Characterization of Novel Riverside Human Stem Cell Lines

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ABSTRACT

Induced pluripotent stem cells (iPSCs) can be derived by transducing somatic cells with transcription factors expressed in embryonic stem cells (ESCs). We have derived novel lines of human iPSCs, designated as Riv (Riverside) from adult fibroblast cells. Riv human iPSCs were able to self-renew in culture, displayed morphology similar to that of human ESCs, and expressed human ESC-specific markers. Here we report in vitro differentiation potential from two different Riv lines, Riv9 and Riv19. Two tested Riv lines differed in their efficiency in generating lineage-specific cell types. Further differences were evident when primordial germ cell (PGC) specific transcription factors, BLIMP1 and STELLAR were found to be highly expressed in Riv9 embryoid bodies (EBs). Hence, differences in developmental potential amongst human iPSC lines have warranted screening and characterization of other Riv clones. We further demonstrated that bone morphogenetic proteins (BMPs) induced differentiation of premigratory and migratory PGCs in Riv9. Human iPSCs can therefore produce germ cells in vitro, despite their fibroblast origin.

MENTOR

Duncan Liew
Academic Coordinator, UCR Stem Cell Core

Yuri Cheung joined the Stem Cell Core as a CNAS STEM Pathway Scholar. I had an excellent first impression of Yuri; as an undergraduate, she exhibited, or even surpassed capability and quality of many graduate students. Her previous research experience in molecular biology and cell culture facilitated her progress in stem cell research. She consistently expressed strong enthusiasm in learning new techniques in the exciting field of human embryonic stem cells. UCR Stem Cell Core has derived various human induced pluripotent stem (iPS) cell lines from fibroblast cells, bypassing the use of human embryos in deriving stem cell lines. She actively participated in characterizing these novel Riverside (Riv) iPS cell lines, and also attempted to differentiate these cell lines to functional cell types, including neurons and germ cells with great success. Her work published in this volume of the UCR Undergraduate Research Journal provides data on our Riv human iPS cell lines and demonstrates that germ line cells can be derived from iPS cell lines, despite their fibroblast origin. Her hard work and dedication also landed her a second authorship in one of the articles that our lab has published in a peer reviewed scientific journal.

AUTHOR

Yuri Cheung
Biology

Yuri Cheung is a graduating senior majoring in Biology. She is a member of UC LEADS and CNAS STEM Pathway Program. Prior to joining the UCR Stem Cell Core, Yuri studied water strider acrosome formation and sperm motility with Dr. Richard Cardullo, and interned with Dr. Jane Gitschier at University of California, San Francisco (UCSF). In the Core, she assisted in deriving multiple human induced pluripotent stem (iPS) cell lines (designated as Riv, Riverside iPS cell lines), and has induced their differentiation into three germ layers and germ line cells. Before graduation, she will participate in deriving neuronal progenitors from Riv iPS cells, which have a great potential in cell-based therapy for neurodegenerative diseases. Next year, Yuri will join the UCR/UCLA Thomas Haider Program in Biomedical Sciences.
INTRODUCTION

One of the breakthroughs in regenerative medicine is the ability to reprogram adult fibroblast cells back to the embryonic state, bypassing the use of human embryos to derive embryonic stem cells (ESCs; Takahashi and Yamanaka, 2006). Like ESCs, human induced pluripotent stem cells (iPSCs) are truly remarkable cells because of their dual abilities to proliferate indefinitely and give rise to all cell types in the body. Their ability to self-renew and differentiate defines them as pluripotent. Human ESCs and iPSCs offer substantial opportunities for providing an unlimited renewable source for cell transplantation targeted to treat several diseases. In addition, human iPSCs, being autologous cells, open up new opportunities for disease and patient modeling, toxicity screening, and patient-specific cell replacement therapy.

Although widely perceived as cell types of equivalent developmental stage, the precise relationship and similarities between human ESCs and iPSCs remain uncertain. Several recently published studies have pointed out marked differences between human ESCs and iPSCs. For example, iPSCs potentially retain their epigenetic memory during in vitro differentiation (Polo et al., 2010). Furthermore, human ESCs express several transcription factors commonly found in germ cells, leading to the notion that human ESCs closely resemble germline cells in early embryo (Zwaka and Thomson, 2005). Previous studies have reported that human ESCs are capable of differentiating into germ cell lines (Kee et al., 2009). However, it is unclear whether iPSCs, due to their somatic cell origin, have the same potential to differentiate into germ cells.

Here, we sought to characterize the newly derived Riverside (Riv) human iPSC lines. We show that Riv lines employ a similar pluripotency regulatory network as human ESCs. Further, our results confirmed the hypothesis that Riv iPSCs are capable of differentiating into three germ layers and germline cells.

MATERIALS AND METHODS

Derivation of Human Induced Pluripotent Stem Cells (iPSCs)

Foreskin fibroblast cells (ATCC) were propagated in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1mM Glutamax-I and 1mM non-essential amino acid (NEAA). 293FT cells were used as a packaging cell line for generating retroviruses. 293FT were transfected with FuGENE HD with pMXS-OCT4, -SOX2 or -KLF4 plasmid, pHIT60 packaging and pVSV-G envelope construct. Media containing retroviruses were collected two days post-transfection. Foreskin fibroblast cells were infected with retroviruses and maintained in 5% O2 incubator. Medium was changed to human iPSC medium (KnockOut DMEM/F12 supplemented with KnockOut Serum Replacement, 1mM Glutamax-I, 1mM NEAA, 55mM 2-mercaptoethanol and 10ng/ml FGF2). Human iPSC colonies were picked using 200ul pipette tips four weeks post-transduction and maintained on matrigel as feeder-free cultures in StemPro medium (Invitrogen). For subcultivation, human iPSCs were treated with accutase (Invitrogen) for 1 min, harvested by centrifugation, and replated onto new matrigel-coated dishes in StemPro medium. All cell lines were maintained in 37°C and 5% CO2.

Differentiation of Human iPSCs

iPSCs were first detached from monolayer and were differentiated in non-tissue culture grade petri dishes. Alternatively, iPSCs were differentiated using known growth factors to direct differentiation to a specific lineage. For definitive endoderm differentiation, iPSCs were cultured in RPMI1640 medium with 50 ng/ml Activin A and 50 ng/ml Wnt3a (both from R&D Systems). Bone morphogenetic protein-4, -7, and -8b were included in DMEM and 10% FBS for primordial germ cell differentiation.

Quantitative Real Time Polymerase Chain Reaction (Q-PCR) Analysis

Total RNA was isolated from undifferentiated human iPSC (day 0) and embryoid bodies (day 5 and day 10)
using BioRad RNA kit. Extracted RNA was treated with DNaseI to remove genomic DNA contamination from samples. First-strand cDNA synthesis was performed using Oligo dT primers and the iScript (BioRad). Q-PCR was performed using the Assay-on-Demand technology (Applied Biosystems). Each reaction consisted of 5 μL of 2x TaqMan master mix, 1 μL of gene-specific probes, 3 μL of water, and 1 μL of diluted cDNA (1:20). PCR amplifications were initiated at 95°C for 10 min followed by 42 cycles of 95°C for 15 seconds and 60°C for 60 seconds. PCR reactions for each sample were performed using 384-well real-time thermocycler (BioRad) and were repeated three times. The Q-PCR data were analyzed using the comparative Ct method.

**Immunocytochemistry Analysis**

Cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 10 mins. Cells were then washed three times in PBS and blocked with PBS with 0.1% Triton-X and 1% blocking serum for 30 min. Following blocking, cells were incubated with primary antibodies in blocking solution overnight: anti-FOXA2, anti-NANOG (Cell Signaling Technology; 1:100), anti-β-III tubulin (Covance; 1:1000) or anti-OCT4 (Santa Cruz; 1:50). Cells were then washed in blocking solution and incubated with Alexa488 or Alexa594 secondary antibodies (Molecular Probes; 1:500) for one hour at room temperature, followed by two washes with blocking solution and third wash with PBS. Cells were mounted with DAPI mounting reagent (Vectorshied). Images were captured with a Nikon Eclipse Ti microscope.

**RESULTS AND DISCUSSION**

**Characterization of Novel Riv Human Induced Pluripotent Stem Cell (iPSC) Lines**

Human foreskin fibroblasts were infected with retroviruses encoding transcription factors OCT4, SOX2 and KLF4 (Fig. 1A). We eliminated the use of retrovirus encoding cMYC due to its role as an oncogene (Nakagawa et al., 2008). Five days after transduction, fibroblast cells were transferred to feeder layers and cultivated in human iPSC culture media supplemented with fibroblast growth factor-2 (FGF2) to enhance reprogramming process. Human iPSC colonies started to appear approximately two weeks post-transduction.

![Figure 1. Generation of iPSCs from human foreskin fibroblast.](image)

(A) Foreskin fibroblasts were infected with retroviruses encoding transcription factors OCT4, SOX2, and KLF4. TRA-1-60+ colonies appeared two weeks post-transduction. (B) iPSCs can be identified by alkaline phosphatase activity and staining with an antibody recognizing stage specific embryonic antigen-3, SSEA3. These iPSCs displayed morphology indistinguishable from that of human ESCs. (C) Immunocytochemistry analysis of OCT4 and NANOG in Riv human iPSCs. Scale bars: 100 um.

We performed live cell staining with an antibody recognizing human embryonic stem cell surface antigen, TRA-1-60. These TRA-1-60+ colonies were handpicked and propagated in culture (Fig. 1B). We have named these iPSC lines ‘Riv’ (Riverside) lines. We have maintained these Riv iPSC lines for over ten passages. Riv iPSCs demonstrated morphology indistinguishable from that of human embryonic stem (ESC) cells: iPSC colonies have distinct borders and tight cell-to-cell junctions within the colonies. Human iPSCs also demonstrated high nuclear:cytoplasm ratio with prominent nucleoli. Fully reprogrammed iPSCs can also be identified by alkaline phosphatase activity.

To further confirm our iPSCs are *bona fide* pluripotent stem cells, we verified the immunoreactivity to antibodies
recognizing the human ESC-specific marker, stage specific embryonic antigen-3, SSEA3. Immunocytochemistry analysis also revealed the expression of human ESC-specific transcription factors OCT4 and NANOG in the cell nuclei of Riv human iPS Cs. Taken together, our data demonstrate that newly derived Riv iPS Cs closely resemble undifferentiated human embryonic stem cells.

**In vitro Differentiation of Riv Human iPSC Lines**

Next, we sought to elucidate differentiation potential of our human iPS Cs. We have used two human iPSC lines, Riv9 and Riv19 in this study. We first employed embryoid body (EB) differentiation model, whereby iPS Cs were differentiated in non-tissue culture-treated petri dishes in the absence of FGF2 following their detachment from monolayer cultures. When iPS Cs are transferred as small clumps into suspension cultures, they develop into aggregates called embryoid bodies (EBs; Fig. 2A). EB formation in many ways recapitulates the three-dimensional complexity of the embryo, resulting in the formation of progenitors representative of the three germ layers (Liew et al., 2005). Withdrawal of FGF-2 and suspension cultures resulted in the rapid differentiation of iPS Cs.

Quantitative real time polymerase chain reaction (Q-PCR) analysis indicated the loss of OCT4, SOX2, cMYC and NANOG, all of which are iPS markers, upon differentiation, and upregulation of neuroectodermal (paired box gene 6, PAX6), liver (α-fetoprotein, AFP), and cardiac (myosin heavy chain-6, MHC6) lineages (Fig. 2B). We detected β-III tubulin-expressing cells, indicative of the presence of neurons in differentiated cultures (Fig. 3A). Activin A and Wnt3a induced the formation of FOXA2-expressing cells (Fig. 3B), suggesting that Riv9 cells were responsive to Nodal signaling and were able to give rise to definitive endoderm cell types (Liew, 2010). Thus our data indicate that Riv human iPSCs are capable of generating cell types representative of three germ layers.

![Figure 2](image_url). In vitro differentiation of human iPS Cs in embryoid bodies (EBs). (A) Morphology of undifferentiated human iPSCs, and differentiating EBs at day-5 (d5) and -10 (d10) derived from Riv iPS Cs. d5 EBs typically consisted of hundreds of cells. d10 EBs grew larger in size and contained thousands of cells. (B) Q-PCR analysis during differentiation. Data are presented as relative expression levels in EBs compared to undifferentiated cells (day 0). Scale bars: 100 um.

![Figure 3](image_url). Immunocytochemistry analysis of differentiated progeny derived from human iPS Cs. (A) β-III tubulin-immunoreactive neurons derived from human iPSCs. (B) Activin A and Wnt3a-induced iPS Cs gave rise to FOXA2-expressing definitive endoderm cells. There was a reduction of SOX2+ cells in the differentiated culture. Scale bars: 100 um.
Primordial Germ Cell Derivation from Riv Human iPS Cells

Primordial germ cells (PGCs) are the founding population of germline cells and gametes. Various studies have revealed several key transcription factors involved in early germline development in mouse embryo. Nevertheless, germ cell specification in human is not well understood due to the scarcity of human embryos available for research and potential ethical problems.

We thus sought to elucidate germ cell differentiation in Riv9 iPS by examining PGC-related transcript expression in EBs. In the mouse embryo, B-lymphocyte induced maturation protein-1 (BLIMP1) is shown to be the master regulator of the earliest germ cell formation (Ohinata et al., 2005). PGCs migrate into the genital ridge and can be identified by SSEA1 and cKIT expression. Q-PCR analysis revealed an upregulation of BLIMP1 mRNA at day 5, suggesting the appearance of PGCs during iPSC differentiation (Fig. 4A). BLIMP1 expression was later downregulated in d10 EBs. Genes encoding cKIT receptor and STELLAR nuclear protein, essential to the process of germ cell migration, were significantly upregulated in d10 EBs. Our data thus support the notion that differentiated germ cell progenitors have undergone maturation at the later stage of EB differentiation. In addition, we tested whether PGCs can be derived from Riv9 iPSCs with bone morphogenetic proteins (BMP4, 7 and 8b), known inducers of PGC formation in mouse embryo. We noted expression of BLIMP1 and SSEA1 proteins in BMPs-induced iPSCs, consistent with the roles of BMPs in inducing differentiation of iPSCs into PGCs (Fig. 4B).

Figure 4. Generation of primordial germ cells (PGCs) from iPSCs. (A) Q-PCR analysis during EB differentiation. There was a dramatic increase in BLIMP1 mRNA expression with the highest level in d5 EBs. STELLAR and cKIT were upregulated at a later stage of differentiation (d10 EBs). (B) Small number of BLIMP1-expressing pre-migratory PGCs emerged in BMPs-induced culture (arrowheads). Arrows indicate BLIMP1 and OCT4 co-expressing cells, indicative of putative migratory germ cells. (C) Appearance of PGCs is further confirmed by positive immunoreactivity of SSEA1 in BMPs-treated cells (arrows). DAPI is used to counterstain the cell nuclei. Scale bar: 100 um.
CONCLUSIONS

Riv human iPSC lines exhibit essential characteristics of human embryonic stem cells. These cells are pluripotent: Riv iPSCs were able to self-renew, express embryonic stem cell-specific genes and surface markers, and maintain the developmental potential to differentiate into tissue-specific cell types. Furthermore, Riv iPSCs were capable of producing germline cells despite their origin outside the germline. Derivation of germ cells from Riv iPSCs could provide a platform for studying epigenetic modification and genomic imprinting in germ cells and thus facilitating our understanding of the mechanisms underlying germline and gamete generation in adult and in human fetus.

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REFERENCES


