as defined by the Food and Drug Administration (FDA) and Center for Biologics Evaluation and Research (CBER) that are widely adopted by the pharmaceutical industry [24, 25]. These guidelines mandate that during product manufacturing, only animal substance-free culture media, matrix, passaging, and all cryopreservation procedures be used in order to avoid immunological reactions against non-human antigens in the cells, and also prevention of infection due to animal microbes, while ensuring that downstream products meet stringent preset specifications [25]. This is mandated

to protect patients' safety to ensure that any investigational new stem cell-based drug meets minimum safety profile requirements [26]. Another limitation is implementation of a broadly reproducible methodology of either deriving or converting hiPSCs under clinical grade culture conditions to meet cGMP mandates, as many academic facilities lack such cGMP suites and standardized/validated conversion techniques would be extremely relevant [27, 28]. The Code of Federal Regulations outlines what specifically cGMP conditions encompass such as the physical characteristics of the manufacturing facility and also guidelines for the procedures and protocols used for downstream cellular products of pluripotent stem cells [29]. To this end, not only did we present data on how a factor-free and fully characterized hiPSC line was derived that could be applied to this goal in Chapter 2, but also presented in this chapter is a broadly applicable, and reproducible, clinical-grade transition protocol into cGMP compatible conditions for induced pluripotent stem cells as any therapeutically relevant cell replacement therapy must be completely free of measurable levels of non-human contaminants [30]. We therefore demonstrate the derivation of a fully characterized, factor-free hiPSC line that can be differentiated into clinically relevant derivatives and converted into cGMP conditions. This is of critical importance to have completed before the FDA will consider approval of clinical-grade hiPSC derivatives for human clinical trials.

Evolution of hiPSC reprogramming technology and mRNA degradation via innate immune system

Although lentiviral based reprogramming has been proven robust and highly reproducible for a variety of cell types [31, 32], the concern of potential insertional mutagenesis is still relevant and thus other reprogramming methodologies have been invented such as episomal plasmids [33], minicircles [34], non-integrating miRNAs [35, 36], cell permeable proteins [37], and sendai viruses [38], although these techniques suffer from low reprogramming efficiencies, having to

dilute out reprogramming vectors, and random genomic integration or persistent viral infection [39]. One of the most recent reprogramming approaches was introduced using synthetic mRNA that allows for virus and integration free hiPSC derivation although significant innate immune responses were seen not only in the original paper [39], but also upon trying to repeat this experiment in our own lab, as detailed in Chapter 3. As a direct result of this innate immune response based degradation of transfected mRNA the original authors discovered that special modifications were needed to prevent interferon- and NF-κB dependent degradation of the single-stranded RNA [39], although based on our results mRNA transfection still results in degradation and heterogeneous expression of the transfected mRNAs as presented in Chapter 3. Therefore, we came up with a small chemical compound screen of a variety of different drugs that could inhibit specific aspects of the innate immune response machinery important in the degradation of single stranded mRNAs, and postulated that the subsequent increased mRNA stabilization of an important reprogramming transcription factor could be validated as being critical for increased mRNA based reprogramming efficiency with further testing.

Somatic cell nuclear transfer and finding novel reprogramming factors

Nuclear transplantation, or more commonly referred to as somatic cell nuclear transfer (SCNT), is a term used to describe the genome wide epigenetic reprogramming of a differentiated cell nucleus, by specific factors in ooplasm, and subsequent derivation of nuclear transfer ESCs (ntESCs) from these cloned blastocysts [8, 40, 41]. Key to personalized therapeutics is the fact that SCNT allows for the derivation of autologous, or genetically identical, ESC lines from a variety of somatic cell types from potentially diseased patients, a methodology already proven in animal models [42]. Importantly, adult cells were originally thought of as maintained in a terminally differentiated state and reversion back into a pluripotent state was not possible, or even that genetic elimination was needed for lineage specific gene expression in a particular

tissue [12]. This was of course proved incorrect when adult cells, specifically mature

lymphocytes that have undergone immune-receptor rearrangements, were successfully cloned

[43]. The molecular process of reprogramming during SCNT involves complete erasure of the

donor cell epigenetic pattern after the donor nucleus is introduced into the oocyte and the re-

establishment of pluripotent epigenetic marks and embryonic characteristic gene expression

[44]. This reprogramming step is then followed by ESC derivation from the inner cell mass and

subsequent differentiation into various derivatives that can be used for tissue generation or cell

replacement therapy upon transplantation [44]. This argument for cell replacement therapy that

could be used in human clinical trials has become stronger with the recent successful SCNT

based derivation of hESCs [45].

In order to explain further why SCNT is important to this thesis, and specifically Chapter 4, a

detour into potential differences between hESCs and hiPSCs must be taken into consideration.

When hiPSCs were first derived they were immediately introduced as being almost identical to

ESCs, considered the gold standard, in regards to morphology, pluripotency markers,

methylation status of defined promoter regions, differentiation potential, and teratoma formation

[13, 46]. Soon after this landmark discovery, the degree of similarity between ESCs and iPSCs

started to be questioned for differences in epigenetics, genomic integrity, transcriptional

differences, immunological differences (as discussed in Chapter 5), and in differentiation

potential [4]. Specifically, multiple publications have cited small, but significant gene expression

signature differences between hESCs and hiPSCs [47] and transcriptional profiling has even

shown heterogeneity in single hESC and hiPSC analysis [48] that cannot be attributed to

random viral insertions throughout the genome [49]. Epigenetic differences have also been cited

in regards to hypermethylation and hypomethylation of cytosine-phosphate-guanine (CpG)

island shores termed differentially methylated regions [50-52]. Additionally, megabase-scale

regions of aberrant non-CG methylation was investigated between hESCs and hiPSCs and

shown to differ significantly between lines [51]. Another study identified specific aberrant

reprogramming hotspots at subtelomeric regions that bear incomplete CG hydroxymethylation in

hiPSCs at a higher frequency than hESCs potentially indicating that hiPSCs are more

epigenetically variable than their ESC counterpart [53]. This topic is still quite controversial and

many publications have provided evidence against specific ESC and iPSC differences, and

instead argue for more of an intrinsic variability amongst iPSC lines. To provide more evidence

towards this intrinsic variability argument, data has shown that some hiPSCs can harbor a

residual epigenetic memory of their tissue of origin due to specific epigenetic marks established

by the parental donor cell [54]. Interestingly, these epigenetic marks, that persist through

pluripotent reprogramming during iPSC factor-based reprogramming, also confers a selective

differentiation propensity towards the original cell donor lineage [55]. However, in support of the

intrinsic variability, all iPSC lines tested gradually resolved all transcriptional and epigenetic

differences upon continued *in vitro* passaging of the cells, indicating that this epigenetic,

transcriptional, and differential differentiation propensity are transient features of early

reprogrammed iPSCs [55]. This data indicates that previous studies may have seen such

differences between ESCs and iPSCs because of early passage cell analysis. This high degree

of passaging ameliorating any differences seen between ESCs and iPSCs was also shown in

other newly derived lines made from polycistronic reprogramming vectors, although this

publication indicates there are a few select differentially expressed genes that specifically

encode several noncoding transcripts and miRNAs [56]. Coincidentally, transcriptional studies in

the mouse identified only two genes that mapped to the imprinted *DLK1-DIO3* gene cluster that

were found to be aberrantly expressed in low developmentally competent mouse iPSC lines

[57]. Interestingly, extensive passaging, as discussed, does not affect the incorrect expression

of the *DLK1-DIO3* gene cluster, indicating this is one possible locus that may not be part of the

intrinsic variability between clones [55]. Therefore, while there is much debate still occurring

about this subject, it seems that a more likely scenario is the argument made for slightly more epigenetic and transcriptional deviation inherent in hiPSCs that is not unique or shared by all iPSC lines [58]. Therefore a comprehensive hiPSC "report card" idea was introduced that scored pluripotent stem cell line quality and utility by detecting cell-line outliers as indicated by DNA methylation and transcriptional analysis so that lines can be chosen that are most relevant to the specific intended application [58]. This increased inherent variability in the hiPSC lines yield a specific niche in the stem cell field to innovate a better reprogramming methodology that can more faithfully and reproducibly make hiPSCs, with decreased epigenetic and transcriptional variation.

SCNT was introduced in this thesis as one methodology of deriving patient specific stem cells. However, as indicated in the last section, increased intrinsic variability in hiPSCs can make the standardization of hiPSC-based differentiation almost impossible, and therefore establishing a possible impediment to full realization of cellular therapy in human clinical trials. Therefore, it is important to note that, while reprogramming is less efficient than factor-based reprogramming, SCNT based derivation of ESCs more closely resemble fertilized ESCs in regards to DNA microarray profiles, gene expression profiling, tissue-dependent differentially DNA methylated regions, differentiation propensity, and overall erasure of any epigenetic memory from the donor source cell [40, 59, 60]. It is clear that nuclear reprogramming reprograms somatic cells to a more ESC like state that is essentially indistinguishable, whereas factor-based approaches suffer from a host of potential epigenetic and transcriptional differences, among others, indicating that there are specific factors that have yet to be identified in the ooplasm that could be utilized to augment current factor-based reprogramming approaches. To this end, it has been shown, before the discovery that caffeine was the key factor needed in successful derivation of a SCNT hESC line, that human SCNT reprogramming could only be accomplished if the oocyte genome was not removed [61]. This potentially indicates that important reprogramming factors

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in human MII oocytes are connected somehow with the chromosome or spindle apparatus and are lost during enucleation, indicating a potential means of finding these factors for augmentation in factor-based reprogramming approaches. The focus of Chapter 4 is finding oocyte derived factors that are important in epigenetic reprogramming during normal development and that are involved in nucleohistone replacement, active demethylation, and nuclear decondensation to allow for genome based transcription and histone modifications to keep the chromatin in a euchromatic state [62]. Additional factors that could be involved in this reprogramming process include proteins and RNAs that act on specific genomic loci that are involved in regulating or facilitating the expression of key reprogramming genes or inhibiting lineage specific genes involved in differentiation [63]. We postulate that since the exposure time to active and passive demethylation of the donor cell nucleus during SCNT is normally disrupted before completion and therefore incomplete chromatin remodeling results, utilizing these previously uncharacterized factors augmented with current factor-based reprogramming approaches could lead to more fully reprogrammed hESCs [62]. Therefore Chapter 4 explains an *in silico* approach to identifying these key factors that we describe as being candidate oocyte reprogramming factors that could be used to augment current factor-based reprogramming protocols.

Immunogenicity profile of hESCs and hiPSCs

One of the central goals of the hiPSC field is personalized cellular therapeutics, or autologous therapy, whereby the donor cells would be genetically identical to the recipient and theoretically should not elicit an immune response. After ESCs were first derived, they were almost immediately postulated to be a promising tool for cell replacement therapies as data provided evidence of an ESC-based immune-privilege due to low expression of major histocompatibility complex (MHC) class I, MHC class II, and costimulatory molecules [64-67]. It was reasoned that

low expression of these proteins would be the basis for evading the immune surveillance machinery and subsequent MHC mediated rejection [64]. Additionally, this immune-privilege was also thought to extend to ESC differentiated derivatives and eventually multiple cell types including oligodendrocyte progenitor cells, embryoid bodies, and insulin-producing cell clusters were shown to elicit minimal immune responses *in vitro* or *in vivo* [66, 68-70]. Further evidence to support this claim in the human came from using a human peripheral blood mononuclear cell reconstituted Trimera mouse whereby only minor activation of a direct allospecific response to different cell types at varying levels of differentiation was seen [71]. Only after strong stimulation of hESCs to upregulate MHC-I expression by IFN-γ challenge and introduction of a human cytotoxic T lymphocyte line that is specific for a HLA-A antigen only expressed on hESCs could T-cell mediated killing be observed [71]. There is also data supporting that the immune-privilege seen by ESCs and their derivatives are more fragile and these cells can be induced to elicit an immune response upon *in vivo* maturation and subsequent MHC-I upregulation or addition of passenger antigen presenting cells (APCs) to potentiate the immunogenicity of an ESC graft [72]. Interestingly, MHC-II is not upregulated even in the presence of IFN-γ, indicating that if hESCs are able to elicit an immune rejection, indirect recognition through antigen-presenting cells most likely triggers this immune reaction [73]. This is especially important considering that although these are pluripotent stem cells capable of potentially differentiating into immune cells proficient in stimulating a direct pathway immune response with cytotoxic T cells, this is unlikely [69]. More likely, however, is the presentation of hESC associated allopeptides that may have been introduced during the cell stress inducing differentiation protocols that can subject these cells to a wide variety of foreign antigens, and can subsequently be processed and presented via APC mediated direct presentation to naïve T-cells in secondary lymphoid organs [69, 72, 74]. These data provide evidence that the immunostimulatory nature of ESCs and their derivatives are lower than wildtype grafts,

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indicating the possibility of mild immunosuppressive therapy would be sufficient for allogeneic transplantation [71]. Due to this putative immune-privilege, pluripotent stem cell banking has been proposed to create a human stem cell bank from donated surplus embryos and that 150 human ES cell lines would provide a 100% match at HLA-A, HLA-B, and HLA-DR for 20% of patients in need of cellular replacement therapy [64]. Although only 3 out of 9 MHC loci need to be matched to be considered for kidney and heart transplantation, it cannot be readily predicted if these same percentages of mismatch would be relevant to stem-cell derived grafts for human transplants [64, 73]. The overall cost of deriving this many hESC lines coupled with material constraints due to ethical concerns for providing surplus embryos for hESC derivation likely precludes this approach from ever reaching clinical potential and patient treatments [73]. Countering this notion of ESC immune-privilege has been numerous studies detailing the observed immune response upon ESC transplantation into injured myocardium and finding significant T-cell based infiltration [75]. Additionally, ESC intramuscular transplantation of firefly luciferase labeled ESCs and monitoring with bioluminescent imaging found complete regression to basal levels of luminescence after 28 days after transplantation, again indicating an alloantigen-specific rejection of these stem cells [76]. In a model that allows for non-directed differentiation, allogeneic EBs elicited a substantial inflammatory response that included macrophages and T cells when transplanted under the kidney capsule of immunocompetent mice [77]. Additionally, *in vivo* matured ESCs that underwent heterotopic transplantation containing allogeneic ESC grafts were found to elicit an immune response even faster than undifferentiated ESCs, providing evidence that ESC-based derivatives may have more of an immune response upon transplantation [78]. Although the data establishing if ESCs and their derivatives are immunogenic is controversial and far from complete, it is reasonable to assume that the mechanisms of potential immune-privilege or immunogenicity seen in ESCs could be extrapolated to iPSCs and should be analyzed in a similar fashion.

This potential immunogenicity response associated with ESCs and their derivatives is of critical importance for trying to implement ESC-based cellular therapeutics for human trials. This idea becomes even more important when considering that an immune response was found in mouse iPSCs when transplanted into syngeneic mice and allowed to form teratomas whereas mouse ESC derived teratomas saw no such immune response [79]. This is notable as iPSCs can be derived from the original diseased host and any therapeutic derived from iPSCs made from the donor tissue is genetically identical to the host and should not elicit an immune reaction. However, this differential immune response seen in the mouse has already been contradicted by other publications claiming that it is irreproducible, thereby fueling the controversial debate on whether hESCs and/or hiPSCs are immunogenic [14, 80]. Thus, Chapter 5 investigates the potential immunogenicity differences between hESCs and hiPSCs by analyzing two key immunogenic genes, previously identified in mouse experiments, and their expression profiles for a variety of different levels of differentiation across many hESC and hiPSC lines.

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CHAPTER 2: GENERATION AND CHARACTERIZATION OF TRANSGENE-FREE HUMAN INDUCED PLURIPOTENT STEM CELLS AND CONVERSION TO PUTATIVE CLINICAL-GRADE STATUS

Abstract

Introduction

The reprogramming of a patient's somatic cells back into induced pluripotent stem cells (iPSCs) holds significant promise for future autologous cellular therapeutics. The continued presence of potentially oncogenic transgenic elements following reprogramming, however, represents a safety concern that should be addressed prior to clinical applications. The polycistronic stem cell cassette (STEMCCA), an excisable lentiviral reprogramming vector, provides, in our hands, the most consistent reprogramming approach that addresses this safety concern. Nevertheless, most viral integrations occur in genes, and exactly how the integration, epigenetic reprogramming, and excision of the STEMCCA reprogramming vector influences those genes and whether these cells still have clinical potential are not yet known.

Methods

In this study, we used both microarray and sensitive real-time PCR to investigate gene expression changes following both intron-based reprogramming and excision of the STEMCCA cassette during the generation of human iPSCs from adult human dermal fibroblasts. Integration site analysis was conducted using nonrestrictive linear amplification PCR. Transgene-free iPSCs were fully characterized via immunocytochemistry, karyotyping and teratoma formation, and current protocols were implemented for guided differentiation. We also utilized current good manufacturing practice guidelines and manufacturing facilities for conversion of our iPSCs into putative clinical grade conditions.

Results

We found that a STEMCCA-derived iPSC line that contains a single integration, found to be located in an intronic location in an actively transcribed gene, *PRPF39*, displays significantly increased expression when compared with post-excised stem cells. STEMCCA excision via Cre recombinase returned basal expression levels of *PRPF39*. These cells were also shown to have proper splicing patterns and *PRPF39* gene sequences. We also fully characterized the post-excision iPSCs, differentiated them into multiple clinically relevant cell types (including oligodendrocytes, hepatocytes, and cardiomyocytes), and converted them to putative clinical-grade conditions using the same approach previously approved by the US Food and Drug Administration for the conversion of human embryonic stem cells from research-grade to clinical-grade status.

Conclusion

For the first time, these studies provide a proof-of-principle for the generation of fully characterized transgene-free human iPSCs and, in light of the limited availability of current good manufacturing practice cellular manufacturing facilities, highlight an attractive potential mechanism for converting research-grade cell lines into putatively clinical-grade biologics for personalized cellular therapeutics.

Introduction

Previous research demonstrated that human somatic cells can be directly reprogrammed back into an induced pluripotent stem cell (iPSC) state through exogenous expression of a small number of transgenic factors [1]. The ability of these cells to differentiate into any human cell type highlights their promise for future autologous cellular therapies [2,3]. Nevertheless, the continued presence of potentially oncogenic transgenic elements following reprogramming

represents a safety concern that must be addressed prior to clinical applications [4-7]. Various integration-free approaches have been investigated to address this safety concern. Of the various techniques tested to date – that is, episomal plasmids [8], minicircles [9], nonintegrating miRNAs [10,11], cell-permeable proteins [12], sendai viruses [13], synthetic mRNAs [14] and the removable polycistronic stem cell cassette (STEMCCA) – and despite each having published reprogramming success (Table 1), only the STEMCCA-based reprogramming approach, in our hands, has consistently and successfully reprogrammed dermal fibroblasts from multiple different adult donors into iPSCs.

Advantages of the STEMCCA reprogramming approach include the following: lentiviruses can transduce both dividing and nondividing cells; the STEMCCA polycistronic cassette was engineered for efficient production of multiple protein products from a single lentivirus and allows a characteristic stoichiometry of protein expression that reproducibly promotes consistent reprogramming success [15,19]; the STEMCCA approach involves only a single transduction event, making it less labor intensive than more involved reprogramming methods such as synthetic mRNAs; the STEMCCA cassette is excisable, eliminating residual transgene expression that reportedly compromises differentiation potential [20]; and iPSCs can be generated to contain only one integration event and accurately mapped in the genome [16,20,21]. To date, a variety of cell types have been reprogrammed through polycistronic lentivirus-mediated reprogramming, including human keratinocytes, bone marrow cells, skin fibroblasts [22], and T cells from peripheral blood [23] and also from patients with diseases such as Huntington's disease [24], heart failure [25], immunodeficiency disorders [26], lung disease [16], and neurodevelopmental disorders [27]. Nevertheless, the majority (approximately 70%) of lentiviral integrations occur in actively transcribed genes [28,29]. Because current safe-harbor criteria discard iPSC lines that result from a viral integration

occurring in a gene [30], this greatly reduces the feasibility of STEMCCA-iPSC-based therapeutics. We and others have previously relied solely on microarray transcriptional analysis to assess the expression of genes following insertion of STEMCCA into the introns of genes [30,31].

In this study, we use both microarray and sensitive real-time RT-PCR to investigate gene expression changes following both intron-based integration and excision of the STEMCCA cassette during the generation of human induced pluripotent stem cells (hiPSCs). We also fully characterized the post-excised iPSCs, differentiated them into four therapeutically useful cell types, and converted them into putative clinical-grade conditions.

Materials and methods

Ethics statement

Written approvals for human skin biopsy procedures and human fibroblast derivation, culture, and experimental use were obtained from the Stanford University Institutional Review Board (Stanford IRB protocol #10368) and the Stanford University Stem Cell Research Oversight Committee (Stanford SCRO protocol #40), and written informed consent was obtained from each individual participant. Cells used in this study were initially derived at Stanford University and transferred to UCLA through a material transfer agreement (UCLA MTA #2011-00000147). Written approvals for the experiments performed in this study were obtained from the UCLA Institute Biosafety Committee (UCLA IBC protocol #123.10.0-f), the Animal Research Committee (UCLA ARC protocol #2006-119-21) and the Stem Cell Research Oversight Committee (UCLA SCRO protocol #2010-010-02).

In vitro culture of primary human skin cells

The human skin-derived (HUF1) primary cell line used in this study was obtained from a 4-mm adult skin punch biopsy and was cultured as described [32]. Two other fibroblast lines were also used in this study: an infant fibroblast line (MGM2) and a fibroblast line from Fibrocell Science, Inc. (Exton, PA, USA) (azficel-T (LAVIV) part #DR01/RMS-5519v00). All human biopsy-derived cells and fibroblast lines were cultured in complete DMEM/F-12 media consisting of DMEM nutrient mixture/F-12 supplemented with 10% fetal bovine serum (FBS), 1× minimum essential medium nonessential amino acid, 1× Glutamax, and 100 IU/ml penicillin–streptomycin (all from Invitrogen/Gibco, Grand Island, NY, USA) and maintained at 37°C in a 5% CO_2 incubator. Culture media were changed every 2 days. Cells were allowed to expand to 80 to 90% confluency before passaging with 0.05% trypsin–ethylenediamine tetraacetic acid (Invitrogen) and replating at a 1:3 ratio. A large bank of early-passage HUF1 cells was cryopreserved in culture media supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA). All research adhered to National Academy of Sciences guidelines.

In vitro culture of stem cell lines

Human-1, human-2, and human-9 embryonic stem cell (ESC) lines were provided by the UCLA Broad Stem Cell Research Center-Stem Cell Core. Multiple integration iPSCs were derived as previously published [31]. The mRNA hiPSCs were derived using Stemgent's mRNA reprogramming factor set (Stemgent, San Diego, CA, USA). The adult pre-excision line (termed C-8, or pre-excised iPSC) and the adult post-excision line (termed 2.3, or post-excised iPSC), derived as explained below, were all initially maintained on 0.2% gelatin-coated six-well plates covered with 35,000 cells/cm^2 irradiated mouse embryonic fibroblasts (MEFs) (GlobalStem, Rockville, MD, USA) with standard ESC media consisting of DMEM/F-12 supplemented with 20% Knockout Serum Replacement, 1× Glutamax, 1× nonessential amino acid, 100 IU/ml penicillin–streptomycin (all from Invitrogen), 1× β-mercaptoethanol (Millipore, Billerica, MA,

USA), and 10 ng/ml recombinant human basic fibroblast growth factor (Globalstem). All cells were transitioned into a feeder-free system and subsequently maintained on reduced growth factor Matrigel (BD Biosciences, San Jose, CA, USA) in mTeSR1 medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 10 ng/ml basic fibroblast growth factor (Globalstem) and 1× Primocin (InvivoGen, San Diego, CA, USA). Media were changed daily. Cells were passaged every 4 to 5 days, depending on colony density and size. Differentiation was removed daily from colonies using pulled glass pipettes. To passage the pluripotent stem cells, an 18-gauge needle was used to cross-hatch colonies in a grid format, with subsequent gentle agitation to remove the pieces with a P200 pipette. Usually, 4 to 8 colonies were passaged onto freshly coated Matrigel plates.

Lentivirus production and infection

For pre-excised and post-excised iPSC lines, lentiviral human STEMCCA vector was synthesized and packaged as published [15] and was concentrated to 100×. The day before infection, 100,000 cells/well were plated in a six-well plate grown in standard DMEM/F-12 media without antibiotics. On the day of transduction, 100× lentiviral supernatant was thawed, and 2 ml MEF conditioned media from each well of fibroblasts to be infected was taken out and mixed with 2× and 4× viral supernatant concentrations, respectively, with 8 μg/ml polybrene (Millipore). This virus-containing mixture was quickly added to the cells to avoid drying, shaken gently, and placed at 37°C in a 5% CO_2 incubator overnight. From day 2 through day 6, media were changed every day with DMEM/F-12 medium with antibiotics. Irradiated xCF1 fibroblasts harvested from day 8 mouse embryos were plated on day 6, and 50,000 and 100,000 cells from one well in a six-well plate were plated on day 7 onto an MEF-plated 10-cm plate and left to sit at 37°C in a 5% CO_2 incubator overnight. The next day, MEF media were replaced with human ESC medium for the duration of the reprogramming and changed daily. Colonies were picked

on the parental plate when colonies reached the size of 60 to 70% of 5✕ field view or became three-dimensional/differentiated into cell aggregates. Each parental colony was cut into two or three pieces and seeded onto a 24-well plate preseeded with xCF1 mouse feeders, one clone per well. Colonies were grown and further subcloned out according to optimal growth and colony morphology (flattened, very little differentiation, and high nucleus-to-cytoplasm ratio) and when colonies reached 60 to 70% of 5✕ field. Subcloning into a 12-well plate required 8 to 10 pieces from each clone per well from a 24-well plate be placed into an xCF1 MEF precoated 12-well plate. The pieces were then eventually subcloned out to a six-well plate for further characterization.

Vector integration site analysis by nonrestrictive linear amplification PCR

DNA was isolated from iPSCs using the PureLink Genomic DNA Mini Kit (Invitrogen). Approximately 100 ng genomic DNA was used to perform nonrestrictive linear amplification (nrLAM) PCR [33]. Briefly, 100 cycles of linear amplification were performed with primer HIV3linear (Biotin-agtagtgtgtgcccgtctgt). Linear reactions were purified using 1.5 volumes of AMPure XP beads (Beckman Genomics, Indianapolis, IN, USA) and captured onto 100 µg of M-280 Streptavidin Dynabeads (Invitrogen Dynal), prepared in accordance with the instructions of the manufacturer. Captured ssDNA was ligated to read 2 linker (Phos-agatcggaagagcacacgtctgaactccagtcac-3C Spacer) using CircLigase II (Epicentre, Madison, WI, USA) in a 10 µl reaction at 65° for 2 hours. PCR was performed on these beads using primer HIV3right (aatgatacggcgaccaccgagatctacactgatccctcagaccctttttagtc) and an appropriate indexed reverse primer (caagcagaagacggcatacgagat-index-gtgactggagttcagacgtgt). PCR products were mixed and quantified by probe-based quantitative PCR, and appropriate amounts were used to load Illumina v3 flow cells (Illumina, San Diego, CA, USA). Paired-end 50-base-pair sequencing was performed on an Illumina HiSeq 2000 instrument using a custom read 1 primer

(ccctcagacccttttagtcagtgtggaaaatctctagca). Reads were aligned to the hg19 build of the human genome with Bowtie [34], and alignments were condensed and annotated using custom Perl and Python scripts to locate vector integrations.

Infection of induced pluripotent stem cells with adeno-Cre

Excision of STEMCCA was performed by transient transduction of a defective adenoviral vector expressing Cre-recombinase-puromycin (Adeno-Cre-puroR), which was generated by Vector BioLabs (Philadelphia, PA, USA) to express Cre recombinase and puromycin resistance, into the parental pre-excised iPSC line. We used 45 and 5 µl concentrated Adeno-Cre-puroR virus with 8 µg/ml polybrene (Millipore) in standard ESC media for 24 hours. After 24 hours (on day 1), the mixed viral supernatant was removed, and the cells were washed twice with ESC media and then cultured in fresh ESC media containing 2 µg/ml puromycin (Invitrogen) for a period of 5 days. Individual colonies still growing after 5 days were subcloned into 12-well plates and expanded as described above.

Genomic and RT-PCR analysis

Genomic DNA was isolated from pluripotent stem cells (PSCs) grown in feeder-free conditions with the PureLink Genomic DNA Mini Kit (Invitrogen) in accordance with the instructions of the manufacturer. PCR was performed using the KAPA HiFi Hotstart ReadyMix PCR kit (KAPA, Woburn, MA, USA) with a five-step PCR protocol as follows: initial denaturation at 95°C for 3 minutes; 35 cycles of each of the following: denaturation at 98°C for 20 seconds, primer annealing at 62°C for 15 seconds, and extension at 72°C for 15 seconds; followed by a single cycle final extension at 72°C for 3 minutes. Ten nanograms of template DNA were used. Primers specific for exogenous integrations of the STEMCCA lentivirus are listed as follows: gDNA-hendo-MycS-forward, 5′-acgagcacaagctcacctct-3′; gDNA-hWPRE-reverse, 5′-tcagcaaacacagtgcacacc-3′. gDNA PCR was normalized to beta-actin: gDNA-hACTB-forward, 5′-

ggagaatggcccagtcctc-3′; and gDNA-hACTB-reverse, 5′-ggtctcaagtcagtgtacagg-3′ [20]. Total RNA was isolated using PSCs grown only on feeder-free conditions to prevent MEF mRNA contamination issues with Roche's High Pure RNA Isolation Kit in accordance with the instructions of the manufacturer (Roche, Indianapolis, IN, USA). Then 700 ng PSCs and 300 ng all fibroblast lines' RNA were reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit, using anchored-oligo(dT)$_{18}$ and random hexamer primers (Roche). PCR was performed using the KAPA HiFi Hotstart ReadyMix PCR kit (KAPA) with a five-step PCR protocol: initial denaturation at 95°C for 5 minutes; 28 cycles of each of the following: denaturation at 98°C for 20 seconds, primer annealing at 64°C for 15 seconds, and extension at 72°C for 15 seconds; followed by a single cycle final extension at 72°C for 5 minutes. In total, 75 ng RNA was used per reaction, and 12 μl with 3 μl loading dye was loaded into a 3% agarose gel in accordance with the recommendations of the manufacturer. Primers specific to exon 4/5 splice junction analysis were: RT-hexon4/5-forward, 5′-tgagcatgctgttctagctgcagga-3′; and RT-hexon4/5-reverse, 5′-accaggaggaccatcatcaccac-3′. RT-PCR gene expression was normalized to beta-actin: RT-hACTB-forward, 5′-ggagaatggcccagtcctc-3′; and RT-hACTB-reverse, 5′-ggtctcaagtcagtgtacagg-3′.

Global transcriptional meta-analysis

Pre-excised and post-excised iPSCs were grown in standard feeder-free culture conditions as stated above and harvested for total mRNA using a High Pure RNA Isolation Kit in accordance with the instructions of the manufacturer (Roche). Microarray analysis was carried out as published[35]. Affymetrix data adhered to the standards proposed by the Functional Genomics Data Society and were deposited in a MIAME-compliant format into the Gene Expression Omnibus [36] [GEO:GSE48830]. Each CEL file was uploaded to GeneSifter (VisX Labs, Seattle, WA, USA) using the Advanced Upload Method and normalized using the Affymetrix Microarray

Analysis Suite (MAS) 5.0 (Santa Clara, CA, USA) algorithm. GeneSifter pairwise analysis between samples was performed using all mean normalization and *t*-test statistical analysis (*P* <0.05). For each pairwise analysis, two replicates from each cell line were compared. Probe sets were considered significantly different when *P* <0.05 and fold change ≥2.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using PSCs grown only on feeder-free conditions to prevent MEF mRNA contamination issues as stated above. Primers and probes were designed and ordered from Roche's Universal ProbeLibrary. Quantitative PCR relative expression experiments used a LightCycler 480 Real-Time PCR System (Roche), and data were further analyzed with LightCycler 480 Software release 1.5.0. Primers for the genes are listed as follows – primers specific for pre-loxP site analysis: QRT-h*PRPF39*-forward, 5′-caggattttacaggctgggta-3′ and QRT-h*PRPF39*-reverse, 5′-tcctggcagccatcaagt-3′, probe #2; QRT-hPOU5F1-forward, 5′-gaagttaggtgggcagcttg-3′ and QRT-hPOU5F1-reverse, 5′-tgtggcccaaggaatagt-3′, probe #13; QRT-hSOX2-forward, 5′-gggggaatggaccttgtatag-3′ and QRT-hSOX2-reverse, 5′-gcaaagctcctaccgtacca-3′, probe #65; QRT-hNANOG-forward, 5′-cagtctggacactggctgaa-3′ and QRT-hNANOG-reverse, 5′-cacgtggtttccaaacaaga-3′, probe #55; and gene expression was normalized using HPRT1 and GAPDH primers: QRT-hHPRT1-forward, 5′-tgaccttgatttattttgcatacc-3′ and QRT-hHPRT1-reverse, 5′-cgagcaagacgttcagtcct-3′, probe #73; and QRT-GAPDH-forward, 5′-gctctctgctcctcctgttc-3′ and QRT-GAPDH-reverse, 5′-acgaccaaatccgttgactc-3′, probe #60. Five nanograms per sample were used in a 20 µl reaction that consisted of 10 µM UPL probe, 2× LightCycler 480 Probes Master, and 20 µM forward and reverse primers. Triplicate experimental samples were paired using the all-to-mean pairing rule with two housekeeping genes run in duplicate for advanced relative quantification.

Sequencing

Total RNA was extracted as stated above and amplified with the hexon 4/5 primers and purified with a PCR purification kit (Qiagen, Valencia, CA, USA). Samples were sent for full-service sequencing at UCLA's Genotyping and Sequencing Core (Los Angeles, CA, USA) using Invitrogen/Applied Biosystems 3730 Capillary DNA Analyzers, and sequence results were analyzed on ApE by (M. Wayne Davis; [37]).

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde/1× PBS for 15 minutes, washed twice with 1× PBS supplemented with 100 mM glycine for 5 minutes, and then incubated, when needed, with permeabilization buffer consisting of 0.1% Triton X-100 (Sigma-Aldrich) in 1× PBS for 30 minutes at room temperature. Blocking was performed with 4% goat serum in Blocker Casein in PBS (Thermo Scientific, Rockford, IL, USA) for 60 minutes at room temperature. The cells were then incubated for 2.5 hours with primary antibody at room temperature. Cells were washed with PBS after primary antibody staining and following each subsequent step. Following primary antibody incubation, the coverslips/wells were incubated with Alexa Fluor secondary antibodies (Invitrogen) at room temperature for 1 hour and mounted in Prolong Gold with 4′,6-diamidino-2-phenylindole (Invitrogen). Cultures were visualized with an AxioCam MR Monocolor Camera and AxioVision Digital Image Processing Software (Axio Observer Inverted Microscope; Carl Zeiss, Jena, Germany).

The primary antibodies used for PSC characterization are mouse anti-Oct-3/4 (C-10) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat anti-SSEA-3 (1:200; Millipore), mouse anti-SSEA-4 (1:200; Millipore), mouse anti-TRA-1-60 (1:200; Millipore), mouse anti-TRA-1-81 (1:200; Millipore), and rabbit anti-NANOG (1:100; Abcam, Cambridge, MA, USA) [32]. For

oligodendrocyte progenitor and oligodendrocyte cells, the following primary antibodies were used: mouse anti-NG2 (1:25; eBioscience, San Diego, CA, USA), rabbit anti-PDGFRα (1:20; Abcam), rabbit anti-SOX10 (1:20; Abcam), mouse anti-OLIG1 (1:200; Millipore), mouse anti-A2B5 (1:50; Millipore), mouse anti-O4 (1:40; R&D Systems, Minneapolis, MN, USA), mouse anti-O1 (1:40; R&D Systems), and rat anti-Myelin Basic Protein (1:40; Abcam). To analyze oligodendrocyte and neuronal co-culture, and to ensure oligodendrocyte human origin, rabbit anti-TUJ-1 (1:2500; Covance, Inc., Emeryville, CA, USA) and mouse anti-human mitochondria (1:40; Millipore) antibodies were used, respectively. For hepatocyte cells, the following primary antibodies were used: mouse anti-CK18 (1:50; Dako, Carpinteria, CA, USA), mouse anti-serum albumin (1:50; R&D Systems), and mouse anti-alpha-fetoprotein (1:100; Invitrogen). For cardiomyocytes, the following primary antibodies were used: mouse anti-Troponin I (1:50; Millipore) and mouse anti-alpha-actinin (Sarcomeric) (1:100; Sigma-Aldrich). For fibroblast differentiation, the following primary antibody was used: mouse anti-COL3A1 (1:40; Santa Cruz Biotechnology).

Induced pluripotent stem cell-directed differentiation

For oligodendrocyte progenitor and mature oligodendrocyte differentiation, embryoid bodies (EBs) were made on day 1 by 1 mg/ml collagenase treatment for 10 minutes, followed by gentle scraping with a 5-ml serological pipette. Detached colonies were collected and transferred to low-adhesion plates (Sigma-Aldrich) in a 50:50 combination of mTeSR1 and Glial Restrictive Media and differentiated as published [38]. For co-culture experiments, rat dorsal root ganglion (DRG) neurons were dissected and cultured as previously described, except for the substitution of rat DRG neurons [39]. DRG neurons were cultured on Matrigel (BD Biosciences) for a period of 7 days before post-excised derived oligodendrocyte progenitor cells were plated on top of the DRG neurons at a density of 15,000 cells/well in a 24-well plate. All cells were cultured in Glial

Restrictive Media. Co-cultured cells were cultured for a period of 7 days before fixation and immunostaining.

For EB-directed beating cardiomyocyte differentiation, post-excised iPSCs were incubated with 1 mg/ml collagenase for 10 minutes and then quenched with standard differentiation media consisting of standard DMEM as listed above but with 20% FBS and also with inclusion of 50 µg/ml ascorbic acid (Sigma-Aldrich), followed by making strips of iPSCs with a 5-ml serological pipette and subsequent placement into low-adhesion plates (Sigma-Aldrich). Media were changed every day with fresh media until day 5, when EBs were plated onto 0.2% gelatin-coated plates. The FBS concentration was reduced to 5% on day 10, and media were changed every 4 to 5 days with fresh ascorbic acid [40].

For non-EB-directed cardiomyocyte differentiation, post-excised iPSCs cultured on Matrigel were changed to DMEM/F-12 (Invitrogen) supplemented with 1× N2, 2 mM L-glutamine, 1 mM nonessential amino acid, 1× B27 supplement (all from Invitrogen), 0.5 mg/ml bovine serum albumin (Fraction V; Sigma-Aldrich), and 0.11 mM 2-mercaptoethanol (Millipore) (N2/B27-CDM) supplemented with 50 ng/ml recombinant human BMP-4 and 50 ng/ml recombinant human activin A (both from PeproTech, Rocky Hill, NJ, USA) for 3 or 4 days and cultured in N2/B27-CDM without additional factors for an additional 8 to 10 days. The medium was changed daily [41].

For hepatocyte differentiation, post-excised iPSCs were grown on Matrigel as stated above until reaching a 60 to 70% confluence upon which endoderm induction was initiated by replacing the post-excised iPSCs for 24 hours with RPMI 1640 medium (Invitrogen/Gibco, Rockville, MD, USA), supplemented with 0.5 mg/ml albumin fraction V (Sigma-Aldrich), and 100 ng/ml Activin A (PeproTech). On the following 2 days, 0.1 and 1% insulin–transferrin–selenium

(Invitrogen/Gibco) were added to the medium, respectively. Post-excised iPSCs were then cultured in hepatocyte culture medium (Lonza, Walkersville, MD, USA) containing 30 ng/ml fibroblast growth factor-4 and 20 ng/ml BMP2 (PeproTech) for 4 days. The now-differentiated cells were then incubated in hepatocyte culture medium containing 20 ng/ml hematopoietic growth factor and 20 ng/ml keratinocyte growth factor (PeproTech) for 6 days, in hepatocyte culture medium containing 10 ng/ml oncostatin-M (R&D Systems) plus 0.1 μM dexamethasone (Sigma-Aldrich) for 5 days, and in DMEM containing N2, B27, 1× Glutamax, 1× nonessential amino acid, and 1× β-mercaptoethanol (all from Invitrogen/Gibco) for 3 more days. Media were changed daily during differentiation [42].

For fibroblast differentiation, EBs were cultured in adherent conditions on 0.2% gelatin using standard fibroblast media with 10% FBS and were passaged until typical fibroblast morphology was seen [43].

Karyotype analysis

Post-excised iPSCs were passaged onto a 25-cm^2 flask to 60 to 70% confluency and sent out for G-band karyotyping analysis (Cell Line Genetics, Madison, WI, USA).

Teratoma formation

Teratomas for the pre-excised and post-excised iPSC lines were generated by injecting 8 × 10^{6}cells resuspended in Hanks' balanced salt solution (Invitrogen) into the two testes in a severe combined immunodeficient adult male beige mouse. All tumors were dissected 6 to 8 weeks after injection and fixed in 4% formaldehyde, and sections were paraffin-embedded and then stained with H & E for further analysis at the UCLA Translational Pathology Laboratory. All

animal experiments were performed in accordance with the UCLA Animal Research Committee and the UCLA Division of Laboratory Animal Medicine.

Good manufacturing practice conversion and analysis

Post-excised iPSCs were slowly transitioned from mTeSR1 media conditions to a 1:1 ratio of mTeSR1 and NutriStem (Stemgent) and finally to a 1:1 ratio of TeSR2/NutriStem (STEMCELL Technologies, Vancouver, BC, Canada) supplemented with 1× Primocin (InvivoGen) and 1× basic fibroblast growth factor (GlobalStem), which are both defined xeno-free media (containing no animal proteins). This conversion used 0:100, 20:80, 50:50, 80:20, and 100:0 mTeSR1/NutriStem:TeSR2/NutriStem ratios, with each condition lasting for 3 days. Regular passaging was maintained every 4 or 5 days based on cell morphology and density. Once cells were converted to the 1:1 TeSR2/NutriStem, the cells were mechanically passaged with an 18-gauge needle in the presence of 1× ROCK inhibitor (Stemgent), preconditioned in the media for 1 hour, and then transferred to a xeno-free substrate (Synthemax; Sigma-Aldrich). Cells were initially fibroblastic in nature, and continual differentiation of the iPSCs had to be taken out with a hand-pulled glass pipette. Specific selection of proper iPSC colonies over a period of 2 or 3 weeks generated morphologically homogeneous and standard-looking iPSCs. Cells that were converted to xeno-free conditions were then transferred to the UCLA good manufacturing practice (GMP)-compatible facility and underwent extended cultivation (for over 3 months) under xeno-free conditions. The cells were then subjected to standardized quality-control testing to ensure viability, sterility, and appropriate cellular composition, which included immunocytochemical analysis of stem cell markers, confirmation that the cells were free from nonhuman contaminants, including bacteria, fungi, mycoplasma or sialic acid (Neu5Gc) contamination, and confirmation they possessed a normal karyotype, and were cryobanked for potential future clinical applications as previously described [31]. To further show the broad

applicability of our slow transition methodology across media and synthetic matrices, we also converted the post-excised cells to a fully defined, synthetic matrix called CELLstart (Invitrogen) and cultured in NutriStem media alone.

Flow cytometry-based detection of sialic acid contamination

Flow cytometry was performed on the BD LSRII flow cytometer and all data were analyzed with BD FACSDiva Version 6.1.3 Software (BD Biosciences). The cell surface expression of nonhuman sialic acid Neu5Gc (*N*-glycolylneuraminic acid) was detected utilizing the chicken anti-Neu5Gc IgG (1:200) (Sialix anti-Neu5Gc Basic Pack Kit; Sialix San Diego, CA, USA) and labeled with FITC-conjugated donkey anti-chicken IgG (H + L) (1:200; Jackson ImmunoResearch, West Grove, PA, USA). 4',6-Diamidino-2-phenylindole (Invitrogen) was included as previously published [35]. Standard conditions and experimental controls were performed as per manufacturer recommendations (Sialix). hiPSCs that were derived and maintained under xeno-free clinical grade conditions and mouse embryonic fibroblasts (Globalstem) served as negative and positive controls, respectively. Additionally, post-excised iPSCs in mTeSR1 plated on Matrigel and post-excised iPSCs in xeno-free NutriStem plated on CELLstart were utilized for this assay.

Statistical analysis

Results are presented as means ± standard deviations. The statistical significance of differences for *PRPF39* gene expression was evaluated using SPSS 20 (IBM Corporation, Chicago, IL, USA). Analysis of variance, a *t* test for independent samples, and Kruskal–Wallis nonparametrical one-way analysis of variance tests were considered statistically significant with P <0.05.

Results

Induced pluripotent stem cell generation and characterization

Previous work has shown that adult somatic human dermal fibroblasts can be efficiently reprogrammed into iPSCs through exogenous expression of four transcription factors (OCT4, KLF4, c-MYC, and SOX2) with a single polycistronic lentivirus, or STEMCCA, flanked by loxP sites (hSTEMCCA-loxP) [16]. We reprogrammed low-passage adult human dermal fibroblasts through transduction of hSTEMCCA. To induce reprogramming, 1×10^5 fibroblasts were transduced with a constitutively active hSTEMCCA-loxP. From these 100,000 cells, 60 colonies with ESC-like morphology were observed, providing a reprogramming efficiency of just over 0.05%, an efficiency which parallels that seen in the literature (Table 1). Twenty colonies were picked and iPSC lines were derived. The 16 iPSC lines with the best morphology were expanded and cryopreserved. Three iPSC lines were thawed and expanded for further analysis for this study. All three iPSC lines possessed typical human ESC-like morphology, including large nucleoli, a high nucleus-to-cytoplasm ratio, and tight compact colonies (Figure 1A). All three iPSC lines, which we define here as parental pre-excised C-3, C-8, and C-11, were originally cultured on MEF layers and standard ESC media conditions for over 20 passages, representing the most commonly used research-grade conditions for iPSC derivation and culture.

Nonrestrictive linear amplification PCR genomic mapping of integration into *PRPF39* and pre-excised induced pluripotent stem cell characterization

Third-generation lentiviruses are capable of integrating into the host genome of primitive human repopulating cells multiple times, initially seeming to limit the practicality of using these viruses for reprogramming (for personalized cellular therapeutics) and warranting the need for new

reprogramming methodologies that yield transductions with fewer copies per cell [44]. Optimization of the multiplicity of infection to between 0.1 and 10, however, recently demonstrated that over 94% of iPSC colonies had a single stable integration [16]. Extensive and site-specific genomic mapping to identify potential insertional mutagenesis and elucidate adverse gene expression effects is needed to establish therapeutically relevant and factor-free iPSC lines. To verify a single integrated STEMCCA line and specifically sequence and map the vector integration, nrLAM-PCR was used to analyze the vector-human genome location. Two lines (C3 and C8) demonstrated single intron-based integrations, and the third line (C11) demonstrated multiple integrations. The C3 iPSC line displayed one integration located in intron 5 of the lysosomal enzyme alpha-*N*-acetylgalactosaminidase (*NAGA*), and the C8 (pre-excised) iPSC line displayed one integration located in intron 4 of pre-mRNA-processing factor 39 (*PRPF39*), a protein known to interact with the spliceosome and play a role in pre-RNA processing [45]. Mutations in *NAGA* have been associated with Schindler disease [46], whereas mutations in *PRPF39* have not been correlated with any specific disease. We therefore focused our characterization and transcriptional analysis on the C8 line.

The C8 pre-excised iPSC line expressed the pluripotency markers alkaline phosphatase, NANOG, OCT4, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 as determined by immunocytochemistry (Figure 2A). Also, to demonstrate pluripotency, iPSCs were injected into the testes of a severe combined immunodeficient mouse. The pre-excised iPSC line successfully formed teratomas representative of all three germ layers: neural tube (ectoderm), gut epithelium (endoderm), and cartilage (mesoderm) (Figure 2B). These results demonstrate that our single integrated hiPSC line is pluripotent and able to contribute to representatives of all three germ layers.

Adeno-Cre-puro excision

We next sought to generate a factor-free line void of any exogenous transgenic factors by expression of a nonintegrating adenovirus expressing both Cre-recombinase and puromycin resistance for selection of post-excised iPSC colonies. C3 and C8 iPSC cells were transduced for 24 hours with the Adeno-Cre-PuroR adenovirus and exposed to puromycin for 5 days, and then colonies were picked to establish three subclones from each colony (C3 subclones 1.1, 1.2, and 1.3 and C8 subclones 2.1, 2.2, and 2.3) after 2 weeks of recovery growth. We determined that successful Adeno-Cre-mediated excision of hSTEMCCA-loxP reprogramming construct occurred in only subclone 2.3, now called the post-excised iPSC subcloned line, as determined through PCR of genomic DNA with primers against endo-Myc-s and A-WPRE (Figure 1B). Expanded post-excised iPSCs were re-exposed to puromycin for 5 days, resulting in 100% cell death of all subcloned colonies and demonstrating that the Adeno-Cre-PuroR did not integrate into the genome following excision. Following Cre-mediated excision, post-excised iPSCs displayed a stable, uniform human ESC-like morphology for over 10 passages on MEFs and maintained pluripotent markers (alkaline phosphatase, NANOG, OCT4, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) at a level comparable with that of the pre-excised iPSC line and control human ESCs (Figure 2A). Importantly, to avoid any MEF mRNA contamination issues in later applications, both the pre-excised and post-excised iPSC lines were transitioned into feeder-free conditions on Matrigel with mTeSR1 media. Post-excised cells also were able to maintain their pluripotency, as shown through their successful contribution to all three germ layers in teratoma formation (Figure 2B). Also, the post-excised iPSC line was able to maintain genomic stability for over 37 passages during the transition from pre-excised to post-excised hiPSCs as demonstrated by the normal karyotype maintained (Figure 2C). The completely factor-free post-excised iPSCs were therefore able to maintain pluripotency markers and a

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normal karyotype and to retain the ability to differentiate to representatives of all three germ layers in the teratoma assay.

PRPF39 gene expression and splicing analysis

Successful excision of the hSTEMCCA-loxP site [16,47] and specific loci mapping of the virus integration were reported [31], but this study did not examine gene expression and splicing analysis of a post-excised hiPSC line in detail with sensitive real-time RT-PCR. We therefore sought to investigate the differential gene expression due to the lentiviral integration of hSTEMCCA-loxP into the integrated gene (that is, _PRPF39_). Even after excision, approximately 200 base pairs of exogenous DNA from the inactive long terminal repeat (LTR) remain integrated into the genome in intron 4 of _PRPF39_. This finding emphasizes the importance of gene expression analysis [48]. First, we performed microarray analysis on the pre-excised and post-excised iPSCs, and like previous investigators [31] we found no statistically significant difference between the gene expression for the integrated gene (_PRPF39_) (data not shown). Next, we used quantitative PCR to analyze the expression of _PRPF39_ with exon-spanning primers across exons 2 and 3. The pre-excised iPSC line demonstrated a statistically significant increase in the expression of _PRPF39_ compared with the post-excised iPSC line and every other cell line tested (Figure 3A). We confirmed this higher expression by running three different fibroblast lines as controls and showing very low expression of _PRPF39_ in fibroblasts (Figure 3A). To investigate whether this expression difference was due to random insertional positional events in the genome, the multi-integration line iPSCs that had three integrations were also analyzed, and they yielded a nonstatistically significant difference with the post-excised iPSC line of _PRPF39_ gene expression. This suggests that the expression difference of the pre-excised iPSC line is specifically due to the insertion of the hSTEMCCA-loxP viral construct into the _PRPF39_ intron 4 and that, following excision, endogenous and homeostatic

levels of *PRPF39* gene resumes. To further confirm correct splicing patterns, primers spanning exon 4 and 5 were used, and proper splicing was confirmed throughout all of the lines tested, including both pre-excised and post-excised iPSC lines (Figure 3B). Also, to confirm the splicing product homogeneity and proper PCR amplification, all lines tested were sequenced and yielded identical sequences. Importantly, although aberrantly increased *PRPF39* expression is seen in the pre-excised iPSC line, the post-excised line was able to revert back to normal levels of expression, indicating that the leftover LTR region was being properly removed during splicing.

Differentiation into clinically relevant cell types

To determine whether our transgene-free iPSC line was capable of differentiating into therapeutically relevant cell types, four different cell lineages were derived. First, oligodendrocyte progenitor cells (which could prove useful for treatment of spinal cord injuries) were differentiated and shown to express characteristic oligodendrocyte progenitor cell markers using immunocytochemistry. Oligodendrocyte progenitors express A2B5, NG2, OLIG1, SOX10, PDGFRα, and O4 (Figure 4A). After *in vitro* maturation, O1 and myelin basic protein were detected, indicating that we derived functionally mature oligodendrocytes (Figure 4B) [38]. However, it is well known that *in vitro* maturation is inefficient and minimal myelin production is produced without addition of further cytokines [38,49]; thus oligodendrocyte progenitor cell maturation is normally presented *in vivo*, where three-dimensional myelination formation is easier to achieve [50]. Therefore, to increase the efficiency of the *in vitro* model, the oligodendrocyte progenitor cells were co-cultured with rat DRG neurons to show an enhanced ability of the oligodendrocytes to produce myelin and myelinate DRG axons; human mitochondria were also stained to prove no contamination of rat oligodendrocytes in the co-culture (Figure 4C). This is an important step in developing an *in vitro* co-culture system that can

allow oligodendrocytes to wrap around axons and display myelination capabilities. Second, functional hepatocytes were derived, as indicated by positive staining for glycogen synthesis following the periodic acid–Schiff test (which could prove useful for treatment of liver diseases such as urea cycle disorders). Cytokeratin 18, serum albumin, and alpha-fetoprotein also were localized with these hepatocytes (Figure 4D). Third, post-excised iPSCs were differentiated into fibroblasts (which could prove useful to generate large numbers of therapeutically useful autologous fibroblasts following gene correction, such as for Epidermolysis Bullosa) that stained positive for a characteristic fibroblast marker, Col3A1 (Figure 4E, left image), at levels comparable with those of control fibroblasts (Figure 4E, right image). Lastly, we derived cardiac myocytes [41] (which could be useful for treatment of heart disease) that were able to beat in culture [40] (see Additional file 1) and also stain positively for alpha-actinin and troponin1 (Figure 4F). Post-excised iPSCs therefore not only maintained pluripotency following hSTEMCCA-loxP excision and proper *PRPF39* expression levels, but also were able to differentiate into four functionally useful cell types that have direct therapeutic applications.

Transition from research-grade to putative clinical-grade induced pluripotent stem cells
We slowly transitioned our research-grade lines from a xeno-containing substrate and media to xeno-free conditions that maintained the pluripotent capability and functionality of the hiPSCs. Transitioning of post-excised iPSCs to xeno-free media conditions consisting of a 1:1 blend of NutriStem/TeSR2 was carried out over a period of 30 days, and this was considered a slow conversion methodology. After the post-excised iPSC line was stably passaging in the xeno-free media, the cells were passaged onto a xeno-free substrate called Synthemax and passaged multiple times, and only the best colonies were selected for each passage (Figure 5A). Next, we performed an extended cultivation (for more than 3 months) of our transgene-free iPSCs in defined xeno-free conditions (free from nonhuman serum, proteins, and cells) under current

GMP manufacturing facilities (inspected and licensed by the state of California) and used qualified defined reagents and a standardized protocol [51]. The cells were also subjected to standardized quality-control testing to ensure viability, sterility, and appropriate cellular composition, including expression of standard stem cell markers (*NANOG*, *OCT4*, and *SOX2*) as indicated by quantitative PCR analysis (Figure 5B). We also converted the post-excised line from Matrigel and mTeSR1 to xeno-free and chemically defined CELLstart and NutriStem media under the same slow transition methodology as that used for the Synthemax and mTeSR1/NutriStem conversion. We used a different xeno-free substrate, and NutriStem alone, to show the robustness of the methodology across substrates and reagents. Both GMP grade conversions (Synthemax and CELLstart) yielded comparable expression of standard pluripotency markers (Figure 5B). Additionally, the pre-converted and post-converted iPSCs were tested, through flow cytometry, for the sialic acid/*N*-glycolylneuraminic acid (Neu5Gc), indicative of a nonhuman animal product contamination [52]. The iPSC line, xHUF-1, which was derived under completely defined and xeno-free conditions, and MEFs, which are of mouse origin, were used to show the negative and positive specificity of the antibody towards Neu5Gc, respectively. As expected, the MEF cells and the GMP iPSC line were 98.8% and 0% positive for Neu5GC, respectively (Figure 5C). We found that the post-excised line kept on Matrigel and in mTeSR1 media still possessed significant Neu5Gc, detectable on 1% of cells, but that this Neu5Gc was subsequently completely lost during the GMP conversion process. We did not define whether this 1% Neu5Gc detection was attributed to contaminating nonhuman epitopes from the original MEFs, serum and/or Matrigel. Regardless, this Neu5Gc detection approach is a stringent assay to determine iPSCs and their derivatives are free from animal epitopes that could lead to an immunogenic response [53].

Discussion

In this study, we show successful derivation of hiPSCs from human adult somatic dermal fibroblasts that contain a single hSTEMCCA-loxP lentiviral integration. We used nrLAM-PCR technology to analyze both the number of integrations in each line and the site in the genome where the lentiviral provirus integrated. One pre-excised line was derived with a single integration found to map into intron 4 of *PRPF39* (a gene not associated with any disease). Following Adeno-Cre-PuroR-mediated excision, a factor-free line, termed post-excised iPSCs, was derived and propagated.

Because previous studies using the polycistronic human STEMCCA lentivirus did not analyze the expression and splicing patterns of an integrated and subsequently excised hSTEMCCA construct in detail, we sought to characterize the expression and splicing patterns of our post-excised iPSC line. Small inactive viral LTRs left in the genome are thought to cause a small risk of insertional mutagenesis [16]. A recent paper, however, argues that only transcriptionally active LTRs, and not transcriptionally inactive LTRs, are capable of forming myeloid tumors, even when multiple LTR copies are present [54]. Previous studies also showed that HIV-based vectors have a clear correlation between increased gene activity hotspots and integration site preference [28], although not specifically into transcriptional start sites as seen with retroviruses [54]. Therefore, despite the fact that oncogenic risk from an inactive LTR is low, the possibility of integration into a transcriptionally active location and gene is high, and therefore target gene expression and splicing data on the integration site are critical. Although we show abnormally increased gene expression in the pre-excised iPSC line, the gene expression levels were reduced to basal levels upon excision, and the post-excised line maintained a normal pluripotent stem cell phenotype. Fortunately, the post-excised iPSC line had proper splicing

of *PRPF39* mRNA, although this is probably due to the wild-type nonintegrated allele properly expressing *PRPF39*. It is important to show that the lentiviral integration does not cause dominant negative interactions with the wild-type allele, allowing normal expression. *PRPF39* gene expression was therefore increased, probably due to an enhancer element like the woodchuck post-transcriptional regulatory element coded by the lentivirus causing a post-transcriptional increase in gene expression [55]. If current safe-harbor criteria are expanded to include intron-based reprogrammed cells that have been characterized to demonstrate a normal post-excision integrated gene expression profile, such as the cells described in this study, this will increase the proportion of generated iPSC lines being considered for therapeutic applications and thereby increase the feasibility of iPSC-based therapeutics. We also demonstrated that the post-excised iPSC line was able to differentiate into multiple therapeutically important cell types, such as hepatocytes, cardiomyocytes, and oligodendrocyte progenitor cells [56].

Finally, we sought a regulatory path to convert these research-grade transgene-free hiPSCs into cells that could be used in future clinical therapeutics (clinical grade). This transition from research grade to clinical grade was previously performed for human ESCs initially derived in the presence of nonhuman serum, proteins, and cells [57]. Geron converted their research-grade ESCs to clinical-grade ESCs by extended cultivation of their cells in defined xeno-free conditions free from nonhuman serum, proteins, and cells under current GMP manufacturing facilities. These facilities involve clean-room suites that are inspected and licensed by the state of California and use of qualified defined reagents and a standardized protocol, followed by standardized quality-control testing. In this study, we used the same approach and converted our research-grade transgene-free iPSCs into putative clinical-grade iPSCs. We discovered that while a small percentage (1%) of the research-grade cells still demonstrated detectible

nonhuman sialic acid (which may induce an immunogenic response if these cells had been used for autologous cellular therapeutics [53]), the post-converted cells no longer demonstrated any detectible sialic acid, suggesting that these cells were now clean and could be used without risking an immunogenic response. However, several caveats must be kept in mind. First, the previous US Food and Drug Administration-approved conversion of research-grade human pluripotent stem cells to clinical-grade cells involved ESCs, not iPSCs, and it is not guaranteed that the same conversion criteria will apply for iPSCs. Second, the US Food and Drug Administration approval technically applied to one specific derivative (oligodendrocyte precursor cells) derived from the converted clinical-grade ESCs, and suggests that a separate US Food and Drug Administration approval would be required for each iPSC-derived, differentiated therapeutic product. How the US Food and Drug Administration will ultimately judge the clinical applicability of these iPSCs, their derivatives, and other future iPSC-based therapeutics initially derived under research-grade xeno-containing conditions remains to be determined.

Conclusions

In summary, we have demonstrated the derivation of a factor-free hiPSC line using a polycistronic human STEMCCA reprogramming virus. nrLAM-PCR-based genomic mapping showed that the line had a single integration into a relatively safe location in intron 4 of the *PRPF39* gene. We then demonstrated proper expression levels following excision of the viral construct, correct splicing patterns, differentiation of the post-excised iPSCs into therapeutically relevant cell lineages, and transition into putative clinical-grade conditions.

Abbreviations

DMEM: Dulbecco's modified Eagle's medium; DRG: Dorsal root ganglion; EB: Embryoid body; ESC: Embryonic stem cell; FBS: Fetal bovine serum; GMP: Good manufacturing practice; H &

E: Hematoxylin and eosin; hiPSC: Human induced pluripotent stem cell; iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast; miRNA: MicroRNA; nrLAM: Nonrestrictive linear amplification; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; PSC: Pluripotent stem cell; RT: Reverse transcription; STEMCCA: Stem cell cassette.

Table 2.1: Human iPSC Reprogramming Efficiencies from Human Dermal Fibroblasts								
	Lenti virus	Polycistronic STEMCCA Lentivirus	Retrovirus	Episomal/Mini circle	Micro-RNAs	Cell-permeable Proteins	Sendai Virus	mRNA
Efficiency (%)	.022%	.01-1.5%	<.01%-.02%	.003-.006%	.002%	.001%	.01-1%	>1%
Integrating	Yes	Yes	Yes	No	No	No	No	No
Reference(s)	[1]	[15, 18]	[54, 55]	[8, 9]	[11]	[12]	[13]	[14]

Figure 2.1
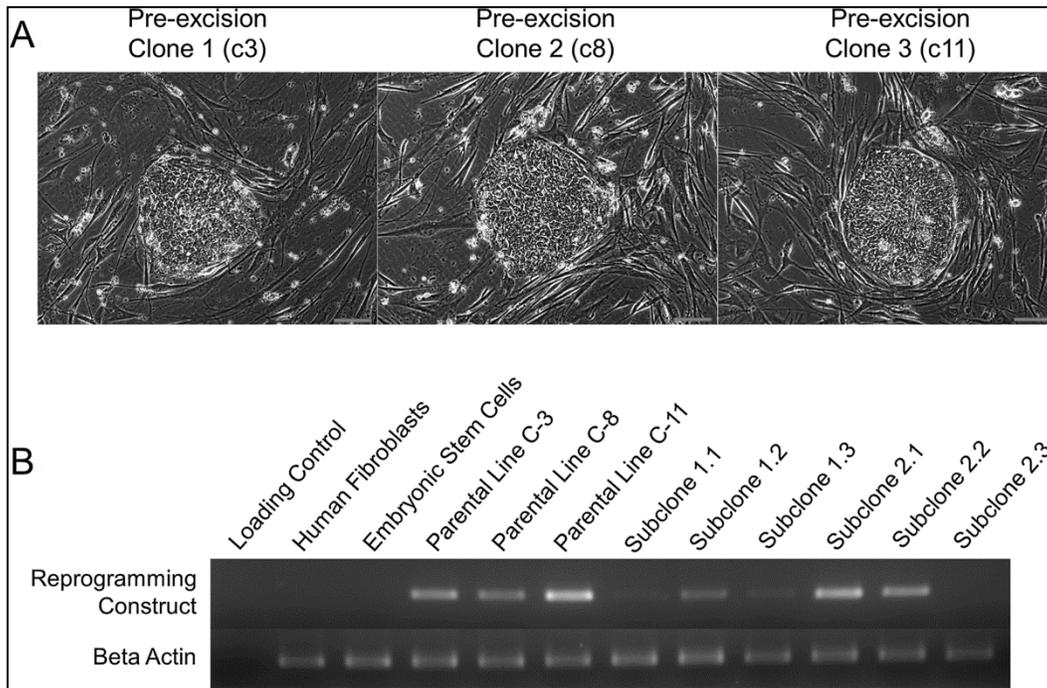
Figure 2.1. Representative colonies from the three hSTEMCCA-derived induced pluripotent stem cell lines. (A) Only the C8 pre-excised induced pluripotent stem cell (iPSC) line was found to have one integration into the *PRPF39* gene and was therefore selected to undergo Adeno-Cre-PuroR selection for removal of the cassette. **(B)** Excision of hSTEMCCA from the pre-excised iPSC adult parental line (C8). RT-PCR of genomic DNA with primers against hSTEMCCA elements endo-Myc-s and A-WPRE, showing that one subclone (2.3 post-excised iPSCs) was free of the integrated provirus. Bars = 100 µM.
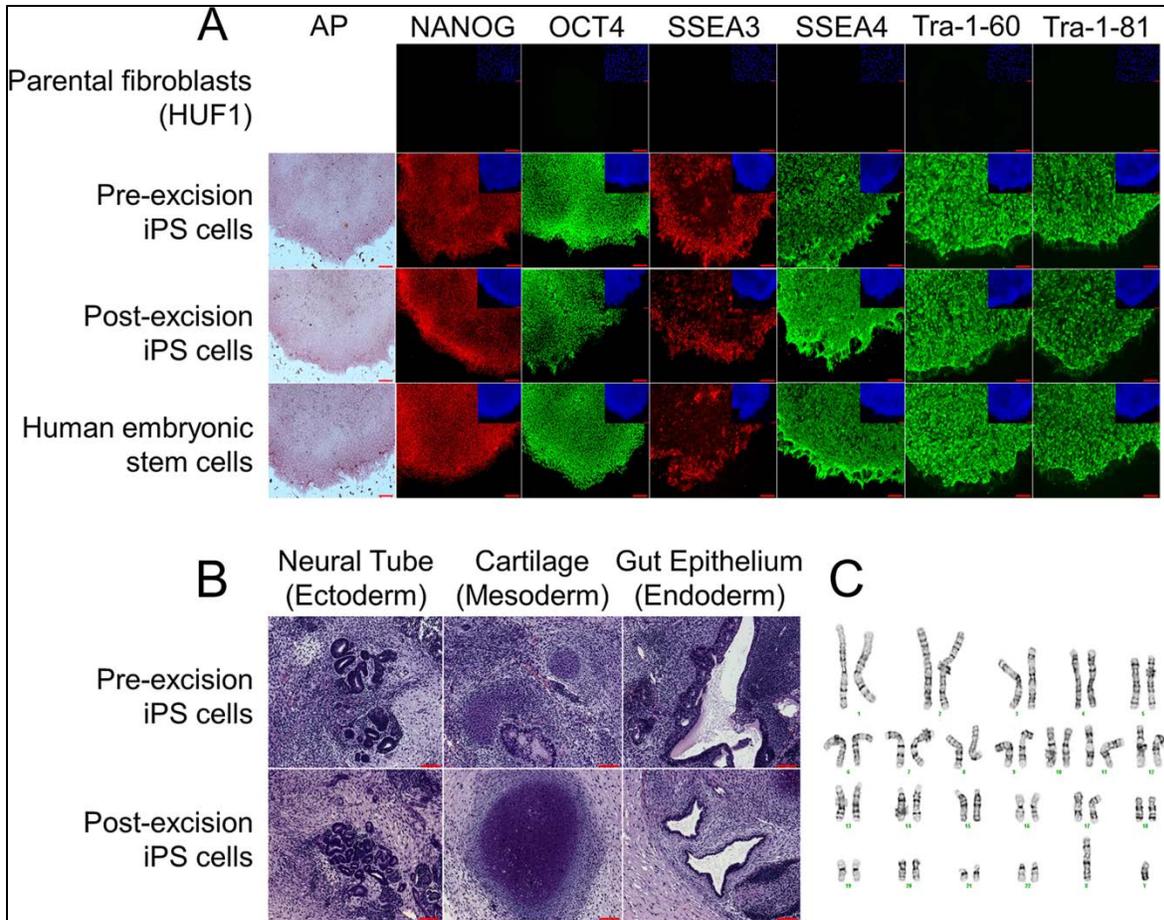
Figure 2.2

Figure 2.2. Characterization of pre-excised and post-excised induced pluripotent stem cell lines. (A) Expression of pluripotency markers from induced pluripotent stem cells (iPSCs) (human embryonic stem cells and parental fibroblasts from which the pre-excised iPSC line was derived serving as controls), showing similar expression of all markers before and after hSTEMCCA excision. **(B)** Histological analysis of teratomas derived from the pre-excised iPSC parental line and post-excised iPSC line. **(C)** G-band karyotyping analysis of the post-excised iPSC line, showing a normal 46XY karyotype following excision of hSTEMCCA. Bars = 100 µM.
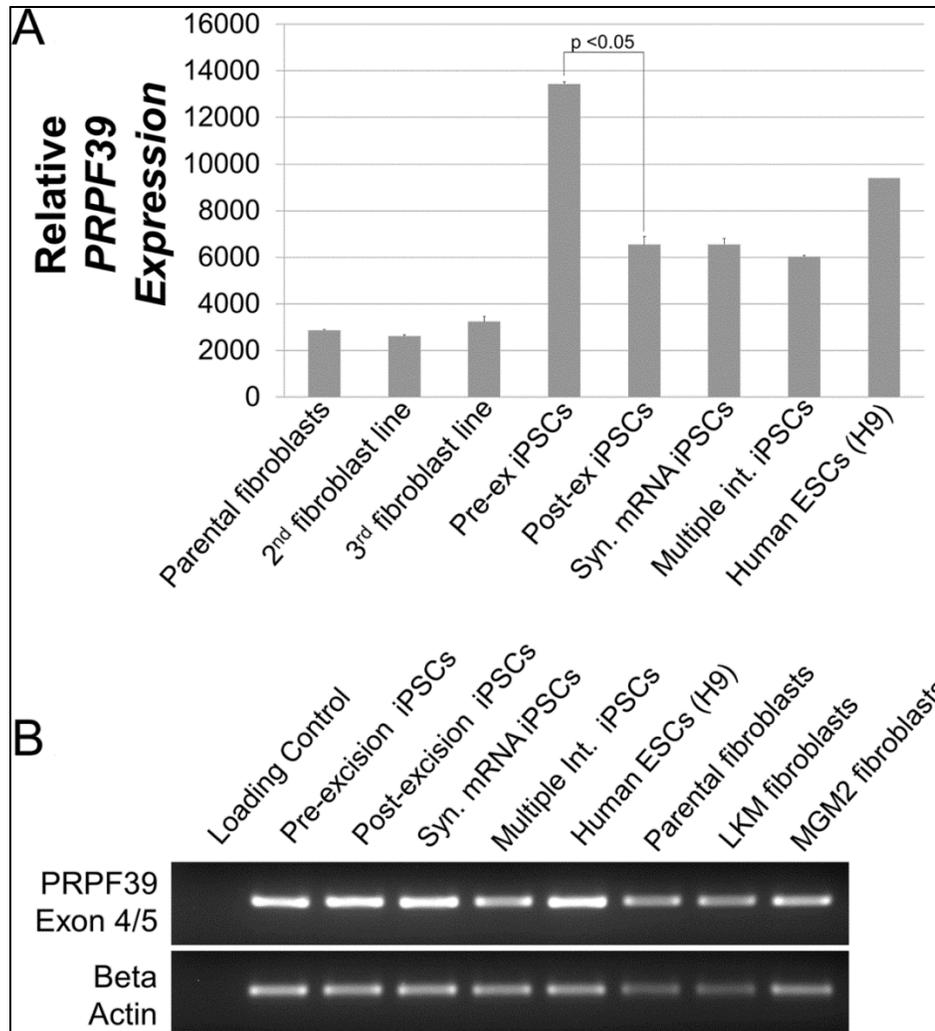
Figure 2.3

Figure 2.3. Expression and splicing analysis of the pre-excised and post-excised lines. (A) Quantitative PCR analysis showed that integration into the *PRPF39* gene caused a statistically significant increase in transcript expression, in which the increased expression was abrogated upon Adeno-Cre-PuroR-mediated hSTEMCCA excision. No statistical differences were seen between all control induced pluripotent stem cell (iPSC) lines, and all fibroblast lines displayed very low expression of *PRPF39*, indicating that this gene is associated with pluripotency. **(B)** To confirm that proper splicing of the transcript is taking place, primers against exons 4 + 5 show that no cryptic splice sites were being introduced and that, upon excision, the transcript correctly spliced itself. P <0.05. ESC, embryonic stem cell; Multiple Int., multiple integration; Syn., synthetic.

Figure 2.4
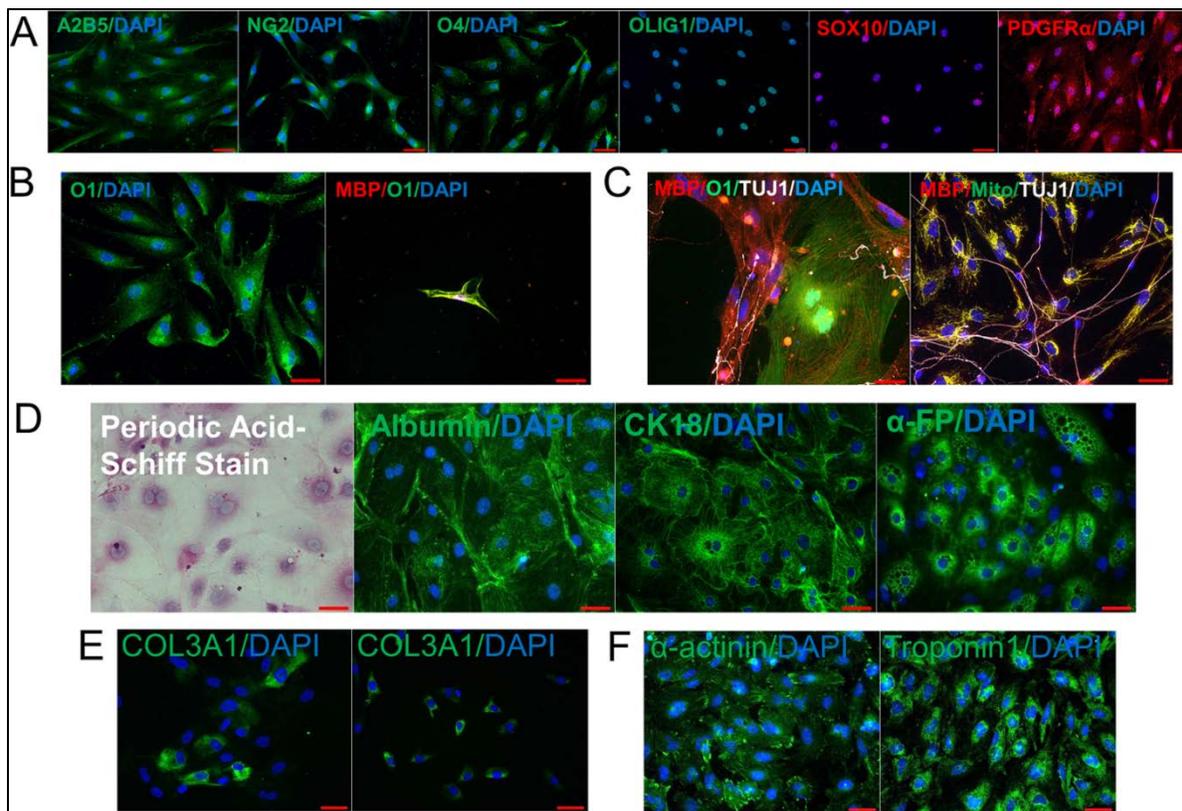
Figure 2.4. Differentiation into therapeutically relevant cell lineages. (A) Post-excised induced pluripotent stem cells (iPSCs) were differentiated into oligodendrocyte progenitor cells expressing characteristic progenitor stage markers. **(B)** Upon terminal differentiation, the progenitor cells matured and displayed the mature antigen O1 and also stained positive for secreting myelin basic protein (MBP), a hallmark of mature oligodendrocytes. **(C)** Due to low efficiency of *in vitro* oligodendrocyte maturation, a co-culture system with dorsal root ganglion neurons was utilized and showed mature oligodendrocytes intimately associated with, and myelinating, neurite outgrowths. Additionally, human mitochondria were stained to display that rat oligodendrocytes were not contaminating the culture. **(D)** Hepatocytes were derived that stained positively for glycogen synthesis as indicated by the periodic acid–Schiff stain, and CK18, albumin, and alpha-fetoprotein. **(E)** Derived fibroblasts stained positive for COL3A1 upon differentiation and at levels comparable with those of control fibroblasts (left picture is iPSC-derived fibroblasts and right are control fibroblasts). **(F)** Cardiomyocytes showed expression of alpha-actinin and Troponin 1. DAPI, 4′,6-diamidino-2-phenylindole. Bars = 50 μM.
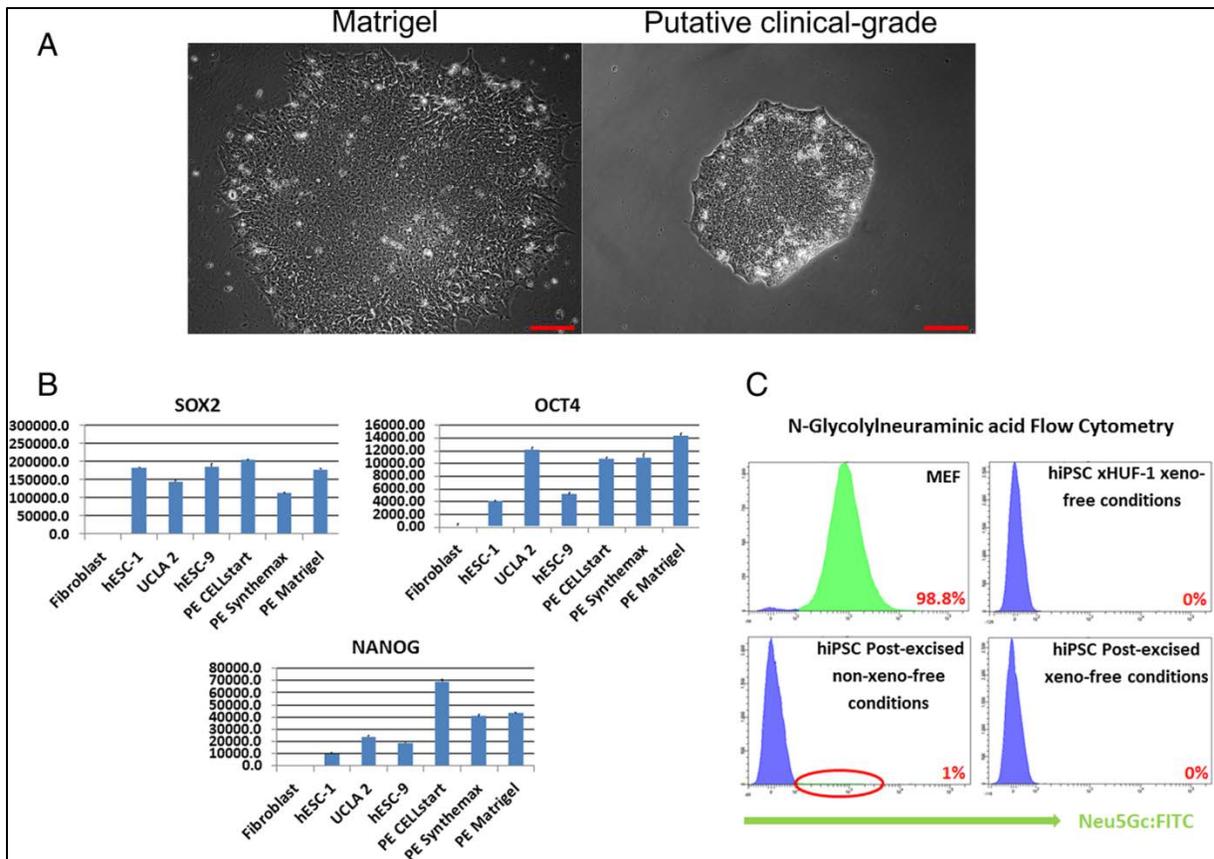
Figure 2.5

Figure 2.5. Morphology of transgene-free induced pluripotent stem cells following conversion to clinical-grade conditions. (A) Conversion of post-excised iPSCs from a xeno-containing substrate, Matrigel, to a xeno-free containing substrate, Synthemax, under current good manufacturing practice (GMP) conditions. **(B)** Quantitative PCR for pluripotency associated genes displays that pre-converted and post-converted induced pluripotent stem cells (iPSCs) retain normal expression levels across multiple synthetic substrates. **(C)** Beyond the standard GMP-grade sterility testing, a flow cytometry-based assay for a nonhuman antigen, *N*-glycolylneuraminic acid, displayed that upon GMP-grade conversion all sialic acid detection was eliminated (1% with post-excised cells on Matrigel down to 0% with post-excised cells in GMP conditions). An iPSC line derived under GMP conditions and mouse embryonic fibroblasts (MEFs) were used as negative and positive controls, respectively. hESC, human embryonic stem cell; hIPSC, human induced pluripotent stem cell; PE CELLstart, post-excised iPSC CELLstart; PE Synthemax, post-excised iPSC Synthemax; PE Matrigel, post-excised Matrigel.

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CHAPTER 3: BAY11 ENHANCES OCT4 SYNTHETIC MRNA EXPRESSION IN ADULT HUMAN SKIN CELLS

Abstract

Introduction

The *OCT4* transcription factor is involved in many cellular processes, including development, reprogramming, maintaining pluripotency and differentiation. Synthetic *OCT4* mRNA was recently used (in conjunction with other reprogramming factors) to generate human induced pluripotent stem cells. Here, we discovered that BAY 11-7082 (BAY11), at least partially through an NF-κB-inhibition based mechanism, could significantly increase the expression of OCT4 following transfection of synthetic mRNA (synRNA) into adult human skin cells.

Methods

We tested various chemical and molecular small molecules on their ability to suppress the innate immune response seen upon synthetic mRNA transfection. Three molecules - B18R, BX795, and BAY11 - were used in immunocytochemical and proliferation-based assays. We also utilized global transcriptional meta-analysis coupled with quantitative PCR to identify relative gene expression downstream of OCT4.

Results

We found that human skin cells cultured in the presence of BAY11 resulted in reproducible increased expression of OCT4 that did not inhibit normal cell proliferation. The increased levels of OCT4 resulted in significantly increased expression of genes downstream of OCT4, including the previously identified *SPP1, DUSP4* and *GADD45G*, suggesting the expressed OCT4 was functional. We also discovered a novel OCT4 putative downstream target gene *SLC16A9* which demonstrated significantly increased expression following elevation of OCT4 levels.

Conclusions

For the first time we have shown that small molecule-based stabilization of synthetic mRNA expression can be achieved with use of BAY11. This small molecule-based inhibition of innate immune responses and subsequent robust expression of transfected synthetic mRNAs may have multiple applications for future cell-based research and therapeutics.

Introduction

Early embryonic development creates an inner cell mass in the developing embryo that, after delamination into the epiblast, initially lends itself exclusively to pluripotent stem cells capable of differentiating into any of over 200 cell types of the human body. The gene expression and transcriptional network that are expressed and regulated are well characterized [1-4]. One of the key pluripotency factors, *OCT4*, a Pou class 5 homeobox 1 transcription factor known as POU5F1, is expressed in human embryonic stem cells (hESCs), induced pluripotent stem cells, early epiblast, and germ cells, including primordial germ cells [5,6]. This transcription factor has been implicated in key pluripotency maintenance functions in both early embryogenesis, including acting as a master regulator in segmentation morphology and organogenesis via activation of key downstream signaling pathways, and activating tissue-specific transcription factors [7]. Interestingly, it has been shown that precise levels of OCT4 are needed during development, as repression leads to loss of pluripotency and subsequent trophectoderm differentiation and overexpression lead to differentiation into primitive endoderm and mesoderm, respectively [8]. It is clear that OCT4 plays a critical function in human developmental biology, and its role has been well defined in that it associates with other pluripotency factors, *SOX2* and *NANOG*, whose mechanism to maintain a pluripotent phenotype involves upregulation and downregulation of over 4,600 genes through a protein network of these three proteins [9-11]. Thus, the delivery and stable expression of synthetic *OCT4* mRNA and other

synthetic mRNAs (synRNAs) may have multiple applications for future cell-based research and therapeutics.

The ability to reprogram easily obtainable human cells, such as skin cells, back into a pluripotent epigenetic state provides exciting new possibilities for *in vitro* research and patient-specific cellular therapeutics to regenerate our bodies following injury, disease, and age-based tissue degeneration[12]. However, the most promising method for reprogramming human somatic cells back into a pluripotent state - referred to as induced pluripotent stem cells - uses viruses to deliver the reprogramming factors (*OCT4, SOX2* combined with *KLF4* and *cMYC* or with *NANOG* and *LIN28*) into human somatic cells [13,14]. As these viruses randomly integrate into the genome, insertional mutagenesis is an important safety concern [15-17]. Alternatives to integrating DNA virus-based reprogramming include the use of episomal plasmids [18] and minicircles [19], protein-based reprogramming [20], and Sendai virus-based reprogramming [21]. Both of the episomal DNA-based reprogramming methodologies, however, still entail some risk of genomic recombination or insertional mutagenesis. The recombinant proteins used in protein-based reprogramming are challenging to generate and purify in the quantities required, and the RNA-based Sendai virus requires an extended period of culture in order to dilute out the viral particles [22]. Perhaps the most promising current integration-free reprogramming methodology for future patient-specific cellular therapeutics involves the direct transfection of RNAs into somatic cells (that is, synthetic whole mRNAs [23] or microRNAs [24] or both). SynRNAs encoding for five of the reprogramming factors (*OCT4, SOX2, KLF4, cMYC*, and *LIN28*) have been shown to reprogram human somatic cells back into a pluripotent state [23]. The most important of these delivered reprogramming factors is *OCT4*, as recent research has demonstrated that *OCT4*, in combination with certain small molecules, can itself

induce a somatic cell to reprogram to pluripotency without requiring assistance from the other factors [25].

Here, we examined the expression of synthetic *OCT4* mRNA following transfection into adult human skin cells, investigated whether various small molecules (B18R, BX795, and BAY11) could significantly increase synthetic *OCT4* mRNA expression, and used transcriptional analysis of OCT4 downstream genes to determine whether the OCT4 protein maintained its functionality as a transcription factor.

Materials and methods

Ethics statement

Written approval for human skin biopsy procedures and human fibroblast derivation, culture, and experimental use was obtained from the Stanford University Institutional Review Board, the Stanford University Stem Cell Research Oversight (SCRO) committee, and written informed consent was obtained from each individual participant. Biopsy material used in this study was obtained and initially analyzed at Stanford University, as previously described [26], and transferred to the University of California at Los Angeles (UCLA) through a material transfer agreement. Written approvals for the experiments performed in this study were obtained from the UCLA Institute Biosafety Committee and the UCLA SCRO committee.

In vitro culture of primary human skin cells

The human skin-derived (HUF1) primary cell line used in this study was obtained from a 4-mm adult skin punch biopsy and cultured as described [26]. Briefly, all human biopsy-derived cells were cultured in complete DMEM/F-12 media consisting of Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 1 ×

minimum essential medium (MEM) non-essential amino acids, 1 × Glutamax, and 100 IU/mL penicillin-streptomycin (all from Invitrogen Corporation/Gibco, Grand Island, NY, USA) and maintained at 37°C in a 5% CO_2 incubator. Culture media were changed every two days. Cells were allowed to expand to 80% to 90% confluency before passaging with 0.05% trypsin-EDTA (Invitrogen Corporation) and replating at a 1:3 ratio. A large bank of early-passage HUF1 cells was cryopreserved in culture media supplemented with 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). All research adhered to National Academy of Sciences guidelines.

In vitro culture of H9 human embryonic stem cells

H9 hESCs (UCLA Broad Stem Cell Research Center-Stem Cell Core) were cultured in standard ESC conditions as published [26]. Briefly, hESCs were cultured in medium consisting of DMEM/F-12 supplemented with 20% knockout serum replacement, 1 × Glutamax, 1 × non-essential amino acids, 100 IU/mL penicillin-streptomycin (all from Invitrogen Corporation), 1 × β-mercaptoethanol (Millipore Corporation, Billerica, MA, USA), and 10 ng/mL recombinant human basic fibroblast growth factor (Globalstem, Rockville, MD, USA) and maintained at 37°C in a 5% CO_2 incubator. Media were changed daily.

Cell proliferation analysis

BAY11 and BX795 were purchased from InvivoGen (San Diego, CA, USA) (catalog code tlrl-b82 and tlrl-bx7). Both inhibitors were diluted initially in DMSO to a stock concentration of 100 mM and stored in aliquots at −20°C. All further dilutions to working concentrations were carried out in filter-sterilized Millipore water. Fresh aliquots were used for each daily transfection. B18R recombinant protein was purchased from eBioscience (San Diego, CA, USA), aliquoted, and stored at −80°C. HUF1 cells were grown as described alone in complete DMEM/F-12 media.

For cell proliferation experiments, HUF1 cells were plated onto gelatin-coated six-well plates (Sigma-Aldrich) at a concentration of 50,000 cells (based on a doubling time of 34 hours) and left to sit for 24 hours to adhere in the presence of BAY11 or BX795 (as indicated in Figure 1), and this was considered day 0. Every day at the 24-hour mark, media were changed with fresh drug and B18R (when applicable). On day 5, cells were washed twice with 1 × phosphate-buffered saline (PBS) (Invitrogen Corporation) and cells detached with 1 mL per well of a six-well plate via 0.05% trypsin-EDTA for 5 minutes at 37°C in a 5% CO_2 incubator. Cells were then quenched with 4 mL of standard fibroblast culture media, centrifuged at 120g for 5 minutes, and resuspended in 1 mL of culture media along with Trypan blue stain (Invitrogen Corporation) for cell counting by using a standard hemocytometer. All cell counts were performed in quadruplicate.

Synthetic mRNA dilutions

For the initial 'mRNA pooling' experiments, synRNA of each of the five reprogramming factors was generated in-house, aliquoted, and stored at −80°C as previously described by Warren and colleagues [23]. Briefly, in accordance with the approach of Warren and colleagues, a 170:160:420:130:120 stoichiometric ratio corresponding to 'KMOSL' factors (which consisted of *KLF4*:*c-MYC*:*OCT4*:*SOX2*:*LIN28*, respectively) was used for the initial 'pooling' experiments. SynRNA stock concentrations for KMOSL were 1,200, 750, 1,500, 650, and 600 ng/µL, respectively, and were all diluted to 100 ng/µL stocks with Tris-EDTA pH 7.0. To pool these together with the same stoichiometric ratios used by Warren and colleagues, 121.4, 114.3, 300, 92.9, and 64.3 µL, respectively, were added together on ice and under sterile conditions, mixed, and immediately placed into 12-µL aliquots for storage at −80°C. Thus, each vial had a cocktail of the five factors at 100 ng/µL to yield 1,200 ng total per aliquot of synRNA, and the synRNA cocktails - except for *OCT4*, which was present at a 3 × molar concentration - were formulated to

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yield equal molarity. For the later *OCT4* mRNA experiments, only *OCT4* synRNA, obtained from a commercial vendor (Stemgent, San Diego, CA, USA), was used.

Synthetic mRNA transfection

All work was carried out in strict RNAse-free conditions. SynRNA was thawed on ice and quickly diluted before degradation. SynRNA was diluted 5 × by using Opti-MEM basal media (Invitrogen Corporation) with 12 µL of synRNA at 100 ng/µL placed into 48 µL of Opti-MEM. RNAiMAX was used at 5 µL per microgram of RNA - 1.2 µg of total synRNA and thus 6 µL of RNAiMAX - and was diluted 10 × by diluting 6 µL of RNAiMAX into 54 µL of Opti-MEM. Each dilution was separate, and the tube was mixed. The two dilutions were pooled, mixed, and incubated at room temperature for 15 minutes. This mixture was added directly into fibroblast culture media without antibiotics, as required by protocol. Final culture volumes in each well were 500 µL. The cells were left to incubate with the synRNA for 4 hours, after which the media were replaced with fresh BAY11 or BX795 along with B18R all in standard fibroblast media. For the *OCT4* mRNA single-factor assay, daily transfections were carried out as above onto 25,000 HUF1 cells plated into a 24-well plate by using synthetic *OCT4* mRNA (Stemgent) over the course of 5 days, with or without the addition of BAY11. B18R was not included during the single-factor transfections.

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde/1 × PBS for 15 minutes, washed twice with 1 × PBS supplemented with 100 mM glycine for 5 minutes, and incubated with permeabilization buffer consisting of 0.1% Triton X-100 (Sigma-Aldrich) in 1 × PBS for 30 minutes at room temperature. Blocking was performed with 4% goat serum in Blocker Casein in PBS (Thermo Scientific, Rockford, IL, USA) for 60 minutes at room temperature. Then OCT4 (C-10) mouse anti-human monoclonal IgG_{2b} 1:200 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA;

catalog code sc-5279) was added to 4% goat serum in Casein-PBS and incubated overnight at 4°C. The next day, cells were washed three times with 1 × PBS before Alexa Fluor 488 goat anti-mouse IgG 1:200 (Invitrogen Corporation) was added to 4% goat serum in Casein-PBS and incubated for 1 hour at room temperature. The cells were rinsed and stained with SlowFade Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen Corporation) for 10 minutes, followed by two 1 × PBS washes. Cultures were visualized with an AxioCam MR Monocolor Camera by using AxioVision Digital Image Processing Software (Axio Observer Inverted Microscope; Carl Zeiss, Jena, Germany). Three images per well were used for luminosity cell counts evaluated with Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). In each replicate, 10 cells located in the top left frame of the picture were characterized with the luminosity tool in Photoshop.

OCT4 quantification

The OCT4 stabilization assay was carried out via a 10 × dilution of the 100 ng/μL synRNA stock across 10 wells for a total of 120 ng per well in a 24-well plate. In total, 10,000 cells per well were plated, and all synRNA dilutions were carried out as stated above. Cells were cultured in standard fibroblast media, without antibiotics, overnight. The day after the initial plating, each combination of BAY11 or BX795, with or without B18R, was diluted directly into cell culture medium for 24 hours to precondition the medium. SynRNA was then transfected in every 24 hours with fresh replacement of media and drug for 5 days. OCT4 was then quantified via immunocytochemical analysis and analyzed as previously mentioned via immunocytochemical and luminosity measurements in Photoshop.

Global transcriptional meta-analysis

In total, 25,000 passage 5 HUF cells were plated into individual wells in a 24-well plate in standard DMEM/FBS media without antibiotics. Twelve hours later, the cells were incubated

with 1 µM BAY11 + 200 ng/mL B18R over two wells, whereas the other two wells received just B18R at 200 ng/mL final concentration. The final volume was 500 µL in each well. After 24 hours of incubation, the cells were transfected with the modified synRNA as detailed above. This was carried out every day for 3 days, and after the last incubation period of 24 hours, the cells were harvested for mRNA by using a Roche High Pure RNA Isolation Kit in accordance with the instructions of the manufacturer (Roche, Indianapolis, IN, USA). hESCs and control fibroblasts were harvested in the same manner. Microarray analysis was carried out as published [27]. Briefly, total RNA was used for an Affymetrix Differential Gene Expression Assay Human Genome U133 Plus 2.0 Array (Genoseq UCLA) for global transcriptional analysis by using standard Affymetrix protocols (Affymetrix GeneChip Expression Analysis Technical Manual, rev. 3. 2001). Uploading and cluster analysis of the CEL files between replicate samples were carried out through GeneSifter (VizX Labs, Seattle, WA, USA[28]) by using the Advanced Upload Method and were normalized by using the Affymetrix Microarray Analysis Suite (MAS) 5.0 algorithm. The following CEL files were used for this analysis: HUF1 cells transfected with synRNA but not treated with BAY11 (GSM994323 and GSM994324), HUF1 cells transfected with synRNA and treated with BAY11 (GSM994325 and GSM994326), HUF1 cells not transfected with synRNA and not treated with BAY11 (GSM994327 and GSM994328) and H9 human embryonic stem cells (GSM994321 and GSM994322). Data from control HUF cells were used as a baseline control to compare the replicates of HUF cells with or without BAY11 and hESCs. All experimental details for the microarray analysis, including all original CEL files, have been made publically available at the Gene Expression Omnibus [29] GSE40444.

Quantitative polymerase chain reaction analysis

Total mRNA that was harvested from the global transcriptional meta-analysis was also used in reverse transcription-polymerase chain reaction (RT-PCR) for quantitative PCR analysis. The RNA yield and quality were determined by using the GE NanoVue Spectrophotometer (GE Healthcare Life Sciences, Piscataway, NJ, USA). For hESCs and for all HUF lines, 1 µg and 180 ng of RNA, respectively, were reverse-transcribed by using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and anchored-oligo(dT)$_{18}$ and random hexamer primers. Quantitative RT-PCR (QPCR) relative expression experiments used a LightCycler 480 Real-Time PCR System (Roche), and data were further analyzed with LightCycler 480 Software release 1.5.0. Primers and probes were designed and ordered from Roche's Universal ProbeLibrary. Primers for the genes are listed as follows: Homo sapiens secreted phosphoprotein 1 (*SPP1*), transcript variant 2, mRNA (NM_000582.2) forward primer: cgcagacctgacatccagt, reverse primer: ggctgtcccaatcagaagg, probe #61; Homo sapiens solute carrier family 16, member 9 (monocarboxylic acid transporter 9) (*SLC16A9*), mRNA, (NM_194298.2), forward primer: gatgcctttggtgaaggaaa, reverse primer: cacagagactgcagacaggact, probe #64; Homo sapiens growth arrest and DNA-damage-inducible, gamma (*GADD45G*), mRNA, (NM_006705.3), forward primer: cagccaaagtcttgaacgtg, reverse primer: cctggatcagcgtaaaatgg, probe #71; *DUSP4*-001 dual-specificity protein phosphatase 4 (ENST00000240100.2), forward primer: tgcatcccagtggaagataac, reverse primer: gcagtccttcacggcat, probe #17; Homo sapiens hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), mRNA, (NM_000194.2), forward primer: tgaccttgatttattttgcatacc, reverse primer: cgagcaagacgttcagtcct, probe #73; *GAPDH*-001 glyceraldehyde-3-phosphate dehydrogenase (ENST00000229239.5), forward primer: gctctctgctcctcctgttc, reverse primer: acgaccaaatccgttgactc, probe #60. cDNA (5 ng) that was reverse-transcribed in the RT-PCR per sample was used in a 20-µL reaction that consisted of 10 µM UPL probe, 2 × LightCycler 480

Probes Master, and 20 µM forward and reverse primers. Duplicate experimental samples were paired by using the all-to-mean pairing rule with two housekeeping genes run in duplicate for advanced relative quantification.

Statistical analysis

Results are presented as mean ± standard deviation. The statistical significance of differences for cell proliferation analysis, immunocytochemical luminosity-based quantification, and QPCR results were evaluated by using Statistical Package for the Social Sciences (SPSS) 20 (IBM Corporation, Chicago, IL, USA). Analysis of variance, *t* test for independent samples, and Mann-Whitney *U* test were considered statistically significant at a *P* value of less than 0.05.

Results

Immunocytochemical detection of OCT4 demonstrating robust stabilization

In our initial attempts to repeat the reprogramming methodology of Warren and colleagues [23] for transfecting synthetic (syn) *OCT4* mRNA into human adult fibroblasts, we observed significant degradation (defined here by low-level and heterogeneous OCT4 expression) of the *OCT4* synRNA. Interestingly, this degradation appeared to be specific to *OCT4* and did not affect *SOX2, KL4, cMYC*, and *LIN28* (data not shown), but the reasons for this are not yet clear. No detectable OCT4 protein was observed in the untransfected human adult fibroblasts (HUF1) (Figure 1a). After transfection of 120 ng of synRNA (as reported by Warren and colleagues [23]), we saw broad heterogeneity and very low expression of OCT4 upon immunocytochemical analysis (Figure 1b). Secondly, addition of Vaccinia virus decoy receptor for type I interferons (IFNs), identified as B18R, resulted in no statistically significant increase in OCT4 expression (Figure 1c), and no significant increase was observed following the use of higher amounts of synRNA (Figure 1d), B18R (Figure 1e), or both (Figure 1f). These results

demonstrate that increasing synRNA concentration and mitigating IFN signaling via B18R are not sufficient to permit robust OCT4 expression from synRNA. Next, we analyzed the immune response pathways to identify alternative potential small-molecule candidates that could potentially block the intra-cellular immune response pathway and thus stabilize OCT4 expression from synRNA (Figure 2a). The degradation of synthetic single-stranded RNA (ssRNA) is elicited through two distinct pathways (Figure 2a). First, interaction between pathogen-associated molecular patterns, including ssRNA [30], leads to activation of the mitogen-activated protein kinases (MAPKs) and the IκB kinase (IKKα and IKKβ), which subsequently turn on nuclear factor-kappa-B (NF-κB), via phosphorylating IκBα [31]. The second pathway uses another subset of pattern recognition receptors - endosomal Toll-like receptors [32], melanoma differentiation-associated gene 5 (MDA-5), and retinoic acid-inducible gene I (RIG-1) - that activate a distinct pathway which requires IKK-related kinases, IKKε, and TANK-binding kinase 1 (TBK$_1$) and which also leads to activation of NF-κB and NF-κB-based gene transcription [31]. Both pathways recognize foreign RNA, and subsequent NF-κB-based activation of IFN regulatory factor (IRF) leads to type I IFN production [33-35]. Thus, inhibitor κB (IκB) proteins, normally sequestering NF-κB in the cytoplasm, must undergo phosphorylation via IκB kinase α (IKK) and subsequent rapid proteasome degradation, allowing NF-κB transcriptionally based regulated IFN production [36]. BAY11 inhibits IκBα phosphorylation (specifically, IRF7 production) [37,38], whereas BX795 inhibits TBK$_1$ and IKKε. Both BAY11 and BX795 ultimately may inhibit many degradation-associated cytokines, RNAse L, and chemokines [31] (Figure 2a). Warren and colleagues found that use of modified ribonucleoside bases and a phosphatase treatment to reduce the signaling through RIG-1, coupled with an IFN inhibitor B18R, led to significantly reduced innate immune responses [39], although it did not completely eliminate this synRNA degradation in our experiments. We then asked whether two other candidate small molecules (BX795 and BAY11) might efficiently block the intracellular

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immune response to synthetic RNA and stabilize OCT4 expression (Figure 2a). When combined with 120 ng of total synRNA, BX795 at the low concentration (0.001 μM) induced broad stabilization and homogenous expression of OCT4 (Figure1g). We then investigated whether increasing the concentration of BX795 to 1 μM would increase OCT4 expression. As shown in Figure 1h, a greater number of cells did express OCT4 but at a level comparable to that of BX795 at 0.001 μM (Figure 1g). Next, we tested a similar compound, BAY11, at a low concentration (0.01 μM) and obtained robust OCT4 stabilization and more uniform expression (Figure 1i). This was followed by one last condition tested with BAY11 at a high concentration (1 μM), and we found dramatically increased OCT4 expression (Figure 1j). In comparison, the original conditions of just synRNA and B18R yielded low and very heterogeneous expression of OCT4 (Figure 1b,c). To further investigate the reproducibility of this phenomenon, we used *OCT4* synRNA alone, bought from an independent commercial manufacturer (Stemgent), and were able to duplicate the response seen in Figure 1, even in the absence of B18R (Figure 3a-c). This indicates that BAY11, and not the modified nucleobases or B18R, is the main contributing factor that is allowing the mRNA stabilization. In an effort to investigate whether the NF-κB-based innate immune response pathway is involved in this *OCT4* stabilization phenomenon (as we hypothesized), we performed an assay without transfection of B18R or multiple pooled mRNAs. Not only did we still observe the stabilization of OCT4 expression but we also observed, through quantitative PCR, a statistically significant decrease in NF-κB expression when in the presence of BAY11 after daily transfection of synthetic *OCT4* mRNA alone (Figure 2b). This correlative evidence suggests that the reproducible and robust increase in *OCT4* mRNA expression we have observed is due, at least in part, to the inhibition of the NF-κB-based innate immune response pathway.

Quantitation of immunocytochemistry and cell proliferation analysis

Luminosity-based measurements were used to quantify which conditions yielded significantly increased relative expression of OCT4. Fluorescent imaging demonstrated that BAY11 at 1 µM yielded the highest statistically significant relative expression of OCT4, followed closely by BAY11 at 0.01 µM (Figure 4a). There were no differences between the expression levels of OCT4 induced by BX795, and both were lower than the BAY11 concentrations (Figure 4a). We then conducted a cell proliferation assay on the HUF cells and found that both concentrations of BX795 and BAY11 at 1 µM have significant cell proliferative defects when compared with controls. However, BAY11 at 0.01 µM had the best compromise between robust OCT4 expression and no statistically significant reduction in cell proliferation compared with controls (Figure 4b). According to these findings, BAY11 used at 0.01 µM yielded optimal OCT4 expression without cell proliferative defects.

Global transcriptional meta-analysis and quantitative polymerase chain reaction

Next, we investigated whether global transcriptional meta-analysis could ascertain any early easily activated downstream transcriptional targets of OCT4 that may also be upregulated because of *OCT4* stabilization in the presence of BAY11. Pairwise analysis of control and BAY11-treated fibroblasts revealed a number of probe sets upregulated (P <0.05, fold change >3). The differentially upregulated genes (when compared with our baseline HUF cells), in the presence of BAY11, were then cross-referenced with these genes in the ESC microarray. Further comparison of the downstream targets of OCT4 was carried out with previously reported results from the chromatin immunoprecipitation paired-end ditags methodology, global expression profiling, and chromatin-immunoprecipitation analysis [9-11]. From these data, four putative gene targets were found to be consistently upregulated: *SPP1, DUSP4, GADD45G*,

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and *SLC16A9* (Figure 5a). These putative genes were confirmed with QPCR; however, the differential levels of gene expression observed via QPCR were, for unknown reasons, generally lower than those observed for microarray analysis. We demonstrated that, in the presence of BAY11, the expression of these four OCT4 early target genes was significantly increased relative to untreated HUF cells (Figure 5b). Interestingly, in the presence of BAY11, HUF cell expression of these four genes more similarly matched expression levels of hESCs, suggesting that BAY11 could be useful in reprogramming by upregulating key pluripotency-associated genes. Additionally, while *SPP1, DUSP4*, and *GADD45G* were previously found to be bound and regulated by OCT4 [9-11], we found a putative novel OCT4 target gene, *SLC16A9*, that has been previously shown through microarray analysis to also be upregulated in ESCs. Thus, through microarray analysis and QPCR, we detected and confirmed four early gene targets that are upregulated in the presence of BAY11, indicative of functional OCT4 expression.

Discussion

In this study, we provide the first significant and reproducible evidence, collected over four independent experiments, in support of the hypothesis that BAY11 can significantly increase the expression of OCT4 in human adult dermal fibroblasts from transfected synRNA without negatively impacting cell proliferation. We demonstrated that this response involves NF-κB-based innate immune responses and is independent of the modified nucleobases and addition of B18R. Also, we were able to reproduce the robust expression of OCT4 by using single-factor mRNA synthesized from an independent company (Stemgent) rather than our original source of synRNA (generated in-house). Importantly, OCT4 can significantly upregulate its putative early target downstream genes, including *SPP1, DUSP4*, and *GADD45G*. For example, dual-specificity phosphatase 4 (*DUSP4*) plays a role in cellular proliferation and differentiation via phosphatase activity in the MAPK pathways, and previous studies reported that deletion of this

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gene causes a significant decrease in the cell proliferation rate [40]. Similarly, *c-MYC* has many downstream targets that enhance cell proliferation [13], and *DUSP4* may have similar functions in reprogramming to increase cell proliferation. We also used global transcriptional analysis to identify a novel OCT4 target gene (*SLC16A9*) and demonstrated that *SLC16A9* was significantly upregulated following BAY11-based treatment of adult human skin cells transfected with synthetic *OCT4* mRNA. *SLC16A9* (or solute carrier family 16, member 9) is a monocarboxylic acid transporter, which is interesting as highly glycolytic cells commonly express monocarboxylate transporters [41]. Interestingly, *SLC16A9* is part of the monocarboxylate transporter family of H^+/lactate symporters capable of bidirectional transport of lactic acid across the plasma membrane [42]. This is an important finding as ESCs have been found to be primarily glycolytic and have very few mitochondria [43]. Thus, ESCs, and most malignant cancers, express this glycolytic phenotype, even in the presence of oxygen, an effect known as the 'Warburg effect', and must efflux lactate to prevent toxic intracellular buildup of lactate [44]. Therefore, upregulation of *SLC16A9* could be another avenue by which to increase reprogramming efficiency not only in mRNA-based reprogramming but also in other reprogramming methodologies.

It is interesting to note that, out of the various reprogramming factors we analyzed (*OCT4, SOX2, KLF4, cMYC*, and *LIN28*), only *OCT4* triggered an intracellular immune response that resulted in very low heterogeneous expression. Whether *OCT4* more closely resembles an evolutionarily recognized single-stranded virus that human somatic cells have evolved defense mechanisms to is an interesting hypothesis but as of yet is unproven. Also, exactly how human fibroblasts recognize and degrade transfected human *OCT4* RNA and whether the polyA or 3′ untranslated region sequences play a role are unclear. We propose that human cells use the same intracellular immune response pathways that degrade viral single-stranded mRNA to

degrade single-stranded *OCT4* mRNA. The difference in recognition and degradation between synthetic *OCT4* and endogenous *OCT4* may reside in the relatively long half-life of viral mRNA and synRNA compared with the relatively short half-life of endogenous *OCT4* mRNA (which is nevertheless continuously transcribed when expressed). This is an interesting area for future study. It should also be noted that, while the immunocytochemical assays we have used in this study do not distinguish between endogenous and exogenous OCT4 protein, we do not consider that this is a significant concern or changes the conclusions of our paper, as no detectable OCT4 protein was observed via the immunocytochemical assay in the human dermal fibroblasts without *OCT4* synRNA transfection. It is also interesting to note that, for unknown reasons, the RT-PCR primers we have previously used to detect endogenous *OCT4* mRNA did not work with synthetic *OCT4* mRNA. Therefore, we focused our quantitative analysis in this study on the increased amount of OCT4 protein generated in the synRNA transfected cells and the significant upregulation of genes downstream of OCT4. In regard to the detection of only four OCT4 putative targets, it is unclear to us why a larger number of OCT4 downstream genes did not get upregulated, although we propose that the four putative OCT4 targets we identified in this study may potentially represent some of the earliest or easiest genes to upregulate (or both), hence their detection in our microarray and QPCR assays, while other OCT4 targets may require additional factors or time (or both) to express to detectable levels. To date, we have observed that BAY11 can significantly and reproducibly stabilize *OCT4* over a 3-day and 5-day culture. It will be interesting to observe whether BAY11 can maintain this stabilized OCT4 expression over longer periods of time without inducing any negative or toxic effects (or both) on the cells. This long-term synRNA stabilization is critical toward almost all future applications of BAY11.

This research represents the first step toward using small molecules to augment the expression of various synRNAs in adult human somatic cells. At present, the molecular mechanism involved in the increased presence of OCT4 protein is not clear and may involve increased mRNA stability, translation, or improved transfection efficiency. Given that BAY11 has been found to suppress IRF7 production [37,38], tempering the innate immune response-mediated decay of exogenous mRNA is a plausible mechanism and likely involves increased mRNA stability of the transfected *OCT4*mRNA. This is an interesting area for future study. In addition, our results indicate that BAY11 may be an important adjuvant to augment reprogramming to pluripotency [23], differentiation to endoderm [8], and/or expression of other potentially useful synRNA-derived factors. One of the most promising aspects of this study is the potential that BAY11 may be useful in stabilizing synthetic *OCT4* mRNA to facilitate the generation of patient-specific induced pluripotent stem cells. This is especially relevant given that past findings have detailed that perhaps one of the biggest roadblocks during reprogramming via frequent mRNA transfections is an innate immune response[45]. Therefore, for the first time, we show that a small molecule, BAY11, can mitigate this innate immune response and partially prevent degradation of transfected synRNA.

Conclusions

We conclude that BAY 11-7082 (BAY11) can significantly increase the expression of OCT4 following transfection of synRNA into adult human skin cells. This small molecule-based stabilization of synRNA expression may have multiple applications for future cell-based research and therapeutics.

Abbreviations

BAY11, BAY 11-7082; DMEM/F-12, Dulbecco's modified Eagle's medium/F-12; DMSO, dimethyl sulphoxide; *DUSP4*, dual-specificity phosphatase 4; EDTA, ethylenediaminetetraacetic acid; ESC, embryonic stem cell; FBS, fetal bovine serum; hESC, human embryonic stem cell; HUF1, human fibroblast; IκB, inhibitor κB; IFN, interferon; IKK, inhibitor κB kinase α; IRF, interferon regulatory factor; KMOSL, *KLF4:c-MYC:OCT4:SOX2:LIN28*; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; NF-κB, nuclear factor-kappa-B; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; QPCR, quantitative real-time polymerase chain reaction; RIG-I, retinoic acid-inducible gene I; RT-PCR, reverse transcription-polymerase chain reaction; SCRO, Stem Cell Research Oversight; *SLC16A9*, solute carrier family 16, member 9; ssRNA, single-stranded RNA; synRNA, synthetic mRNA; TBK_1, TANK-binding kinase 1; UCLA, University of California at Los Angeles.

Figure 3.1
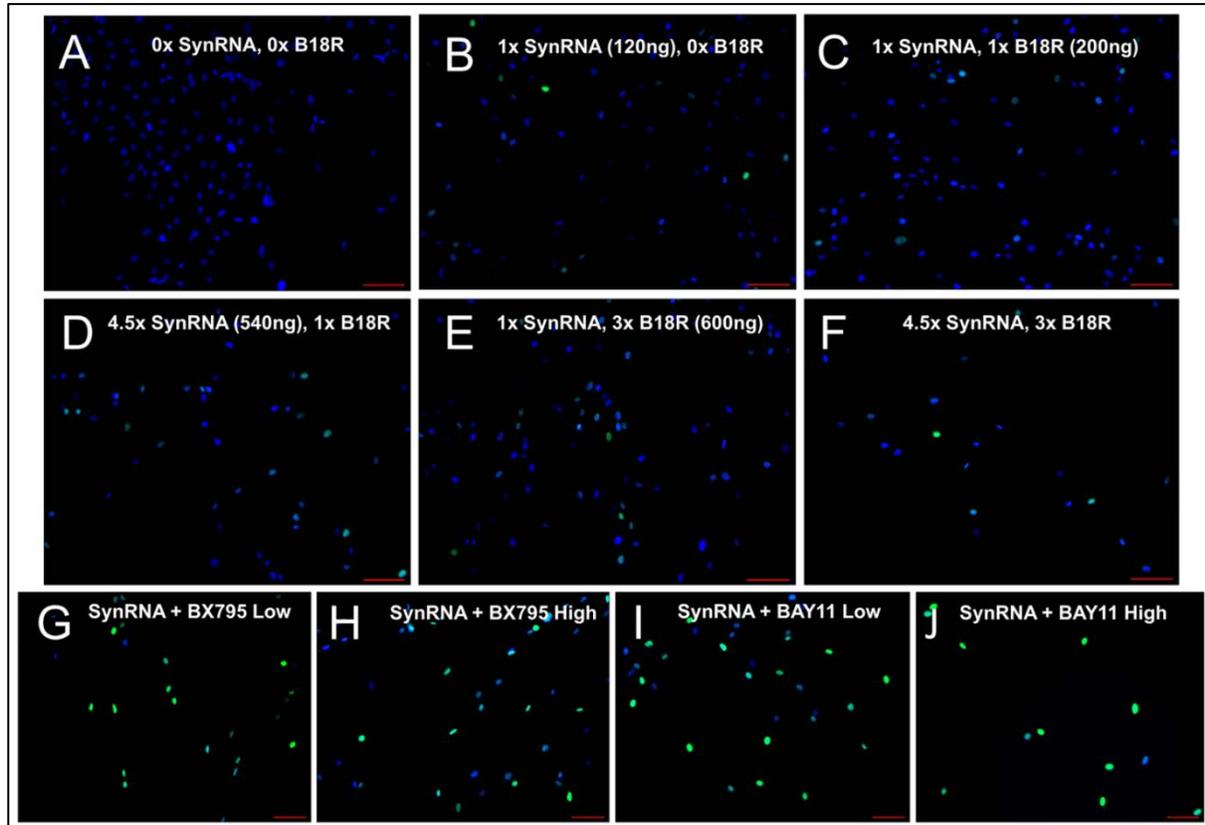
Figure 3.1. Immunocytochemical analysis of OCT4 expression. (a-j) Adult human dermal fibroblasts (HUF1) were exposed to different synthetic mRNA (synRNA) concentrations to stabilize and promote homogenous OCT4 expression. Addition of 1 × or 4.5 × synRNA did not yield significantly higher OCT4 expression (b,d). Addition of B18R was added to assuage interferon (IFN) signaling, although 1 × and 3 × concentrations did not yield significant increases in OCT4 expression (c,e). 4.5 × mRNA at 520 ng and 3 × B18R at 600 ng/mL also did not yield significant stabilization of OCT4 (f). Small-molecule compounds of BX795 at low (0.001 µM) and high (1 µM) concentrations did yield robust expression of OCT4 (g and h, respectively). BAY11 at low (0.01 µM) and high (1 µM) concentrations also stabilized OCT4 to an even greater degree than BX795 (i and j, respectively). Scale bar represents 100 µM. DAPI (4′,6-diamidino-2-phenylindole) staining is represented in blue; OCT4 staining is represented in green. BAY11, BAY 11-7082.

Figure 3.2
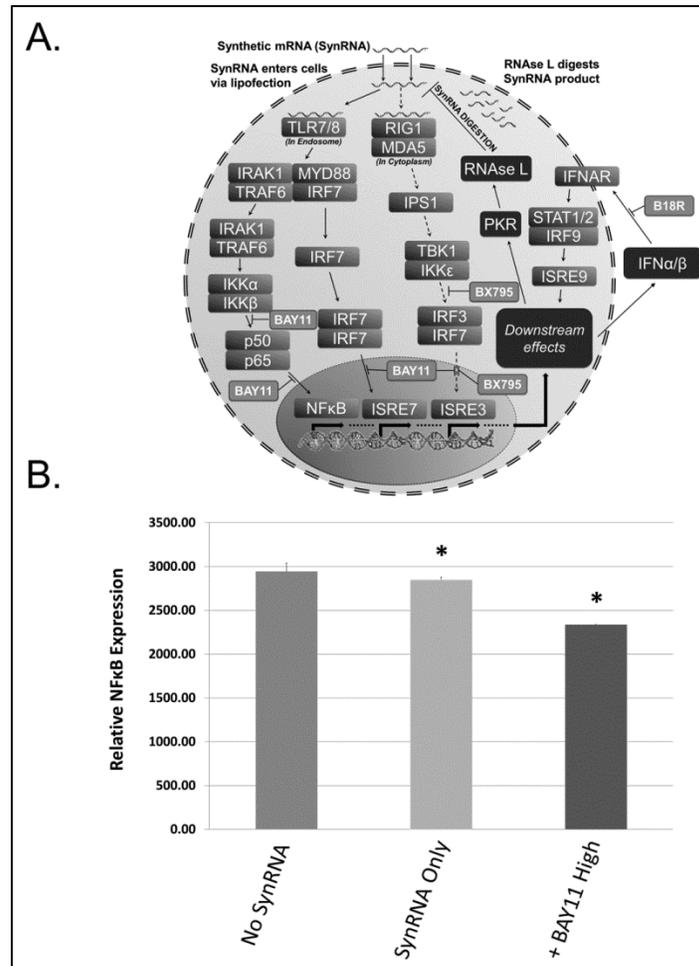
Figure 3.2. **Overview of molecular signaling pathway and quantitative real-time polymerase chain reaction analysis of nuclear factor-kappa-B (NF-κB). (A)** Dotted line represents the molecular pathway that BX795 inhibits, specifically at IKKε. The pathway with non-dotted lines represents the additional pathways that BAY11 inhibits, including the IKKβ and IRF7 pathways. **(B)** Relative NF-κB levels with and without BAY11. Overall levels decrease in the presence of BAY11, contributing to the increase in OCT4 expression previously seen. Asterisks indicate statistically significant changes in expression where P value was less than 0.05. BAY11, BAY 11-7082; IFNAR, interferon-α/β receptor; IKK, IκB kinase; IRAK1, interleukin-1R-associated kinase 1; IRF, interferon regulatory factor; ; ISRE, interferon stimulated response element; MDA5, melanoma differentiation-associated gene 5; MYD88, myeloid differentiation primary-response gene 88; RIG-I, retinoic acid-inducible gene I; STAT1/2, signal transducer and activator of transcription 1/2; TLR7/8; TRAF6, tumor necrosis factor receptor-associated factor 6.

Figure 3.3
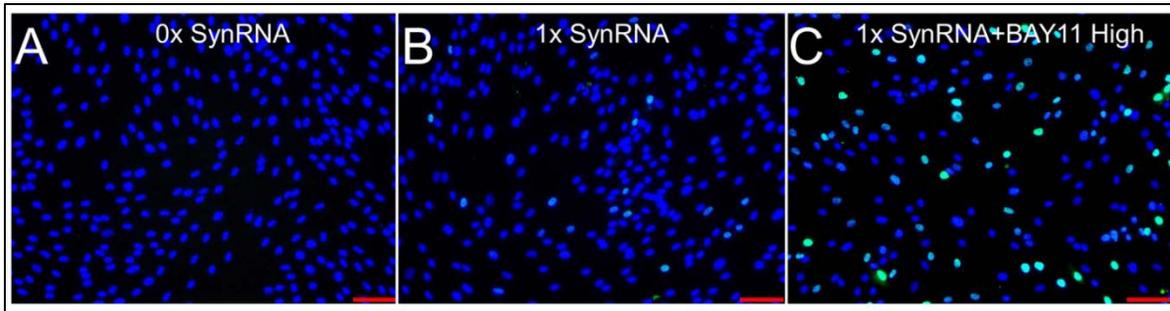
Figure 3.3. Immunocytochemical analysis of OCT4 expression with OCT4 mRNA alone. (A-C) HUF1 cells were exposed to 0 × and 1 × synthetic *OCT4* mRNA without B18R and in the presence or absence of BAY11. The addition of BAY11 was noted to again stabilize and allow homogenous expression of OCT4 without the presence of B18R or any other mRNAs in culture. Scale bar represents 100 μM. DAPI (4′,6-diamidino-2-phenylindole) staining is represented in blue; OCT4 staining is represented in green. BAY11, BAY 11-7082; synRNA, synthetic mRNA.
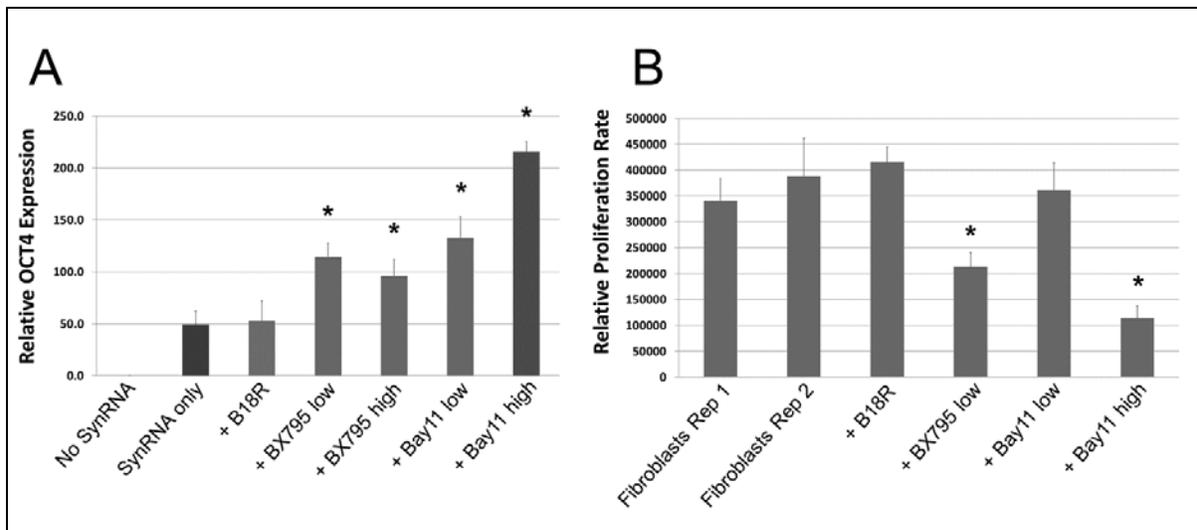
Figure 3.4

Figure 3.4. Relative OCT4 expression levels and relative proliferation rate. (A) Quantitative analysis of immunocytochemically detected OCT4 expression. **(B)** Proliferation analysis following exposure to small molecules. Asterisks indicate statistically significant increases or decreases over controls where P value was less than 0.05. BAY11, BAY 11-7082; synRNA, synthetic mRNA.

Figure 3.5
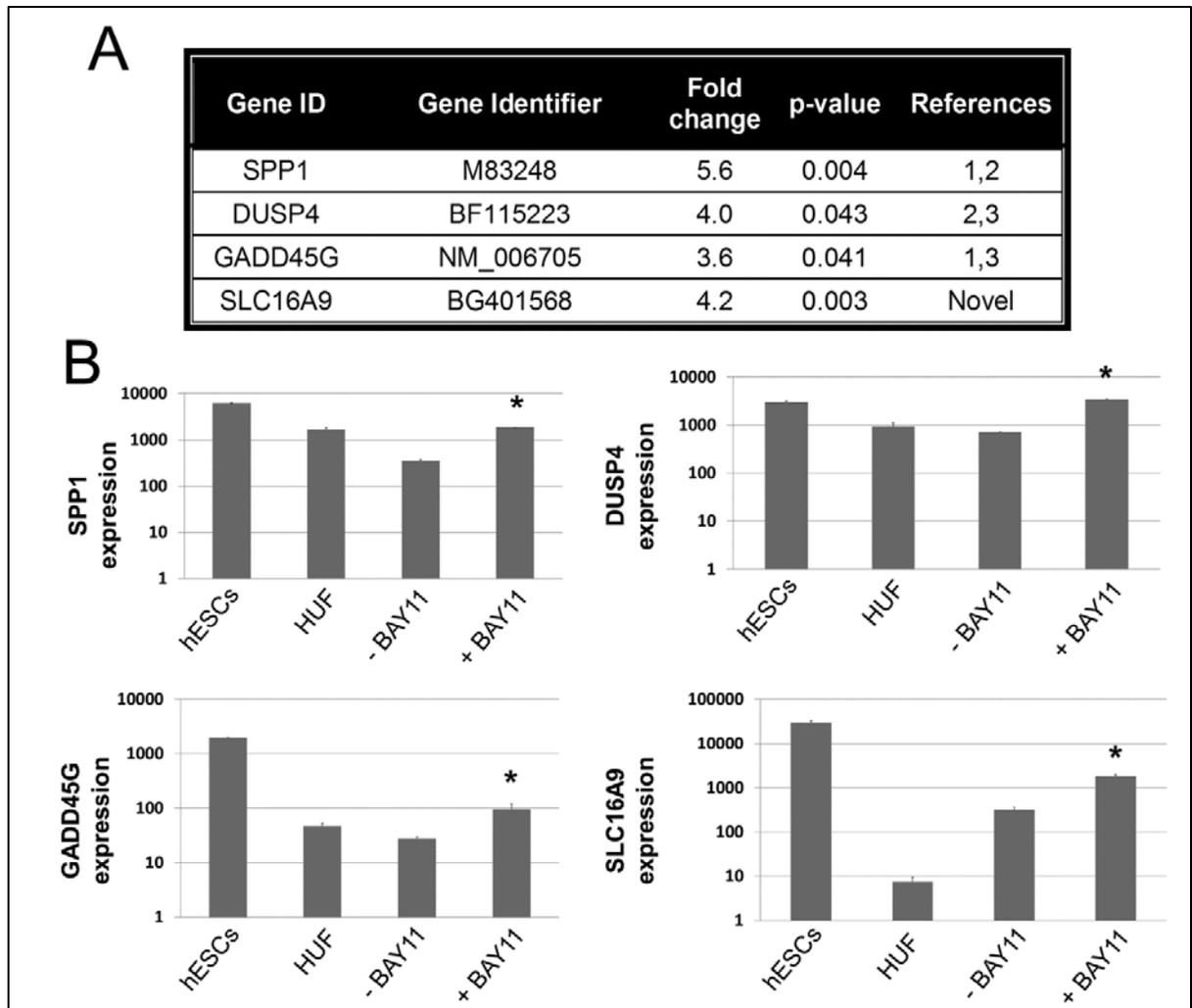
Figure 3.5. Microarray analysis and quantitative real-time polymerase chain reaction (QPCR) of genes upregulated by BAY11. (A) Microarray data show four putative gene targets that were identified by over 3 × fold changes in human fibroblast (HUF) cells treated with BAY11compared with untreated HUF cells over the course of 3 days of daily synthetic mRNA transfections. (B) QPCR analysis confirmed microarray results for the four genes and demonstrated that relative expression of the four genes becomes more similar to human embryonic stem cell (hESC) expression in the presence of BAY11. Asterisks indicate statistically significant increases over control cells not receiving BAY11 where P value was less than 0.05. BAY11, BAY 11-7082; *DUSP4,* dual-specificity phosphatase 4; *SLC16A9,* solute carrier family 16, member 9.

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CHAPTER 4: IDENTIFYING CANDIDATE OOCYTE REPROGRAMMING FACTORS USING CROSS-SPECIES GLOBAL TRANSCRIPTIONAL ANALYSIS

Abstract

There is mounting evidence to suggest that the epigenetic reprogramming capacity of the oocyte is superior to that of the current factor-based reprogramming approaches and that some factor-reprogrammed induced pluripotent stem cells (iPSCs) retain a degree of epigenetic memory that can influence differentiation capacity and may be linked to the observed expression of immunogenicity genes in iPSC derivatives. One hypothesis for this differential reprogramming capacity is the "chromatin loosening/enhanced reprogramming" concept, as previously described by John Gurdon and Ian Wilmut, as well as others, which postulates that the oocyte possesses factors that loosen the somatic cell chromatin structure, providing the epigenetic and transcriptional regulatory factors more ready access to repressed genes and thereby significantly increasing epigenetic reprogramming. However, to empirically test this hypothesis a list of candidate oocyte reprogramming factors (CORFs) must be ascertained that are significantly expressed in metaphase II oocytes. Previous studies have focused on intraspecies or cross-species transcriptional analysis of up to two different species of oocytes. In this study, we have identified eight CORFs (ARID2, ASF1A, ASF1B, DPPA3, ING3, MSL3, H1FOO, and KDM6B) based on unbiased global transcriptional analysis of oocytes from three different species (human, rhesus monkey, and mouse) that both demonstrate significant ($p < 0.05$, FC > 3) expression in oocytes of all three species and have well-established roles in loosening/opening up chromatin structure. We also identified an additional 15 CORFs that fit within our proposed "chromatin opening/fate transformative" (COFT) model. These CORFs may be able to augment Shinya Yamanaka's previously identified reprogramming factors (OCT4, SOX2, KLF4, and cMYC) and potentially facilitate the removal of epigenetic memory in iPSCs and/or reduce the expression of

90

immunogenicity genes in iPSC derivatives, and may have applications in future personalized pluripotent stem cell based therapeutics.

Introduction

Global epigenetic [1-4] analysis provides evidence in support of the hypothesis that the mammalian metaphase II oocyte possesses a superior capacity to epigenetically reprogram somatic cell nuclei towards an embryonic stem cell (ESC)-like state than the current factor-based reprogramming approaches. The significance of this putative incomplete factor-based reprogramming for future patient- specific cellular therapeutics was increased when factor- reprogrammed isogenic cells recently demonstrated a T cell-dependent immune response upon transplantation into a perfectly matched (syngeneic) mouse, a phenomena not seen in syngeneic transplantation of ESCs [5]. One hypothesis for this differential reprogramming capacity is that the oocyte possesses specific factors that are lacking in the current factor-based reprogramming approaches and that it may be possible using factors identified from oocytes to recapitulate the oocyte's putative superior epigenetic reprogramming capacity (Fig. 1). John Gurdon, Ian Wilmut, and others have previously suggested that the key reprogramming factors in the oocyte may be involved in loosening somatic chromatin and thereby providing the transcriptional regulatory apparatus access to repressed genes [6].

Here, we propose that in addition to oocyte-based factors that open up/loosen chromatin, the key candidate oocyte reprogramming factors (CORFs) may also include factors that promote a transformation in cell fate, which we refer to as the "chromatin opening/fate transformative" (COFT) model. Whether CORFs are selected based exclusively on their known ability to loosen chromatin, using our expanded COFT

model, or based on additional considerations, we propose that there are two basic approaches for future us- age of CORFs in reprogramming experiments—the CORF-augmented approach and the CORF-dynamic approach. The CORF-augmented reprogramming model involves including CORFs and induced pluripotent stem cell factors (iPSC-Fs), such as OCT4, SOX2, KLF4, and cMYC [7, 8], together with the hypothesis that the CORFs will augment the reprogramming capacity of the iPSC-Fs, either by opening up the chromatin to be more accessible to epigenetic reprogramming and/or through an as of yet unidentified mechanism, and generate an epigenetic and transcriptional landscape that is closer to ESCs. The CORF-dynamic reprogramming model involves using CORFs initially to reprogram the somatic cell back into an oocyte-specific (totipotent) epigenetic state and then transition to iPSC-Fs to differentiate the totipotent cells into pluripotent stem cells that more closely resemble ESCs (Fig. 1). One of the potential benefits of generating CORF-iPSCs that are fully reprogrammed back into an ESC-like epigenetic state is that their derivatives may not express immunogenicity gene expression, as has been observed for iPSC-derivatives [5]. However, to test these models empirically, a list of CORFs must be ascertained. The COFT model proposed here suggests that the key oocyte factors will play a role in either remodeling the chromatin architecture to an euchromatic state to be accessed by transcriptional regulators and/or through promotion of a transformation in cellular fate, preferentially toward an oocyte/totipotent or stem cell/pluripotent epigenetic state.

Previous research has focused on identifying CORFs based on intraspecies [9] or interspecies analysis using up to two different species [10]. In this study, global transcriptional meta-analysis was performed on human, rhesus monkey, and mouse metaphase II oocytes in comparison to their respective adult dermal fibroblast

transcriptomes, representing the first multispecies global transcriptional analysis of metaphase II oocytes for identification of putative CORFs. We identified a set of 23 CORFs using the COFT model criteria that demonstrated significantly increased expression in oocytes from all three species, and of those 23 CORFs, we propose that eight CORFs (ARID2, ASF1A, ASF1B, DPPA3, ING3, MSL3, H1FOO, and KDM6B) possess a function that most closely correlates with the "chromatin loosening/enhanced reprogramming" concept as previously proposed by Gurdon and Wilmut.

Materials and Methods

Ethics statement

Written approvals for human skin biopsy procedures, human fibroblast derivation, culture, and experimental use was obtained from the Stanford University Institutional Review Board, the Stanford University Stem Cell Research Oversight (SCRO) committee, and written informed consent was obtained from each individual participant. Biopsy material used in this study was obtained and initially analyzed at Stanford University, as previously described [11] and transferred to UCLA through a material transfer agreement. Written approvals for the experiments performed in this study were obtained from the UCLA Institute Biosafety Committee and UCLA SCRO committee.

Cell culture

Human adult dermal fibroblasts were cultured in complete Dulbecco's modified Eagle medium (DMEM)/Mixture F-12, supplemented with 10% fetal bovine serum (FBS), 1 -

minimal essential medium (MEM) nonessential amino acids, 1 · Glutamax, and 100 IU/mL penicillin-streptomycin (all from Invitrogen/Gibco, Grand Island, NY, USA) and maintained at 37°C in a 5% CO_2 incubator. Culture medium was changed every 2 days. Cells were allowed to expand to 80–90% con- fluency before passaging with 0.05% trypsin-EDTA (Invitrogen) and replated at a 1:3 ratio. A large bank of early passage cells was cryopreserved in culture medium supplemented with 10% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). All research adhered to National Acad- emy of Sciences guidelines. H9 human embryonic stem cells (hESCs) (UCLA Broad Stem Cell Research Center-Stem Cell Core) were cultured in standard ESC conditions, as previously described (Byrne et al., 2009). Briefly, hESC medium consisted of DMEM/F12 supplemented with 20% Knockout Serum Replacement (KSR), 1 · Glutamax, 1 · nonessential amino acids, 100 IU/mL penicillin-streptomycin (all from Invitrogen), 1 · b-mercaptoethanol (Millipore, Billerica, MA, USA), and 10 ng/mL recombinant human basic fibroblast growth factor (bFGF; Globalstem, Rockville, MD, USA).

Global transcriptional meta-analysis

All cultured cells (including adult dermal fibroblasts and human ESCs) were harvested for total mRNA extraction using the High Pure RNA Isolation Kit according to manufacturer's instructions (Roche, Indianapolis, IN, USA). Microarray analysis was carried out as published [12]. Briefly, total RNA was used for an Affymetrix Differential Gene Expression Assay Human Genome U133 Plus 2.0 Array (Genoseq UCLA) for global transcriptional analysis using standard Affymetrix protocols (Affymetrix GeneChip Expression Analysis Technical Manual, rev.3. 2001). Up- loading and cluster analysis of the CEL files between replicate samples was through GeneSifter (VizX Labs,

www.geospiza.com) using the Advanced Upload Method and normalized using the Affymetrix Microarray Analysis Suite (MAS) 5.0 algorithm. All microarray data were deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/ projects/geo/). Affymetrix CEL files for all other human, rhesus monkey, and mouse cells analyzed in this study were obtained from GEO. Each CEL file was generated through analysis of the cells' total RNA hybridized to the species- relevant Affymetrix GeneChip Array, with the U133 Plus 2.0 Arrays used for human cells, the Rhesus Arrays used for rhesus monkey cells, and the 430 2.0 Mouse Arrays used for mouse cells. The following CEL files (with their respective GEO accession numbers) were analyzed and compared in this study: human metaphase II oocytes (GSM304261, GSM304262, GSM136512, GSM136513, GSM136519, GSM136525, GSM288812, GSM288876), human adult dermal fibroblasts (GSM994327, GSM994328, GSM301264, GSM301265, GSM288223, GSM288224, GSM288225, GSM288226), human embryonic stem cells (GSM994321, GSM994322, GSM194307, GSM194308, GSM378813, GSM378818, GSM462819, GSM462820), rhesus monkey metaphase II oocytes (GSM300529, GSM300530), rhesus monkey adult dermal fi- broblasts (GSM187389, GSM187390), mouse metaphase II oocytes (GSM132659, GSM132660), and mouse adult dermal fibroblasts (GSM106139, GSM106141).

Data analysis

Each CEL file was uploaded to GeneSifter (VizX Labs, Seattle, WA) using the Advanced Upload Method and normalized using the Affymetrix MAS 5.0 algorithm. Cluster analysis between all human samples was performed through GeneSifter Project Analysis using analysis of variance (ANOVA) statistical analysis ($p < 0.01$, threshold

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> 100, Manhattan distance, ward linkage and gene row centering). GeneSifter pairwise analysis between oocytes and fibroblasts for each species was performed using all mean normalization and t-test statistical analysis (p < 0.01). For each pairwise analysis, between two to eight biological replicates from each cell line or tissue type were used, de- pendent on availability. Probe sets were considered to be significantly upregulated (compared to the species-specific adult   dermal fibroblast   baseline)   when   the p value was < 0.01 and fold change was equal or greater than 3. When duplicate probe sets or genes were identified, the duplicates with the lower fold change were removed. Gene ontology analysis for biological processes was performed in Gene- Sifter on the significantly upregulated   probe   sets.   CORFs were identified on the basis of both demonstrating significantly increased expression in oocytes from all three species and possessing a function that fit within the COFT reprogramming model.

Results

Investigating interexperimental variability

To ensure that interexperimental variability, such as cell line variability and differences manifested in experimental design, would not contribute to false positives in identifying differences in gene expression, analysis of the variability between samples harvested from separate experiments was taken into account.   Cluster analysis was performed using eight biological replicates   each of human metaphase II oocytes, human adult dermal fibroblasts, and human ESCs. Despite the materials being derived from a number of different experiments, we observed cell-type specific clustering for each of the cell types analyzed across all experiments (Fig.   2A)   and   clusters   of   cell- specific   gene   expression (Fig. 2B), suggesting that interexperimental variability was

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significantly lower than the intrinsic similarities for cell type– specific transcriptomes. This result was used as the foundation to justify the use of materials obtained from multiple experiments in subsequent pairwise comparison analyses.

Identifying putative human CORFs

Before cross-species specific analysis was applied, we established a baseline of upregulated genes that would serve as the foundation for putative CORFs in the human. Pairwise analysis of the eight biological replicates of the human metaphase II oocytes was compared to the eight biological replicates of the human dermal fibroblasts. Gene ontological analysis and filtering was performed on the significantly upregulated genes, and 404 human putative CORFs were identified based on their possession of a function in chromatin remodeling, transcriptional regulation, and/or having previously been associated with a stem cell-like state (see Table S1) (Supplementary Data are available at www.liebertpub.com/cell/).

Cross-species analysis of putative CORFs

In an effort to further narrow down potential CORF candidates, the putative human CORFs identified in the gene ontological analysis were subjected to cross-species analysis to identify overlapping upregulated genes that could be identified as cross-species specific CORFs. Pairwise analysis was repeated for both rhesus monkey and mouse metaphase II oocytes in comparison analysis with their respective adult dermal fibroblasts; and following the same gene ontological filtering, a list of 377 rhesus monkey putative CORFs (Table S2) and 399 mouse CORFs (Table S3) were identified. Cross-species analysis of the various putative CORFs from all species was performed, and 48

species-independent putative CORFs were identified (Fig. 3, Table S4). Background research was performed on these putative CORFs, and a final list of 23 CORFs was identified that met all of the CORF-criteria included in the COFT model (Table 1). Specifically, these 23 factors possessed a function in either remodeling the chromatin architecture to loosen/open it up to be accessed by transcriptional regulators and/or through promotion of a transformation in cellular fate, preferentially toward an oocyte/totipotent or stem cell/pluripotent epigenetic state (Table 1). These 23 factors included factors that remodel and open up chromatin. They include:

ARID2, which plays a key role in activating gene expression through the PBAF chromatin remodeling complex [13];

ASF1A and ASF1B, which are histone-remodeling chaperones that cooperate with chromatin assembly factor 1 (CAF-1), which plays a key role in remodeling chromatin in pluripotent embryonic cells [14, 15];

BRDT, which plays a role in the reorganization of acetylated chromatin in germ cells [16]; DPPA3 and DPPA5, which are pluripotency-associated factors, with DPPA3 in particular playing a known role in altering chromatin structure in oocytes [17]; [18];

RPS6KA5, which contributes to gene activation by histone phosphorylation [15];

TADA2L, a component of the ATAC complex, which has histone acetyltransferase (HAT) activity on histones H3 and H4 [15];

ING3, a component of the NuA4 HAT complex that is involved in transcriptional activation of select genes principally by acetylation of nucleosomal histones H4 and H2A [15];

MLL3, which activates transcription through methylation of 'Lys-4' of histone H3 and is essential in maintaining the hematopoietic stem cell state [19];

MSL3, a component of the MSL complex that is responsible for the majority of histone H4 acetylation at 'Lys-16', which is implicated in the formation of a more open chromatin structure, specifically by inhibiting the formation of compact 30-nanometer–like fibers and impeding the ability of chromatin to form cross-fiber interactions [20];

NCOA3, a nuclear receptor co-activator that displays HAT activity [15];

H1FOO, the oocyte-specific linker histone that has greater mobility than somatic histones and plays a key role in gen- erating the increased instability of the embryonic chromatin structure following fertilization and somatic cell nuclear transfer [21]; and

KDM6B, a histone demethylase that specifically demethylates 'Lys-27' of histone H3 and thereby prevents the formation of repressive chromatin through polycomb group (PcG) protein complex PRC1 binding [22].

Also included are oocyte-expressed transcription factors that promote global epigenetic transformation and/or re- programming to a stem cell-like state, such as:

FOXK2, which promotes activation protein 1 (AP-1)- mediated transcriptional regulation throughout the genome [23];

NR5A2, a transcription factor that can replace OCT4 in reprogramming somatic cells into iPSCs [24]; TAF4B, which functions as a gene-selective co-activator in certain cells and is involved in the activation of antiapoptotic genes [15];

HHEX, a transcription factor important for embryonic development [15];

LEF1, which transcriptionally activates MYC and CCND1 expression and enhances cell proliferation [15];

ERG, a transformation-specific transcription factor that promotes and maintains leukemia [25];

NFATC2, which induces lymphocyte proliferation [26];

POU4F1, a transcription factor associated with cancer (acute myeloid leukemia) [27]; and AFAP1L2, which contributes to SRC-regulated transcriptional activation [28].

Discussion

IPSCs have significant promise for cell replacement therapy, but some iPSCs demonstrate epigenetic memory and derivative immunogenicity gene expression that could negatively impact their clinical application. Studies comparing the DNA methylomes of mouse and human iPSCs with their respective species-specific ESCs discovered many iPSC lines retained aberrant iPSC-specific differential methylation patterns, a phenomenon referred to as "epigenetic memory" [2, 3]. The epigenetic memory of iPSCs was observed to impair the differentiation capacity of the iPSCs, with iPSCs demonstrating a reduced capacity to differentiate into cells from lineages different to the donor cell type [2, 3]. There is significant evidence that most, if not all, iPSC lines can gradually resolve at least some, if not most, of their transcriptional and epigenetic differences with ESCs with increased passaging [29]. However, it has also been observed that a subset of iPSC lines nevertheless retain epigenetic memory, even following extended passaging [3].

How significant a challenge this residual epigenetic memory will pose for future autologous cellular therapeutics is unclear. However, it may be prudent to consider erring on the side of caution and continuing to investigate novel augmented nuclear reprogramming approaches that may be able to both help remove residual epigenetic memory, regardless of passage, as well as potentially augment the nuclear reprogramming process, increasing the overall feasibility of the human iPSC-based approach. There is mounting evidence to suggest that the epigenetic reprogramming capacity of the oocyte is

superior to that of the current factor-based reprogramming approaches. One hypothesis for this differential reprogramming capacity postulates that the oocyte possesses factors that loosen the somatic cell chromatin structure, providing the epigenetic and transcriptional regulatory factors more ready access to repressed genes and thereby significantly augmenting epigenetic reprogramming. These CORFs may be able to loosen chromatin during the reprogramming process and thereby result in "CORF-iPSCs" with significantly lower levels of epigenetic memory and derivative immunogenicity gene expression. It is also possible that incorporating CORFs will also speed up the epigenetic reprogramming toward pluripotency and/or enhance the overall percentage of cells that attain pluripotency, although none of these things have been investigated to date. If CORF- iPSCs could be generated with less epigenetic memory and/ or lower/no derivative immunogenicity gene expression, this may make them better sources of autologous pluripotent material than the current iPSC-generation approaches and thus represent a potentially transformative impact upon personalized pluripotent stem cell-based regenerative medicine and an important early translational consideration for future iPSC-based therapeutics.

However, the key first step toward the generation of CORF-iPSCs is the identification of putative CORFs. In this study, global transcriptional meta-analysis was performed on human, rhesus monkey, and mouse metaphase II oocytes in comparison to their respective adult dermal fibroblast transcriptomes and a set of 23 CORFs was identified that were shown to have significantly increased expression in oocytes from all three species and to possess a function that fit within the COFT model. Of these 23 CORFs, eight possess a function that most closely correlates with the "chromatin loosening/enhanced reprogramming" hypothesis.

There are several considerations to discuss in regard to this type of multispecies in silico study. First, we used data that were obtained from several different groups, including our own, and a possible concern is that a potentially significant proportion of the transcriptional differences observed will be due to lab-specific variables not shared across multiple research groups. However, this does not appear to have significantly affected the results of this global transcriptional meta-analysis study because we observed cell-type specific clustering for all samples analyzed. The second consideration is that this type of in silico transcriptional analysis approach lacks the primary biological material to perform quantitative RT-PCRs for these CORFs. Thus, these CORFs must be considered as provisional until they are confirmed using RT- PCR. The third consideration is that we have made several underlying assumptions in our CORF-identification approach that may not be correct, including our hypothesis that CORFs will be strongly expressed and will either open chromatin or transform cellular fate.

We acknowledge that it is certainly possible that some very important reprogramming factors may be expressed at relatively low levels undetectable to microarray-based analysis or may not function in a role that opens chromatin or transforms cellular fate. The CORF-identification approach performed in this study may not be optimal, and alternative approaches, such as those that incorporate embryonic stem cells into the meta-analysis [10] may provide a superior transcriptional approach toward CORF identification. The fourth consideration is that we propose the hypothesis that the underlying reprogramming mechanism will be maintained across oocytes from different mammalian species, specifically human, rhesus monkey, and mouse. However, it is certainly possible that there are species-specific reprogramming molecules that

would be eliminated by our multispecies conserved factor-identification approach, and perhaps the previously reported intraspecies analysis [9] would provide a superior CORF-identification approach.

The final consideration of a transcriptional-based approach toward putative CORF identification is that it does not analyze alternatives to messenger RNA (mRNA) transcripts in the oocyte, such as stable long half-life proteins, microRNAs, and intrinsic physical variables of the oocytes, such as a large amount of ooplasm to quickly dilute somatic cell factors post transplantation [30]. Nevertheless, this study provides an important first step toward a transcriptional-based identification of species-independent CORFs, which represents the first such multispecies transcriptional analysis of potential oocyte-reprogramming factors and an important foundation for testing the CORF- augmented and CORF-dynamic reprogramming models, using either vectors [8] or synthetic mRNAs [31]. With regard to the CORF- dynamic reprogramming model, this will be facilitated by the usage of synthetic mRNAs as the mixture that is transfected into the somatic cell can simply be transitioned over time from a CORF mixture toward an iPSC-F mixture.

We propose that an important next step will be the derivation, global epigenetic analysis [2, 3, 32], and derivative immunogenicity gene expression analysis [5] of CORF-iPSCs in comparison with iPSCs generated using standard approaches [8, 33-35]. If CORF-augmented iPSCs demonstrate significantly lower level of epigenetic memory and derivative immunogenicity gene expression, we would then propose that CORF-iPSCs may have significant promise for future personalized pluripotent stem cell based therapeutics.

In conclusion, we have identified 23 CORFs that are significantly detected in human, rhesus monkey, and mouse metaphase II oocytes, eight of which are associated with chromatin "loosening." These CORFs represent a foundation for future research to investigate the CORF- augmented and CORF-dynamic factor-reprogramming approaches and may provide an important step toward generating immune-compatible patient-specific iPSC-based cellular therapeutics.
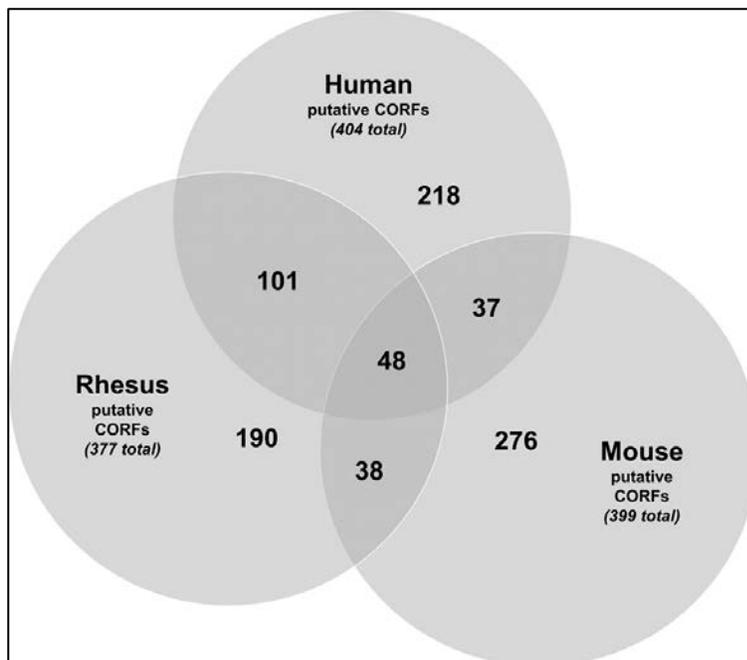
Figure 4.1
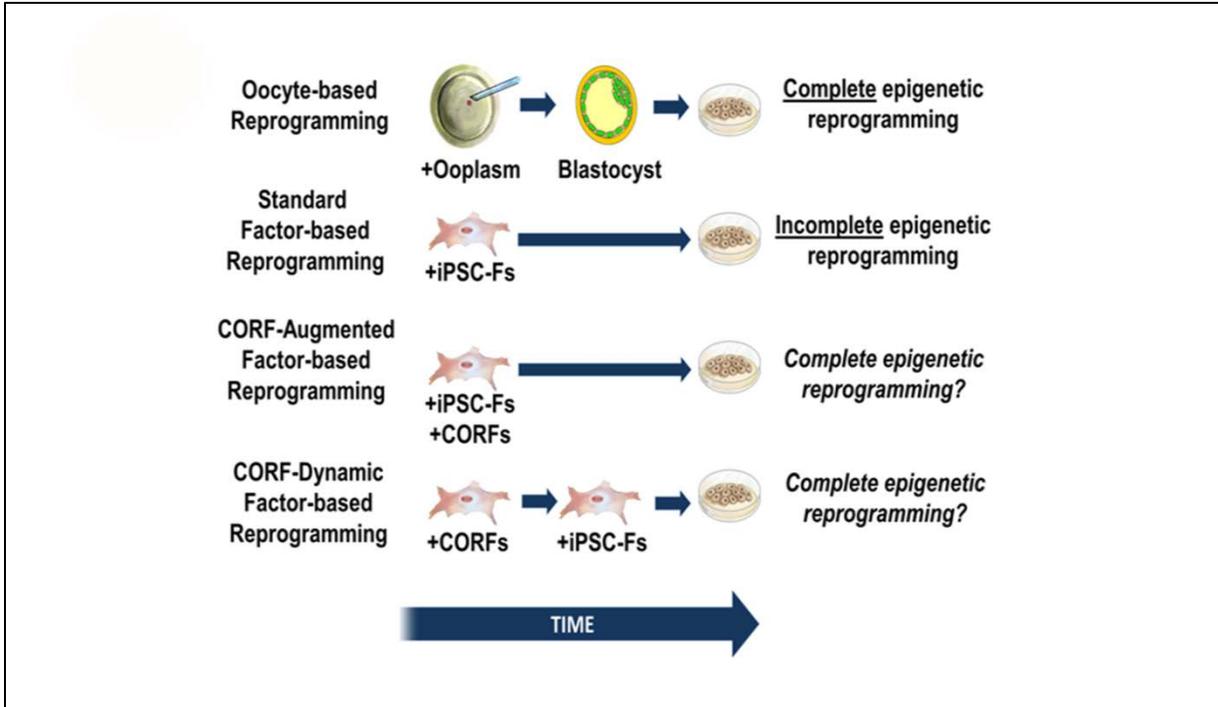
Figure 4.1. CORF-based reprogramming concept. Oocyte-based reprogramming involves placing the somatic cell nucleus into an enucleated metaphase II oocyte. Factors in the ooplasm can induce complete epigenetic reprogramming, and subsequent ESCs derived from oocyte-reprogrammed (somatic cell nuclear transfer) embryos (blastocysts) demonstrate similar epigenetic patterns to ESCs derived from fertilized blastocysts (fESCs). Standard factor-based reprogramming involves exposing the somatic cell nucleus directly to standard iPSC-Fs, such as OCT4, SOX2, KLF4, and cMYC. These factors can induce reprogramming of the somatic cell nucleus into iPSCs, but there is mounting evidence that these cells do not always possess the same epigenetic patterns or differentiation potential as fESCs. The CORF-augmented reprogramming model involves using both iPSC-Fs and CORFs at the same time to investigate if the additional CORF factors will open up the chromatin and significantly increase reprogramming of augmented CORF-iPSCs toward a fESC epigenetic pattern. The CORF-dynamic model involves first using CORFs to reprogram the somatic cell nucleus back into an oocyte-specific (totipotent) epigenetic state, followed by exposure to the iPSC-Fs to differentiate these totipotent cells into CORF-iPSCs to investigate if this approach will significantly increase reprogramming of dynamic CORF-iPSCs toward a fESC epigenetic pattern.

Figure 4.2
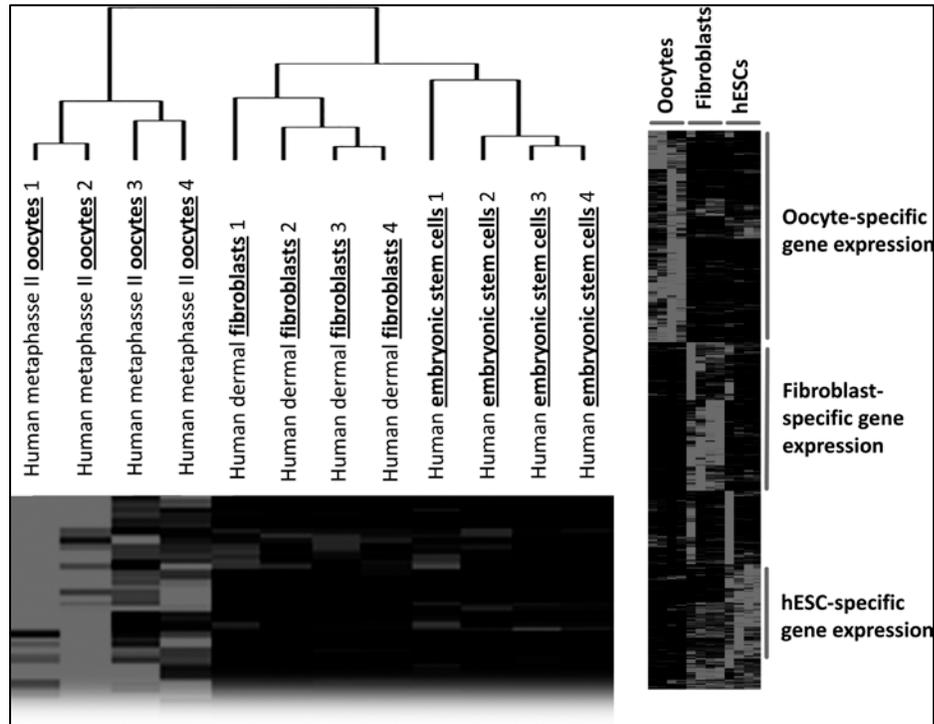
Figure 4.2. Global transcriptional analysis of human samples. Global gene expression cluster analysis of human oocyte, ESC, and adult dermal fibroblast samples from different experiments demonstrating cell type–specific clustering and clustering of cell type specific genes.

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Figure 4.3

Figure 4.3. Identification of species-independent putative CORFs. Analysis of overlap between human, rhesus monkey, and mouse putative CORFs.

Table 4.1

	CANDIDATE OOCYTE REPROGRAMMING FACTORS (CORFs)				
	Fold change in expression[a] compared to fibroblasts in:				
Gene (aka)	Human oocytes	Rhesus oocytes	Mouse oocytes	Function	Reference
AFAP1L2 (XB130)	5.5	155	130	Contributes to SRC-regulated transcriptional activation	Xu et al. (2007)
ARID2 (BAF200)[b]	19	8.2	13	Activates gene expression through the PBAF chromatin remodeling complex	Xu et al. (2012)
ASF1A (CIA)	6.6	17	4.8	Histone chaperone that cooperates with CAF-1 in pluripotent embryonic cells	Houlard et al. (2006); GeneCards.org (2012)
ASF1B (CIA-II)	4.0	12	8.8	Histone chaperone that cooperates with CAF-1 in pluripotent embryonic cells	Houlard et al. (2006); GeneCards.org (2012)
BRDT (BRD6)	7.6	120	280	Reorganization of acetylated chromatin in germ cells	Pivot-Pajot et al. (2003)
DPPA3 (STELLA)	370	650	110	Critical component involved with altering chromatin structure in the oocyte	Liu et al. (2012)
DPPA5 (ESG1)	457	235	3.3	Pluripotency-associated factor	Kim et al. (2005)
ERG (p55)	6.6	65	120	Transformation-specific transcription factors	Tsuzuki et al. (2011)
FOXK2 (ILF1)	14	6.5	4.5	Promotes AP-1–mediated transcriptional regulation throughout genome	Ji et al. (2012)
H1FOO	320	18	480	Replaces linker H1 histones and induces embryonic chromatin structure	Becker et al. (2005)
HHEX	40	190	7.4	Transcription factor important for embryonic development	GeneCards.org (2012)
ING3	11	14	13	Transcriptional activation of select genes principally by acetylation of H4 and H2A	GeneCards.org (2012)
KDM6B (JMJD3)	21	10	17	Histone demethylase that specifically demethylates 'Lys-27' of histone H3	Aoto et al. (2008)
LEF1 (TCF10)	61	45	6.9	Transcriptionally activates MYC and enhances cell proliferation	GeneCards.org (2012)
MLL3 (HALR)	21	31	41	Methylates 'Lys-4' of histone H3 and essential in maintaining stem cell state	Jude et al. (2007)
MSL3	4.4	56	7.2	Acetylation of histone H4 at 'Lys-16', which opens up chromatin	Shogren-Knaak et al. (2006)
NCOA3 (SRC-3)	4.5	9.2	16	Remodels chromatin through histone acetyltransferase activity	GeneCards.org (2012)
NFATC2	10	39	8.1	Induces lymphocyte proliferation	Caetano et al. (2002)
NR5A2 (FTF)	3.6	94	26	Transcription factor that can replace OCT4 in reprogramming	Heng et al. (2010)
POU4F1 (BRN3A)	13	250	143	Transcription factor associated with cancer (acute myeloid leukemia)	Fortier et al. (2010)
RPS6KA5 (MSK1)	9.6	89	8.4	Contributes to gene activation by histone phosphorylation	GeneCards.org (2012)
TADA2L (ADA2A)	5.3	4.7	3.1	Histone H3 and H4 acetyltransferase activity	(GeneCards.org 2012)
TAF4B (TAF2C2)	21	9.2	4.2	Gene-selective coactivator in certain cells and activates antiapoptotic genes	GeneCards.org (2012)

[a]All fold changes represented a significant ($p < 0.05$) three-fold or greater increase in detected gene expression in the relevant species metaphase II oocytes compared to their adult dermal fibroblasts.
[b]The eight genes highlighted in bold represent the CORFs most closely associated with the "Chromatin loosening" concept as proposed by John Gurdon and Ian Wilmut (Gurdon and Wilmut 2011) and others.

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CHAPTER 5: CHARACTERIZING IMMUNOGENICITY DIFFERENCES IN HUMAN EMBRYONIC STEM CELLS, INDUCED PLURIPOTENT STEM CELLS, AND THEIR DERIVATIVES

Abstract

Autologous cellular therapy using human induced pluripotent stem cells (hiPSCs) should, in theory, allow for therapeutic cell replacement while avoiding immunogenicity responses. This concept has been a source of much controversy from the original discovery that undifferentiated syngeneic mouse iPSCs elicit an immune response upon transplantation. However, analysis of any potential immunogenicity response in hiPSCs and their derivatives has yet to be explored. Here, we utilized correlative gene expression analysis of two previously discovered immunogenicity genes, *ZG16* and *HORMAD1*, to assay immunological responses in teratomas, non-directed embryoid body differentiation, semi-directed tri-lineage differentiation, and full *in vitro* differentiation of clinically relevant derivatives. We provide evidence of line-to-line and experimental variation and therefore differential responses to differentiation protocols that cause stochastic expression of *ZG16* and overall low levels of *HORMAD1*. We therefore provide the first analysis that *ZG16* is a fetally associated antigen with heterogeneous expression across multiple hESC and hiPSC derived cell types that varies between lines and experiments. We conclude that autologous hiPSC-based cellular therapeutics should still be viewed upon favorably in light of the lack of correlation between hiPSCs and an immune response, assuaging immunogenicity based autologous cell rejection concerns for cell replacement therapies, although this conclusion is dependent upon the hypothesis that the levels of *ZG16* expressed from hiPSC-derivatives are not immunologically relevant.

Introduction

Cell based therapeutics using hESCs and hiPSCs show great potential as these cells are pluripotent and have the ability to differentiate into any cell type of the human body. Originally, hESCs seemed to have a potential immune privilege when it came to allogeneic transplantation due to their very low MHC-I and non-existent MHC-II cell-surface protein expression [1-3]. There have also been some studies showing that this immune privilege is carried over during differentiation into embryoid bodies, small-differentiated tissue fragments derived from teratomas, oligodendrocyte progenitor cells, and insulin-producing cell clusters [4-8]. However, other studies have countered this evidence by providing data showing that ESC injection into injured mouse myocardium forms allogeneic grafts *in vivo* that are positively correlated to an immune response [9, 10]. Also, transplantation of ESC-derived embryoid bodies into allogeneic mice can elicit tissue damage and necrosis [11]. This is relevant as even syngeneic grafts upregulate chemokines that are chemotactic for CD8+ T-cells and inflammatory cytokines due to transplantation tissue injury, a relevant concern considering that cellular replacement therapy would therefore likely impose a localized inflammatory environment that can upregulate MHC-I proteins, chemokines, and other potentially immunogenic antigens that could lead to an adaptive immune response [12, 13]. ESC-based differentiation into clinically relevant cell types and subsequent introduction of new and potentially antigenic molecules could be applicable to iPSCs as data have shown even minor histocompatibility complex proteins cause ESC graft rejection [8, 11]. Most of these mechanisms of potential immune privilege or incompatibility for hESCs should apply to hiPSCs and subject them to similar scrutiny.

The evolution of reprogramming technology used to derive pluripotent stem cells from adult somatic cells has occurred rapidly since the inception of factor-based reprogramming [14, 15]. In addition, hiPSCs have been heralded to be a viable alternative from controversial hESCs for

autologous cell replacement therapy, disease modeling and gene correction [16, 17]. However, multiple groups have shown evidence of ESC and iPSC differences with regards to transcriptional, epigenetic, genomic imprinting, somatic mutations, and differentiation efficiencies [18-30]. Additionally, more hESC and hiPSC differences were brought forth after a controversial result recently came out showing evidence that syngeneic transplanted mouse iPSCs, but not ESCs, were capable of eliciting an immune response upon teratoma formation [31]. Follow up studies have produced various results with one group utilizing chimeric mice as tissue donors to look at immunogenicity, although this approach has limitations as T-cell based tolerance would have most likely eliminated any response to these tissues during thymic development. This group also correlated developmental competency to an immune response whereby iPSC clones with low developmental competency display T-cell mediated immune rejection [32]. Another laboratory failed to show any type of differential immune response for both ESCs and iPSCs and their differentiated derivatives when transplanted into syngeneic mice [33]. Additionally, a study showing dopaminergic neuron transplantation into the mouse striatum was concluded to have no response upon autograft transplantation, although a significant above baseline level of CD45+ pan leukocytes were found in histology staining; long term immunogenicity problems associated with a basal level of leukocytes is unknown and is not currently associated with immunogenicity concerns [33]. These results provide conflicting data that put into question the traditional view that genetically matched cellular therapy should not elicit immune rejection and potentially tests the foundational basis for personalized cellular therapeutics and at a minimum warrants further studies into immunogenicity issues, especially as no human immunogenicity studies with pluripotent stem cells and their derivatives have been analyzed [34, 35].

Teratoma formation is considered the gold standard for testing pluripotency in hPSCs as teratomas are tumors that can contain tissues from each embryonic germ layer, indicating

pluripotent potential [36]. The initial indication of an immune response in syngeneic teratoma formation was not surprising as previous syngeneic mouse ESC transplantations found T and B lymphocytes as well as macrophages in almost all of the teratomas, which formed at nearly a 100% success rate [37]. This was again shown in a separate lab where data showing that syngeneic iPSCs that undergo *in vivo* differentiation into teratomas when transplanted into mouse ischemic myocardium elicit an immune response [38]. Interestingly, teratomas from both studies, while able to show an immune response, did not regress, indicating that this immune response is attenuated. However, what was somewhat unexpected was the differential immune response in iPSC and ESC derived syngeneic mouse teratomas whereby transcriptional profiling of the iPSC T-cell infiltrated teratomas identified two genes, *ZG16* and *HORMAD1* that were over expressed in the regressing teratomas [31]. Interestingly, only *HORMAD1* has previously been identified as a testis/cancer antigen (CTA) that is immunogenic [39], whereas the level of expression of *ZG16* across human fetal and adult tissues is unclear [40], although *ZG16* overexpression has been significantly correlated to an immunogenicity response in syngeneic organisms [31]. This immunogenicity response correlation was seen upon forced expression into previously non-immunogenic ESCs that then displayed T-cell mediated teratoma regression [31]. Further association of *ZG16* and *HORMAD1* to an immune response was provided when these two genes, upon forced expression into dendritic cells, were able to activate T-cells directly as indicated by IFN-γ release [31].

Results

Therefore, with this association in mind, teratomas were made from five different hESC lines and 7 hiPSC lines (ES1-5 and iPS1-7) that represent a spectrum of pluripotent stem cells (see Supplementary Materials and Methods) and should be a good representation of inherent variability between stem cell lines and any putative differences of hES and hiPS cells. All of the

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tested ESC and iPSC lines formed teratomas containing cells of all three germ layers i.e.

mesoderm, endoderm, and ectoderm (Fig. 1A and Supplemental Fig. 1A), with 100% efficiency

(data not shown). Since teratomas represent such rich tissue diversity and therefore provide

many different types of cells that represent potential targets for an immune response, the

teratomas were sectioned into 10 pieces and analyzed via quantitative PCR for both *HORMAD1*

and *ZG16*. We found that, after averaging the 10 pieces, overall *HORMAD1* expression was

very low with high Ct values across all PSC lines analyzed and had heterogeneous expression

(Fig. 1B top). Conversely, *ZG16* was found to have much higher expression although it too has

heterogeneous expression across all PSC lines (Fig. 1B, bottom). While there were statistically

significant differences between inter-experimental ESC lines and iPSC lines, we also found

statistically significant differences between intra-experimental iPSC and ESC lines, providing

evidence that intra-line variation between specific hiPSC or hESC lines of the PSCs are likely

more important than inter-line differences between hESCs and hiPSC lines. Understanding that

teratomas formed subcutaneously and via intratesticular injection form distinct types of tissues

both as evidenced by solid tissue vs. cyst formation and via histological analysis, we also opted

to take two lines, ES1 and iPS2 and make teratomas with subcutaneous injections [41]. Due to

the low frequency of success of this methodology, only these two lines were able to be

analyzed, although both cell types made teratomas that were indistinguishable histologically

from their testicular counterpart (Fig. 1C). Postulating that the gene expression differences seen

in the testicular teratomas are stochastic, we wanted to see the inherent variability of teratoma

formation from the same line in the same animal. To this end, we transplanted ES1 or iPS2 cells

into the left and right legs of an immunocompromised mouse, and again found overall low

expression of *HORMAD1*, with only the iPS2 cells showing a significant difference between the

left and right hind leg formed teratomas (Fig. 1D, top left). We again noticed that *ZG16*

expression was higher than *HORMAD1*, and both lines analyzed showed a significant difference

between left and right hind leg formed teratomas, indicating induced stochastic differences even when formed in the same animal, in the same location (Fig. 1D, bottom left). When subcutaneous and testicular teratomas from ES1 and iPS2 lines were compared, we found a significant difference for *HORMAD1* expression between the ES1 teratomas, but not the iPS2 teratomas (Fig.1D, top right). However, the higher expressing *ZG16* gene was found to differ not only between ES1 and iPS2 subcutaneous and testicular derived teratomas, but also there was a difference between ES1 and iPS2 subcutaneous teratomas (Fig 1D, bottom right). However, because of the variation we saw between the left and right hind leg teratomas, we conclude that the differences seen between the subcutaneous and testicular teratomas for both lines for *HORMAD1* and *ZG16* are irrelevant, and again display the stochastic expression differences and line-to-line variation.

Understanding that undifferentiated pluripotent stem cells would never be transplanted into patients for concerns of tumor formation, we next sought to look at the expression patterns of *HORMAD1* and *ZG16* across varying levels of non-directed, partially directed and full directed differentiation of PSCs. We hypothesized that based on the gene expression heterogeneity seen in teratomas, we would again see broad expression variance due to experimental variation and potential differential susceptibilities of hPSC lines to differentiation protocols. Therefore, the hESC and hiPSC lines were first differentiated in a non-directed manner i.e. not subjected to specific factors to specify lineage, and made into embryoid bodies (EBs) (Fig. 1E and Supplemental Fig. 1B). EBs are three-dimensional multicellular aggregates that not only mimic early embryogenesis i.e. differentiate into mesoderm, endoderm, and ectoderm, but differentiate spontaneously in suspension culture and in basic serum containing media [42, 43]. Additionally, EB size and time points have been linked to allowing for different differentiation trajectories; we chose to make EBs with 100 cells, 1000 cells, and 10000 cells per EB and also allowed EBs to grow on gelatin coated plates for 1, 2, and 3 weeks before harvesting [44, 45]. Upon examining

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HORMAD1 expression we again found extremely low expression levels that varied across hES and hiPS lines (Fig. 1F, top). We subsequently found high expression levels of *ZG16* compared to *HORMAD1*, although at still relatively low levels compared to teratoma expression, that varied significantly between inter- and intra-experimental hES and hiPS lines (Fig 1F, bottom). Both for *HORMAD1* and *ZG16* statistically significant variance was observed, although levels were inconsistent and no specific correlation between different cell numbers and time points across all lines tested was observed.

Different tissues represent an array of targets that could induce an immune response; we subjected PSCs to a more advanced methodology of differentiation than that of the non-directed EB differentiation, via a more exclusive partial directed differentiation into specific representatives of the three-embryonic germ layers. To this end, hESCs and hiPSCs were differentiated to mesoderm, ectoderm, and endoderm lineages and were characterized to show typical gene expression patterns (Supplemental Fig. 2) and via protein expression by using immunofluorescence microscopy (Fig. 1G and Supplemental Fig. 3) that show specific characteristic markers of mesoderm (Brachyury), ectoderm (Pax6) and endoderm (SOX17) [46, 47]. Once again we found low expression of *HORMAD1*, at comparable levels to EBs that varied across inter- and intra-experimental hESC and hiPSC lines that did not show any specific correlation or pattern from uninduced cells to induced cells (Fig. 1H, top). Additionally, *ZG16* expression was higher than EB expression with specific cell lines seeing higher levels of expression that differed between lineages (Fig. 1H, bottom). Interestingly, certain cell lines in ectoderm, endoderm, and mesoderm differentiation display a statistically significant increase in *ZG16* expression upon induction into each respected lineage (ectoderm: iPS1 and iPS2, endoderm: ES2, ES3, ES5, iPS3 and iPS6, mesoderm: iPS2, iPS3 and iPS4) whereas others show a significant decrease (ectoderm: iPS6, endoderm: ES1, mesoderm: ES1 and ES4) (Fig. 1H, bottom). This shows that different lines, regardless of being hESCs or hiPSCs, show

different susceptibilities to differentiation protocols and start to express *ZG16* aberrantly. Whether or not these expression differences upon induction are also seen upon full directed differentiation was tested next.

Noting that multiple hESC and hiPSC lines had different susceptibilities to differentiation protocols, we postulated that these differences between cell lines arose during the differentiation process because of exposure to new molecules that could potentially cause an immune response and/or interact with *ZG16* [48]. Therefore, we directed the hESC and hiPSC lines under strict directed differentiation protocols into clinically relevant cell types e.g. oligodendrocyte progenitor cells (OPCs), hepatocytes, and cardiomyocytes. These hESC and hiPSC derivatives were assessed by immunofluorescence microscopy to confirm cell type specific staining for OPCs (A2B5, NG2, O4, Olig1, SOX10, PDGFRα, and O1), hepatocytes (Periodic Acid Schiff Stain, Albumin, CK18, and α-FP) and cardiomyocytes (GATA-4, MEF-2, α-actinin, and Troponin1), and by gene expression analysis (Fig. 2A and 2B). We next examined gene expression for *HORMAD1* and *ZG16* and found overall low and heterogeneous expression for *HORMAD1*, although we noticed a non-statistically significant trend that differentiation increased expression across all cell lines for all types of derivatives (Fig. 2C, top). Notably, the fibroblast-2 line had a significant expression level of *HORMAD1* when compared to all PSC derivatives, allowing us to postulate that *HORMAD1* does not seem likely to be involved in causing an immune response if an adult cell type is capable of expressing higher *HORMAD1* levels than the PSC derivatives (Fig. 2C, top). *ZG16* expression was then shown to have a statistically significant increase upon differentiation of every cell line tested and across all derivatives; the human fetal cells (HFCs) had varied levels of *ZG16* in comparison to the PSC derivatives although it is notable that the OPC expression level was significantly higher than all other OPC derivatives (Fig. 2C, bottom). Cardiomyocyte human fetal cells had no ZG16 expression and were thus significantly lower than all derivatives whereas hepatocyte human

fetal cells had an intermediate expression level (Fig. 2C, bottom). Interestingly, all adult

fibroblast lines had no expression of *ZG16*, providing evidence that *ZG16* is a putative fetal

antigen, especially since human hepatocyte and OPC fetal cells had high expression of *ZG16*

(Fig. 2C, bottom). To ascertain whether this significant increase upon differentiation is a

reproducible phenomenon, and thereby potentially a screening tool for lines that have low

projected *ZG16* expression, or just a stochastic process, the hepatocyte differentiation was

repeated with the iPS1 and iPS3 lines at similar passage e.g. within 4 passages. The previously

very low expressing iPS1-differentiated line had a significant increase across all replicates

compared to control iPS1 cells whereas the iPS3-differentiated line had a significant decrease

across all replicates compared to control iPS3 cells (Fig. 2D). The iPS1 replicates, when

averaged together, were again found to be significantly lower than the iPS3-differentiated line

when averaged across all three replicates, although significant variability was also seen

between iPS3-A and iPS3-B replicates and that of the original iPS3 differentiation, indicating a

high level of variability between experiments and intra-experimental replicates (Figure 2D). Thus

we conclude that although *ZG16* expression is definitively and reproducibly increased upon

differentiation, our data suggests that inherent intra- and inter-experimental variability preclude

this as a useful immunogenicity screening tool (Fig. 2D). We hypothesize, in agreement with

previous research [49], that the differentiated state of PSC progeny are reflective of an early

fetal developmental period and different lines, and experimental replicates, have variable

susceptibilities to differentiation protocols that manifest, in part, to differential expression of

ZG16. We find it notable that *HORMAD1*, a previously characterized CTA [39] has overall low

expression levels whereas *ZG16*, previously only shown to be expressed in certain adult tissues

and not identified as a CTA, can now be identified as a putative fetal antigen, with overall

heterogeneous, but high expression in a variety of PSCs and derivatives.
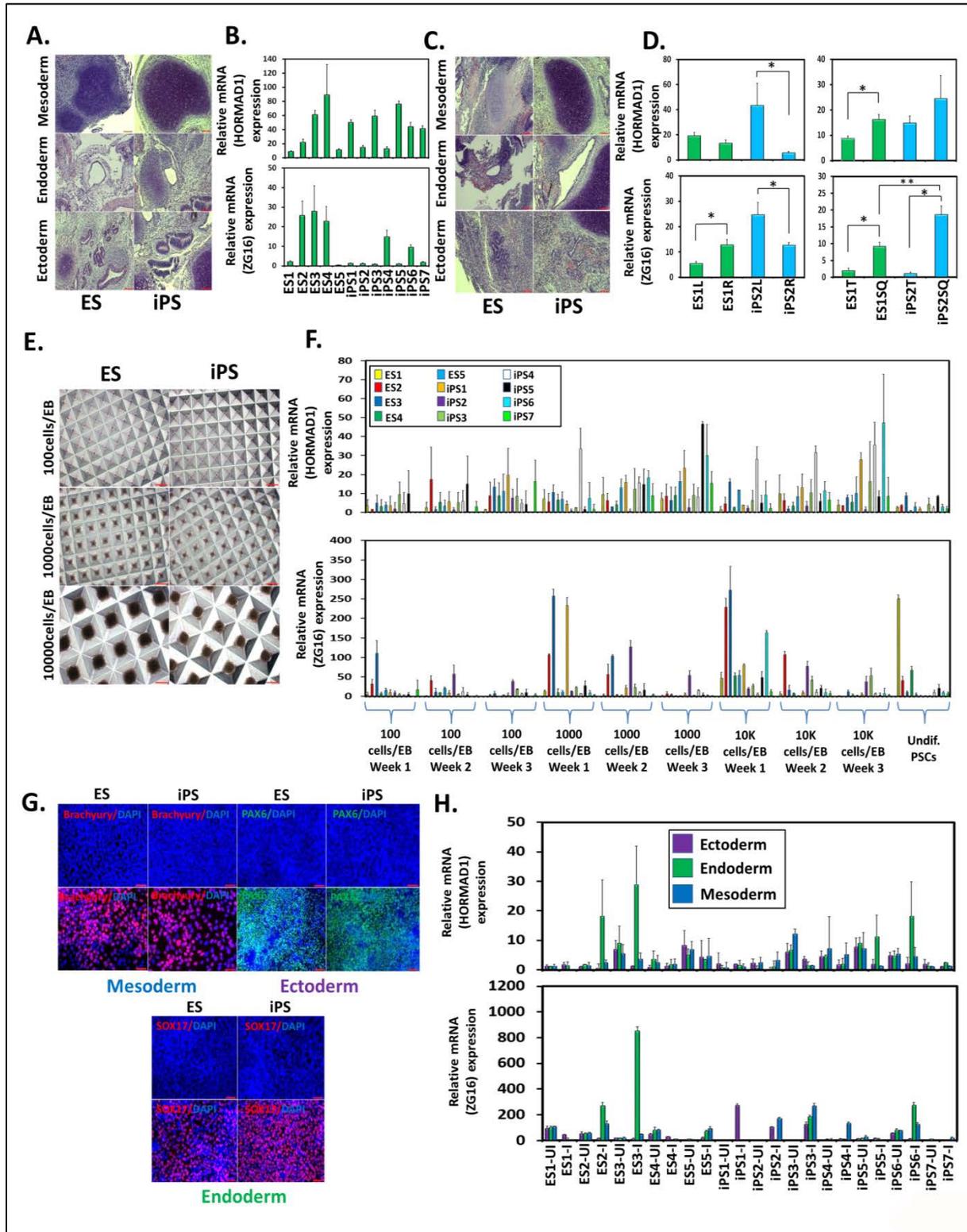
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Discussion

In this study, we show for the first time gene expression analysis across a variety of hESC and hiPSC derivatives with varying levels of differentiation utilizing two putative immunogenicity genes that were previously correlated to an *in vivo* T-cell mediated immune response in the mouse [31]. Although both *HORMAD1* and *ZG16* were implicated in the previously seen immune response in the mouse, we found that *HORMAD1* expression was very heterogeneous and low across all lines and differentiation states tested. More telling was the relatively high expression of *HORMAD1* seen in the fibroblast-2 line; since the differentiated somatic cell fibroblast line is known to be non-immunogenic in the host, we conclude that the overall levels of *HORMAD1* expression seen throughout the different stages of differentiation across all the lines are not great enough to elicit an immune response and should be treated as non-immunogenic relative levels. Interestingly, only *HORMAD1* has been implicated as a CTA, which have a characteristic fetal expression profile and are immunogenic [50]. We thus utilized this fetal expression of *HORMAD1* and its immunogenic correlation and extrapolated this for the potential application to *ZG16*. We speculate that since *HORMAD1* is a known CTA with immunogenic properties, the fact that we saw much higher levels of *ZG16* that were reproducibly increased upon *in vitro* differentiation indicates that current differentiation protocols are not robust or specific enough in differentiating these human pluripotent stem cells into fully differentiated adult cells. Thus, we postulate that current differentiation protocols only differentiate these pluripotent stem cells into a fetal like state, as shown previously [49], and implicate *ZG16*, for the first time, as a fetally associated antigen that may also be a CTA. It will be important in future studies to investigate a functional link between *ZG16* expression and an immune response in a human based assay in order to analyze if the overall *ZG16* expression levels shown here correlate to an immune response with human pluripotent stem cell derivatives. If this correlation is shown, it will be important to investigate and create better

differentiation protocols that eliminate this inherent line-to-line, hESC, and hiPSC variation that causes different susceptibilities to differentiation protocols and possible expression of immunogenic antigens.

Figure 5.1

Figure 5.1. Different Levels of hESC and hiPSC Differentiation Show Variable *HORMAD1* and *ZG16* Expression Levels *In Vitro*

(A) Representative H&E staining from hESC and hiPSC testicular derived teratomas showing representatives of the germ layers e.g. cartilage (mesoderm), gut epithelium (endoderm) and neural tube (ectoderm). Scale bar=100μM. See also Figure S1A.

(B) Whole testicular derived teratoma tumors were extracted and sectioned into 10 pieces followed by total RNA isolation. Total RNA was subjected to reverse transcription and cDNA synthesis followed by Q-PCR for *HORMAD1* and *ZG16* (top and bottom, respectively). Overall low and variable *HORMAD1* expression with very high Ct values was seen (top) whereas substantially higher and heterogeneous expression of *ZG16* was seen across all hESC and hiPSC lines (bottom). Results were normalized to *HPRT1* and *GAPDH*. Data are represented as ± SEM of triplicates of ten different pieces quantified. *ZG16* y-values are in units of hundreds. Scale bar=100μM.

(C) Representative H&E staining from hESC and hiPSC subcutaneously derived teratomas showing representatives of the germ layers e.g. cartilage (mesoderm), gut epithelium (endoderm) and neural tube (ectoderm). Scale bar=100μM.

(D) Whole subcutaneously derived teratoma tumors were extracted, sectioned, and made into total RNA as detailed in (B). Q-PCR yielded low *HORMAD1* expression (top) whereas higher *ZG16* expression was identified with significant differences identified between not only two teratomas taken from the same animal (bottom left) but also between teratomas derived subcutaneously and via testis from the same line (bottom right). Results normalized to *HPRT1* and *GAPDH*. Data are represented as ± SEM of triplicates of ten different pieces quantified. *ZG16* y-values are in units of hundreds. *p < 0.05

(E) Representative hESC and hiPSC derived EBs at three different sizes. Scale bar=200μM. See also Figure S1B.

(F) EBs made at different sizes (100cells/EB, 1000cells/EB, and 10000cells/EB) were plated onto gelatin coated plates and harvested at different time points (1, 2 or 3 weeks post plating) for total RNA. Q-PCR was performed and overall low and heterogeneous expression of *HORMAD1* was observed with no significant trend seen between size or time samples (top). *ZG16* expression was higher, with some lines at specific time points having significant increases or decreases, although no significant trend was observed across size or time points (bottom). Error bars represent ± SD. All PCRs were run in triplicate. Undif. PSCs=undifferentiated pluripotent stem cells.

(G) Representative immunofluorescence staining with lineage specific markers. Top panel left: ESCs and iPSCs were differentiated into early mesoderm like cells and stained for Brachyury (red). Top panel right: ESCs and iPSCs were differentiated into ectoderm like cells and stained for PAX6 (green). Bottom panel: ESCs and iPSCs were differentiated into early endoderm like cells and stained for SOX17 (red). Scale bar=50μM. See also Figures S2 and S3.

(H) Gene expression analysis of *HORMAD1* and *ZG16* was assessed in each hESC and hiPSC derived tri-lineage derivative across all lines for uninduced (UI) and induced (I). Q-PCR for *HORMAD1* gene expression was found to be extremely low and variable across all lines, regardless of induction of lineage (top). *ZG16* expression varied in directionality of differences and some were found to have significant differences (see text), although there was no clear

correlation of certain lines or lineages (bottom). Error bars represent ± SD. All PCRs were run in triplicate.

Figure 5.2
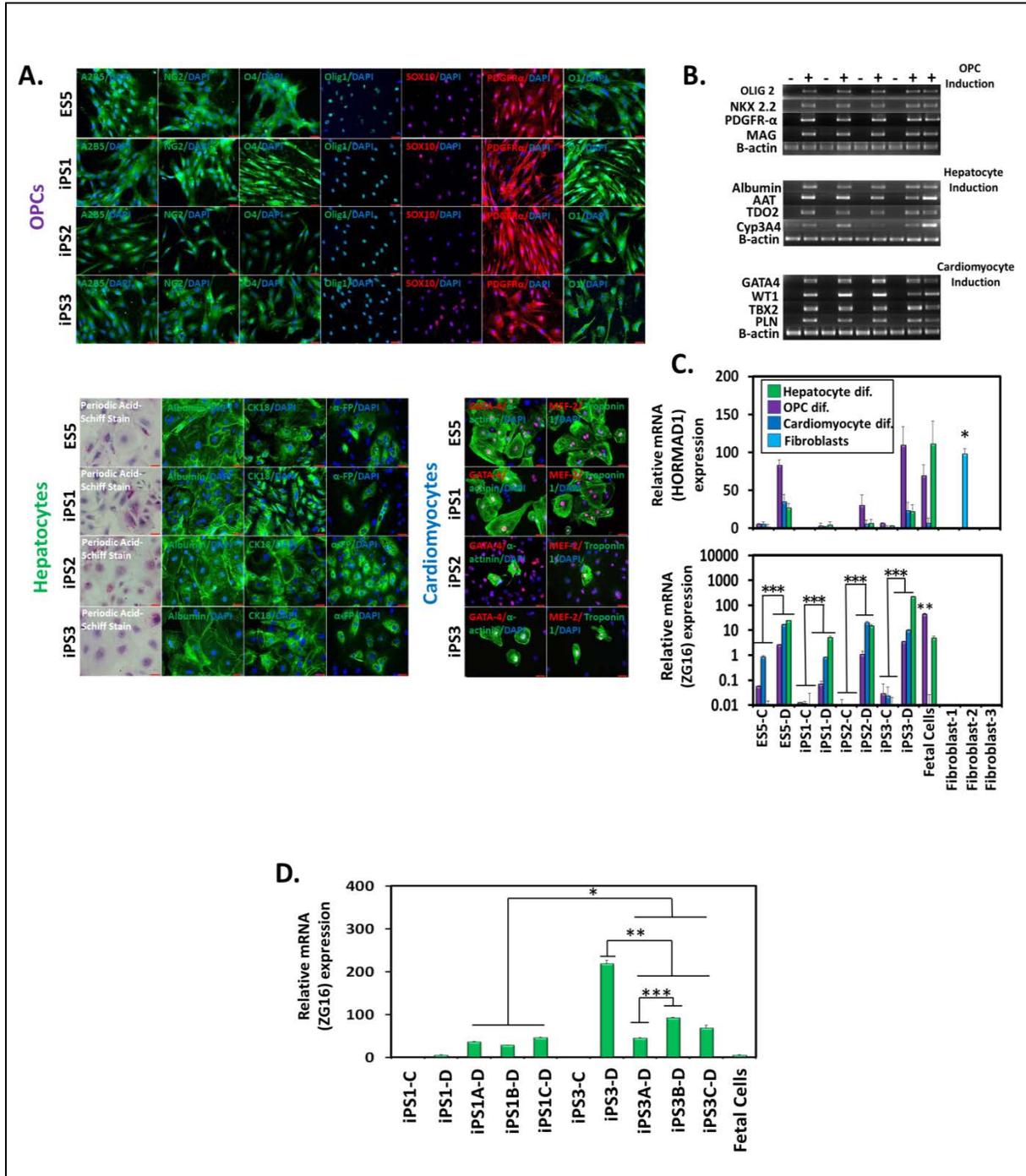
Figure 5.2. *ZG16* **Expression Increases Significantly Upon Differentiation of ESC and iPSCs into Clinically Relevant Cell Derivatives**

(A) Immunostaining of specific hESC and hiPSC lines differentiated into three clinically relevant cell types representative of all three germ layers. Top four rows, from left to right: oligodendrocytes progenitor cells (ectoderm) stained with A2B5 (green), NG2 (green), O4 (green), Olig1 (green), SOX10 (red), PDGFRα (red), and O1 (green). Bottom left four rows, from left to right: hepatocytes (endoderm) stained for glycogen synthesis with periodic acid-Schiff test (pink coloration), albumin (green), CK18 (green), and α-FP (green). Bottom right four rows, from left to right: cardiomyocytes (mesoderm) were stained with GATA-4 (red) and α-actinin (green), and MEF-2 (red) and troponin1 (green). Scale bar=50µM for OPCs, hepatocytes, and cardiomyocytes; scale bar=100µM for periodic acid-Schiff stain.

(B) RT-PCR analysis confirmed the expression of lineage specific genes for differentiated cell types: OPCs (*OLIG2, NKX2.2, PDGFRα,* and *MAG*), hepatocytes (*Albumin, Alpha-1 antitrypsin, Tryptophan 2,3-dioxygenase,* and *Cytochrome P450*), and cardiomyocytes (*GATA4, WT1, TBX2,* and *PLN*). All PCRs were run with *B-actin* control.

(C) *HORMAD1* expression in hESC and hiPSC derivatives was very low and heterogeneous (top). *ZG16* expression analysis resulted in a significant increase across all four lines upon differentiation (bottom). *p < 0.05 for *HORMAD1* expression for Fibroblast-2 line compared to all other PSC derivatives. **p < 0.05 for human fetal OPCs compared to other OPCs derived from all lines. *** p < 0.05 for each PSC line undifferentiated compared to differentiated. Error bars represent ± SD. Y-axis is a logarithmic scale and with display units of 100. All PCRs were run in triplicate.

(D) Gene expression analysis for *ZG16* was assessed in hiPSC1 and hiPSC3 derived hepatocytes to investigate if the significant response seen in (C) is reproducible, or if this increase in *ZG16* is a stochastic process. The significant increase in *ZG16* expression seen during hepatocyte differentiation from control iPSCs is reproducible across both iPS1 and iPS3 lines. A significant increase when all replicates from the second differentiation experiment were averaged (A, B and C replicates) was found between iPS1 and iPS3 lines, although significant differences between iPS3-D and all iPS3 A, B, and C replicates along with a significant difference between iPS3A and iPS3B replicates indicate inherent variability between replicates after hepatocytes differentiation. A, B, and C listed in the cell line annotations indicate the duplicate differentiation protocol across three replicates. * p < 0.05 for iPS1 A, B and C differentiated replicates compared to iPS3 A, B and C differentiated replicates; ** p < 0.05 for iPS3-D compared to iPS3 A, B and C differentiated replicates; and *** p < 0.05 for iPS3A-D compared to iPS3B-D. *ZG16* Y-values are in units of hundreds. Error bars represent ± SD.

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CHAPTER 5: SUPPLEMENTARY INFORMATION

Materials and methods

Ethics statement

Written approvals and informed consent regarding human skin biopsy procedures along with human fibroblast derivation, culture and experimental use are detailed elsewhere [1].

Tissue culture maintenance of primary human skin cells

The human skin-derived (HUF1) primary cell line used in this study was derived and cultured as previously described [1]. Additionally, two other fibroblast lines, MGM2 and LAVIV, used in this study are detailed as previously described [1]. All three fibroblast lines were cultured in standard fibroblast media conditions as detailed previously [1]. Briefly, fibroblast lines were cultured in complete DMEM/F-12 consisting of Dulbecco's modified Eagle's medium nutrient mixture/F-12 (DMEM/F-12) supplemented with fetal bovine serum (FBS), 1x non-essential amino acids, 1x Glutamax, and 100IU/mL penicillin-streptomycin (Invitrogen/Gibco, Grand Island, NY, USA) and maintained in a 37°C in a 5% CO_2 incubator. Regular passaging with 0.05% trypsin (Invitrogen) and banking was done in standard fibroblast medium supplemented with 10% dimethyl sulphoxide (Sigma-Aldrich, St. Louis, MO, USA).

In vitro culture of stem cell lines

Human embryonic stem cell lines 1 and 9 were procured from WiCell (Madison, WI). UCLA embryonic stem cell lines 2, 3 and 6 were procured from the UCLA Broad Stem Cell Research Center-Stem Cell Core. HESC lines 1, 2, 3, 6, and 9 are hereafter referred to as ES1-5, respectively. Multiple integration iPSCs were derived as previously published [2]. mRNA, adult pre- and post-excision hiPSCs, and MGM 2.19, 6.7, and 13.1.0 hiPSCs were derived from patient derived fibroblasts via standard skin biopsy procedures. HiPSCs were derived by using the stem cell cassette (STEMCCA) lentiviral based reprogramming methodology (a kind gift

from Don Kohn's laboratory, UCLA) [1, 3, 4]. HiPSC lines pre- and post-excised hiPSCs (genetically identical lines) are hereafter referred to as iPS1 and 2. The mRNA derived line is hereafter referred to as iPS3. MGM 2.19, 6.7 and 13.1.0 are hereafter referred to as iPS4, 5, and 6, respectively. The multiple integration line is hereafter referred to as iPS7. All hESC lines were originally plated on mouse embryonic fibroblasts and maintained in hESC media as described [1]. Colonies were subsequently passaged into feeder-free conditions with a 18-gauge needle (Fisher Scientific , Waltham, MA, USA) onto reduced growth factor Matrigel (BD Biosciences, San Jose, CA, USA). All further stem cell culture in feeder free conditions was carried out as published for all hESC and hiPSC lines [1]. Briefly, all stem cells, once converted to feeder-free conditions consisting of Matrigel as a substrate, utilized a 50:50 blend of Nutristem (Stemgent, San Diego, CA, USA) and mTeSR1 medium (STEMCELL Technologies Inc., Vancouver, BC, Canada). Cells were regularly passaged either with an 18-gauge needle or STEMPRO EZPassage Tool (Invitrogen) every 4-5 days.

Teratoma formation

One 10cm dish of each individual stem cell line was grown to 95% confluency and cells were removed in clumps with a 25mL serological pipette and the plate was rinsed with DMEM/F-12 (Invitrogen/Gibco). Cells were spun down at 200xg for 5 minutes and resuspended in ice-cold Matrigel diluted at 1:2 in DMEM to a total volume of 50µL. Each 10cm dish was split into two e.g. 7.5 million cells per injection site. For testicular injections both testes in a severe combined immunodeficient (SCID) adult male beige mouse were injected with 50µL of the cell/Matrigel slurry. For subcutaneous injections, 7.5 million cells were injected into the subcutaneous space in each hind-leg of the SCID adult beige mouse. Both for testicular and subcutaneous injections the mice were anesthetized; this was utilized for the non-surgical subcutaneous injections to ensure cells were not immediately dispersed upon movement and adequate time for Matrigel

solidification could take place. Teratomas were harvested at 7 weeks for both testicular and

subcutaneous teratomas by surgery and immediately half of the teratoma was sectioned with a

scalpel into 10 pieces and placed into RNA*later* buffer (Qiagen, Valencia, CA, USA). The other

half of the teratomas were fixed in 4% formaldehyde, and sections were paraffin-embedded and

then stained with hematoxylin and eosin for histological analysis at the UCLA Translational

Pathology Laboratory. All animal experiments were performed in accordance with the UCLA

Animal Research Committee and the UCLA Division of Laboratory Animal Medicine.

Non-directed embryoid body differentiation

Embryoid bodies (EBs) were made by taking 95% confluent 10cm dishes of hESC or hiPSCs

and washing them once with 1x PBS (Invitrogen). This was followed by incubation for 5 minutes

with StemPro Accutase to form a single cell suspension. The plate was rinsed 2x with

nonsupplemented DMEM/F-12 and spun down at 300xg for 5 minutes. This pellet was then

resuspended in AggreWell Medium (STEMCELL Technologies) with Rock inhibitor. Different EB

sizes were created by changing the number of input hES or hiPS single cells from 100cells/EB,

1000cell/EB, and 10000cells/EB into AggreWell 400 or AggreWell 800 plates (STEMCELL

Technologies). EBs were placed in ultra-low attachment multiwall plates (Sigma-Aldrich) for 24

hours and then underwent one media change with AggreWell Medium for 24 hours and then

plated onto .2% coated gelatin wells in a 6-well plate in standard fibroblast containing media

until harvested at the designated time points. Media were changed every 2 days for the duration

of the experiment.

Directed tri-lineage differentiation

Directed differentiation into mesoderm was carried out as previously published [5]. Briefly, hESC

and hiPSC were routinely passaged at a high confluency onto Matrigel with daily media

changes. After 48 hours, stem cell media was replaced with basal differentiation media

(STEMdiff APEL, STEMCELL Technologies) supplemented with 5µM GSKi (CHIR99021, Stemgent) for 24 hours and further differentiated in APEL media supplemented with 25ng/mL human recombinant bone morphogenetic protein 4 (rhBMP4, Peprotech, Rocky Hill, NJ, USA) for 24 hours. HESCs and hiPSCs were differentiated into ectoderm by following the manufacture's protocol (STEMCELL Technologies). Briefly, the stem cell colonies were made into a single cell suspension as detailed above using Accutase. Cells were plated onto Matrigel coated plates or glass coverslips overnight and then rinsed with DMEM/F-12. Appropriate volumes of STEMdiff Neural Induction Media (STEMCELL Technologies) were placed onto cells for a period of 10 days with daily media changes. HESCs and hiPSCs were differentiated into endoderm by following the manufacture's protocol (STEMCELL Technologies). Briefly, the stem cell colonies were made into a single cell suspension as detailed above and plated onto Matrigel coated wells or glass coverslips overnight and then rinsed with DMEM/F-12. The cells were then incubated with the specific media and supplements as indicated by the protocol for a period of 5 days with daily media changes.

Directed differentiation into cardiomyocytes, oligodendrocyte progenitor cells and hepatocytes

For cardiomyocyte differentiation, hESCs and hiPSCs were differentiated as published [6, 7]. Specifically, 1 million cells originally plated onto Matrigel coated wells were found to be the optimal cell density for hESC and hiPSC lines. Oligodendrocyte progenitor cells were differentiated as published [8]. Specifically, EBs were made with 5000cells/EB as it has been shown that larger EBs tend to make better neural lineages [9] Hepatocytes were differentiated as published [1, 10].

RNA isolation and reverse transcription polymerase chain reaction

Total RNA was isolated using a High Pure RNA Isolation Kit as per manufacture's recommendations (Roche, Indianapolis, IN, USA). cDNA was synthesized using 1000ng/μL total RNA using Transcriptor First Strand cDNA Synthesis Kit using both anchored-oligo(dT)$_{18}$ and random hexamer primers (Roche) in accordance to the manufacture's recommendation. Reverse transcription Polymerase Chain Reactions (RT-PCR) were carried out as previously described [1]. Primer sequences, annealing temperatures and PCR conditions are listed in the supplemental online data Table 1.

Quantitative PCR

All PCR reactions were conducted in triplicate and utilized two housekeeping genes, HPRT1 and GAPDH, as normalization controls. Equal amounts of cDNA (10ng), 2x LightCycler 480 Probes Master, specific UPL probe, PCR grade water (all three from Roche), and primers (Valuegene, San Diego, CA, USA) were used per reaction. Reactions were loaded onto LightCycler 480 Multiwell Plate 96 and sealed with sealing foil. Total volume per well was 20μL and amplified on a LightCycler 480 Real-Time PCR System and all data analyzed with LightCycler 480 Software release 1.5.0 using the all to mean pairing rule for advanced relative quantification. A list of primers and probes used are listed in the supplemental online data Table 2.

Immunocytochemisty

All cells for staining were plated onto glass coverslips and fixed for 15 minutes in 4% paraformaldehyde. Cells were washed 2x with 1xPBS supplemented with 100mM glycine for 5 minutes and permeabilized, when needed, with .5% Triton X-100 (Sigma-Aldrich) in 1xPBS for 60 minutes at room temperature. Blocking used 10% goat or donkey serum in Blocker Casein in

PBS (Thermo Scientific, Rockford, IL, USA) for 60 minutes at room temperature. Cells were washed with 1xPBS after primary staining and each subsequent step. Following primary antibody incubation, appropriate secondary Alexa Fluor antibodies (Invitrogen) were incubated for 1 hour at room temperature in the dark and mounted on Prolong Gold with DAPI (Invitrogen). Coverslips were visualized with an AxioCam MR Monocolor Camera and AxioVision Digital Image Processing Software (Axio Observer Inverted Microscope; Carl Zeiss, Jena, Germany). A list of primary antibodies used is listed in the supplemental online data Table 3.

DNA extraction and HLA typing

DNA extraction from peripheral blood mononuclear cells (PBMCs) was performed using PureLink Genomic DNA Mini Kit (Invitrogen) by following manufacturer's instructions. Extracted DNA was sent for HLA-A, -B, and –DRB typing at the UCLA Immunogenetics Center (UCLA, Los Angeles, CA, USA).

Statistics

Results are presented as means ± standard deviations and means ± standard error. The statistical significance of differences for all gene expression analysis was evaluated by using SPSS 20 (IBM Corporation, Chicago, IL, USA). Analysis of variance, *t* test for independent samples, and Kruskal-Wallis non-parametrical one-way analysis of variance tests were considered statistically significant with a *P* value of less than 0.05.

Table 5.1: RT-PCR Primer List

Gene Name	Abbreviation	Forward Primer 5'-3'	Reverse Primer 5'-3'	Amplicon Length (BP)
Oligodendrocyte transcription factor 2	Olig2	CTCCTCAAATCG CATCCAG	ACTTCTCGCTTT TGGTGAGG	212
Platelet-derived growth factor alpha	PDGFRα	CCTGAAAAGGG TCAGAAGGA	GTGGTTGAATG CCAACCCTG	352
NKX homeobox 2	NKx2.2	TGGCCATGTAAA CGTTCTGA	GCCGAATAGCT GAGCTCCAA	312
Myelin-associated glycoprotein	MAG	TATGATTTCAGG TAACGGCTGAC	AAGTACCAGAC ACCATGCAC	203
Albumin	Alb	ACCCCAAGTGTC AACTCCAA	CTGAAAAGCAT GGTCGCCTG	224
Alpha-1-antitrypsin	AAT	AAGGACACCGA GGAAGAGGA	CTTGGAGAGCT TCAGGGGTG	393
Tryptophan 2,3 dioxygenase	TDO2	TGGAGACGATG ACAGCCTTG	TCCAGAAGTGT CTTTTCCTGCT	214
Cytochrome P450	Cyp3A4	GGTGGTGAATG AAACGCTCAG	GGTCCACTTCC AAAGGGTGT	243
GATA Binding Protein	GATA4	TCCAAACCAGA AAACGGAAG	AAGACCAGGCT GTTCCAAGA	352
T-box2	TBX2	AGTGGATGGCT AAGCCTGTG	ACGGGTTGTTG TCGATCTTC	249
Wilms tumor 1	WT1	GGGCAGAGCAA CCACAGCACA	GCCACCGACAG CTGAAGGGC	469
Phospholamban	PLN	ACAGCTGCCAA GGCTACCTA	GCTTTTGACGTG CTTGTTGA	191

Table 5.2: QPCR Primer List

| Gene Name	Abbreviation	Forward Primer 5'-3'	Reverse Primer 5'-3'	Probe #
HORMA domain-containing protein 1	HORMAD1	atgatctttgtgtcaaa atactgagag	tctgtaaagcatcat aacatcctagc	61
Zymogen granule membrane protein 16	ZG16	cctcagcctctggca atg	gttgccagaatga gagaatcg	31
Kinase insert domain receptor	KDR	gacaggaagaccaa gaaaagaca	tccaggtttcctgtg atcg	27
Mix paired-like homeobox	MIXL1	ccggagattatcctca acca	agagatgcccccct ccaac	34
Nodal homolog	NODAL	taaagccattgtctag ttctcctg	ggtcctcttggatg agttcct	13
Snail homolog 1	SNAIL	aggatctccaggctc gaaag	tcggatgtgcatctt gagg	64
Wingless-type MMTV integration site family, member 3	WNT3	tggaactgcaccacc ataga	gtgaacgaaggcc gactc	64
Catenin, beta interacting protein 1	Catenin	gcctttggaagggtct ctct	tgtcccagtggcat ttcata	64
Chemokine receptor 4	CXCR4	gcactcacctctgtga gcag	atgtccacctcgctt tcct	55
Forkhead box A2	FOXA2	cgttccgggtctgaa ctg	tgcccttccatcttc acc	68
Goosecoid homeobox	GSC	cctccgcgaggaga aagt	cgttctccgactcct ctgat	29
Sex determining region Y-box 7	SOX7	ttcctcaccagccag gtc	atttgcgggaagtt gctcta	73
Chordin	CHRD	aggaaatggctccct gatct	ggcttggtctccag tgtcat	77
Neural cell adhesion molecule 1	NCAM1	agacgcagccagtc caag	tgcttgatcaggttc actttaataga	55
Nestin	NES	tgcgggctactgaaa agttc	ctgagcgatctgg ctctgta	2
NOTCH 1	NOTCH	ggcaatccgaggact atgag	ctcagaacgcact cgttgat	2
Paired box 2	PAX2	ttccgtgttttcgctttt c	agcgacagagac ggagagag	55
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	agccacatcgctcag acac	gcccaatacgacc aaatcc	60
Hypoxanthine phosphoribosyltransferase 1	HPRT1	tgaccttgatttattttg catacc	cgagcaagacgtt cagtcct	73

Table 5.3: List of antibodies for ICC

| Protein	Isotype	Clone	Manufacturer
Brachyury	Goat, IgG	Not available	R&D Systems
PAX6	Mouse, IgG1	AD1.5	Santa Cruz Biotechnology
SOX17	Goat, IgG	Not Available, Polyclonal	R&D Systems
A2B5	Mouse, IgM	A2B5-105	Millipore
NG2	Mouse, IgG2a	9.2.27	eBioscience
O4	Mouse, IgM	O4	R&D Systems
Olig1	Mouse, IgG2b	Not available	Millipore
SOX10	Rabbit, IgG	Not available, Polyclonal	Abacm
PDGFRα	Rabbit, IgG	Not Available, Polyclonal	Abcam
O1	Mouse, IgM	O1	R&D Systems
MBP	Rat, IgG2a	12	Abcam
Mitochondria	Mouse, IgG1	113-1	Millipore
Albumin	Mouse, IgG2a	188835	R&D Systems
CK18	Mouse, IgG	DC10	DAKO
α-Fetoprotein	Mouse, IgG	ZSA06	Invitrogen
α-actinin	Mouse, IgG1	EA-53	Sigma
GATA-4	Mouse, IgG	H-112	Santa Cruz Biotechnology
Troponin1	Mouse, IgG2b	C5	Millipore
COL3A1	Mouse, IgG1	B-10	Santa Cruz Biotechnology

Figure 5.3
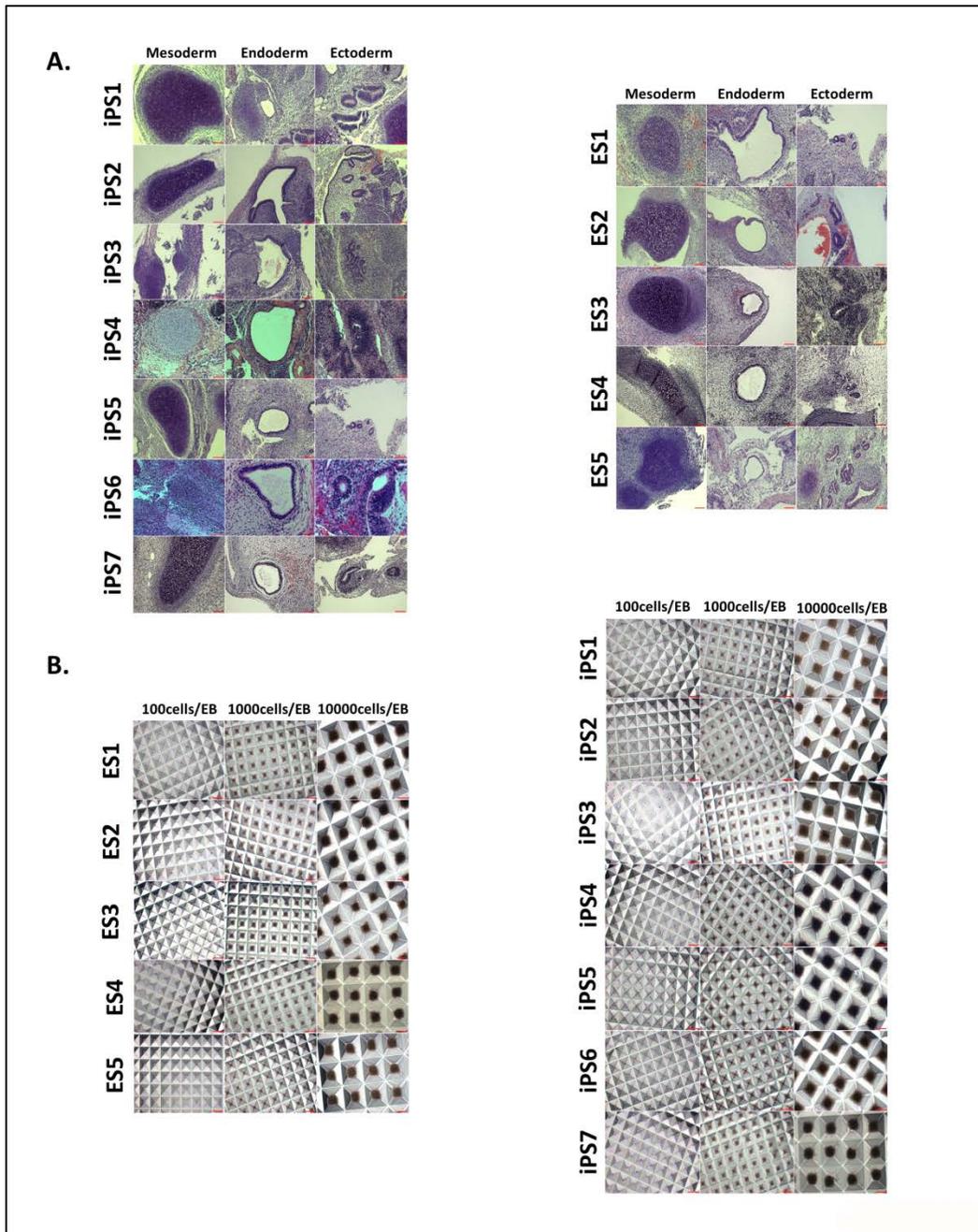
Figure 5.3. Related to Figure 5.1A and 5.1E. (A) Testicular teratomas H&E staining for all hESC and hiPSC lines all displaying key characteristics of the three germ layers e.g. cartilage (mesoderm), gut epithelium (endoderm) and neural tube (ectoderm). Scale bar=100µM. (B) EB images showing three different sizes (100cells/EB, 1000cells/EB, and 10000cells/EB). Scale bar=200µM.
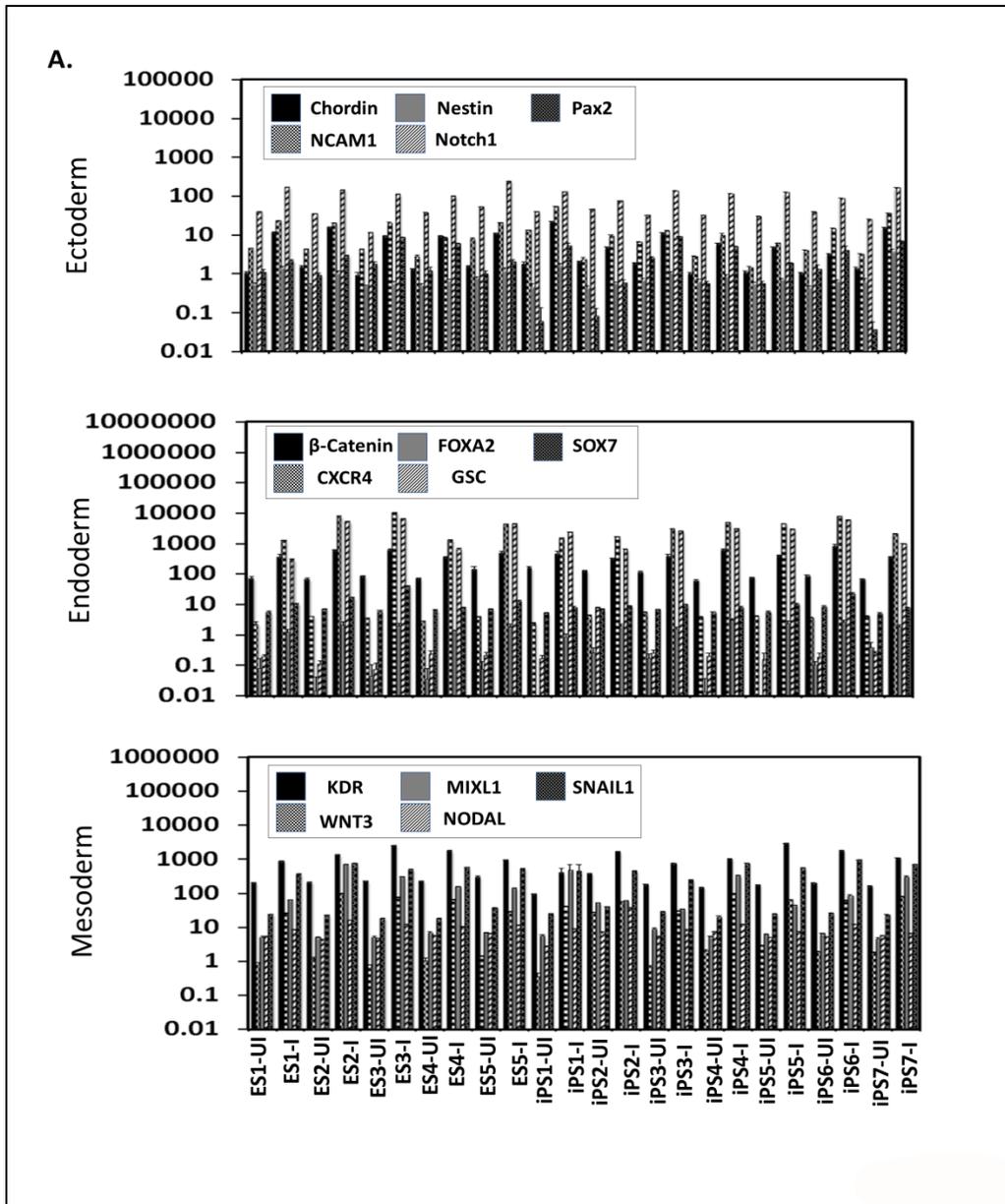
Figure 5.4

Figure 5.4. Related to Figure 5.1G and 5.1H. (A) Quantitative real time PCR analysis was used to show characteristic lineage specific gene expression of ectoderm (Chordin, NCAM1, Nestin, Notch1, and Pax2), endoderm (β-Catenin, CXCR4, FOXA2, GSC, and SOX7), and mesoderm (KDR, WNT3, MIXL1, NODAL, and SNAIL1) of 5 hESC and 7 hiPSC lines. Expression levels were normalized to two housekeeping genes (HPRT1 and GAPDH). Error bars represent ± SD. Y-axis is a logarithmic scale with display units of 10^5, 10^6, or 10^7. All PCRs were run in triplicate.

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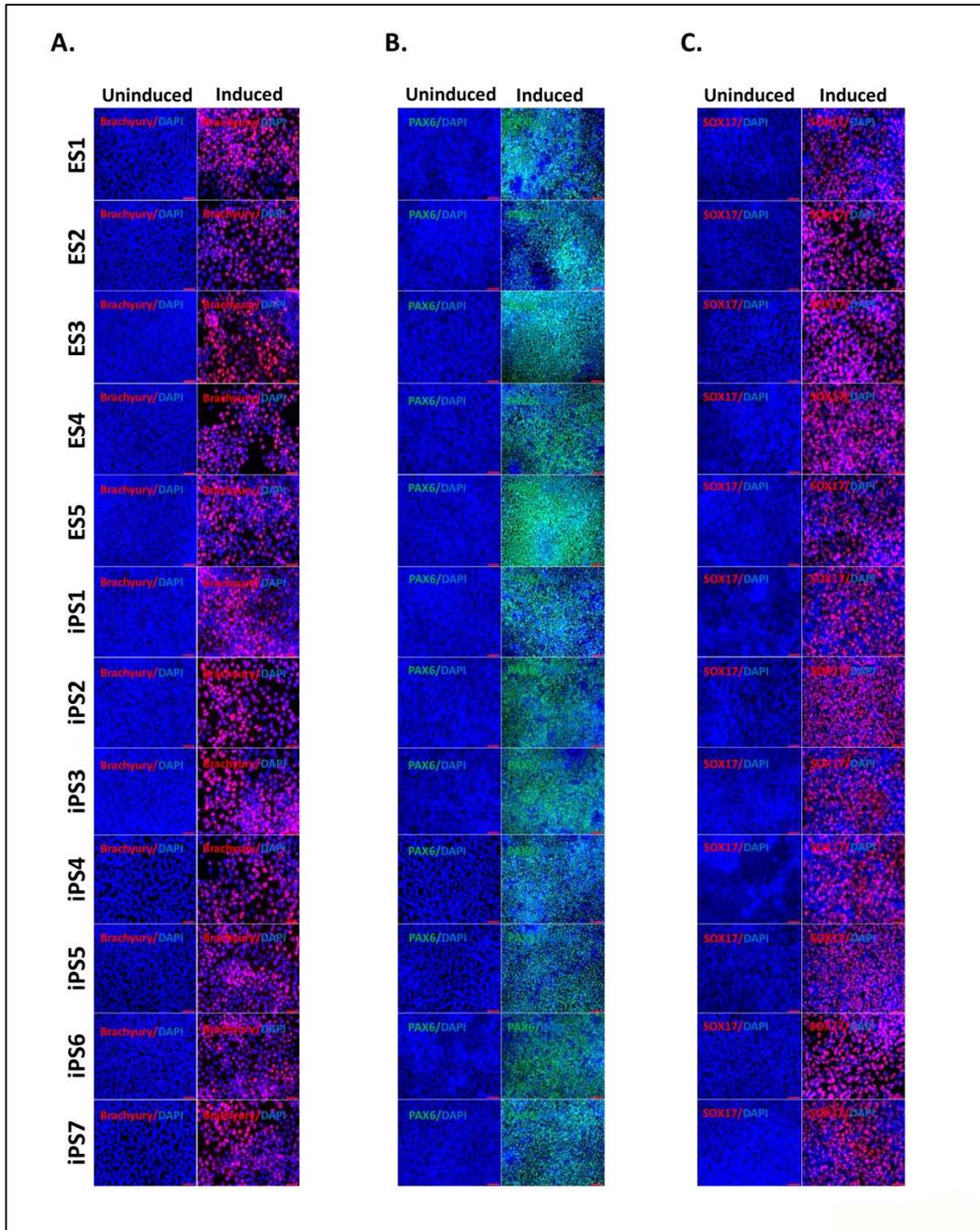
Figure 5.5

Figure 5.5. Related to Figure 5.1G and 5.1H. (**A-C**) Immunofluorescence staining with lineage specific markers after hESCs and hiPSCs were differentiated into representatives of the three germ layers. (**A**) Staining for mesoderm with Brachyury, (**B**) staining for ectoderm with PAX6, and (**C**) staining for endoderm with SOX17. Scale bar=50μM.

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