Please cite this article in press as: Kang et al., iPS Cells Can Support Full-Term Development of Tetraploid Blastocyst-Complemented Embryos, Cell Stem Cell (2009), doi:10.1016/j.stem.2009.07.001

Cell Stem Cell
Brief Report

iPS Cells Can Support Full-Term Development of Tetraploid Blastocyst-Complemented Embryos

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DOI 10.1016/j.stem.2009.07.001

To our knowledge, for the first time, we demonstrate that induced pluripotent stem cells (iPSCs) can autonomously generate full-term mice via tetraploid blastocysts complementation. Differentiated somatic cells can be reprogrammed into iPSCs by forced expression of four transcription factors—Oct4, Sox2, Klf4, and c-Myc. However, it has been unclear whether reprogrammed iPSCs are fully pluripotent, resembling normal embryonic stem cells (ESCs), as no iPSC lines have shown the ability to autonomously generate full-term mice after injection into tetraploid blastocysts. Here we provide evidence demonstrating that an iPSC line induced by the four transcription factors can be used to generate full-term mice from complemented tetraploid blastocysts and thus appears to be fully pluripotent. This work serves as a proof of principle that iPSCs can in fact generate full-term embryos by tetraploid complementation.

Reprogramming of differentiated somatic cells into iPSCs via expression of four defined transcription factors has attracted great scientific and public attention, and the generation of iPSCs from individual patients has raised hopes for treating many degenerative and genetic diseases (Dimos et al., 2008; Ebert et al., 2009; Hanna et al., 2007; Park et al., 2008; Soldner et al., 2009; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2008; Yu et al., 2007). iPSCs can functionally differentiate into all cell lineages within chimeric mice, including germ cells that can ultimately give rise to offspring (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). However, unlike ESCs or even somatic cell nuclear transfer (SCNT)-produced NT-ESCs, no iPSC lines have so far fulfilled the most stringent pluripotency criterion of being able to generate full-term mice via tetraploid blastocyst complementation (Hayashi and Surani, 2009; Jaenisch, 2008; Maherali and Hochedlinger, 2008; Wernig et al., 2007). To understand the reprogramming mechanisms that occur during iPSC generation, it is important to address whether iPSCs are fully pluripotent.

As in previous reports (Brambrink et al., 2008; Stadtfeld et al., 2008), doxycycline (Dox)-controlled Tet-on-inducible lentiviruses carrying cDNAs of the transcription factors Oct4, Sox2, Klf4, and c-Myc were transduced into mouse embryonic fibroblast (MEF) cells from ROSA26-M2rtTA transgenic mice (Figure 1A). After viral transduction, the MEFs were cultured in ES culture medium containing Dox for 12 days until ES-like colonies appeared, and then the Dox was removed from the culture medium (Figure 1B). Four days later, ES-like colonies were individually digested and replated in 24-well plates. After propagation, we selected five iPSC lines that exhibited typical ESC morphology (Figure 1C) for long-term culture and characterization. The ES-like colonies generated were positive for AP activity (see Figure S1 available online). RT-PCR and qPCR analysis demonstrated that the expression of pluripotency marker genes, including Oct4, Sox2, and Nanog, was comparable across all five iPSC lines and normal ESC lines but showed dramatic differences relative to MEFs (Figures 1D and 1E). Silencing of the exogenous lentiviral vectors was also observed (Figure S2). Immunofluorescent staining further confirmed the protein expression of three transcription factors and the surface marker SSEA-1 in the iPSCs (Figure 1F). In addition, other pluripotency-related genes were all expressed at similar levels in the iPSCs and normal ESCs (Figure S3). The karyotypes of all five iPSC lines were normal, with 40 chromosomes (Figure S4). Southern blot analysis was used to examine viral integration into the genome of iPSCs and indicated that there were one or two integrations for each of the four lentiviral constructs into the iPS genome (Figure 1G).

To investigate the pluripotency of the generated iPSCs, we performed in vitro differentiation and teratoma and chimera experiments. The iPSCs formed EBs efficiently in vitro, and upregulation of marker genes for all three germ layers was detected in the plated EBs (Figure S5). We then transplanted the iPSCs subcutaneously into immune-deficient SCID mice and found that all the iPSC lines tested generated teratomas 3–4 weeks after transplantation. Hematoxylin and eosin (H&E) staining indicated that the iPSCs could differentiate into a variety of cell types from all three germ layers (Figure 1H). The LiPS-6 line generated postnatal chimeras with normal appearance that gave rise to germline offspring after mating with ICR female mice (Figure 1I). However, we could not produce full-term mice from this line via tetraploid complementation. Meanwhile, we noticed a very high degree of chimerism in the chimeras generated from one iPSC line, LiPS-8, as indicated by its chimera coat color, which was almost completely contributed by iPSC (Figure 1J). We therefore decided to test whether these iPSCs were capable of generating full-term mice through tetraploid complementation.

We first generated tetraploid embryos by fusing late two-cell-stage embryos to one-cell-stage embryos using an Electro Cell Manipulator (BTX 2001). The embryos were collected from B6D2F1 female mice mated with male mice of the same strain. Ten to fifteen iPSCs were microinjected into the cavity of tetraploid blastocysts, and the complemented blastocysts (n = 200) were transferred into seven pseudopregnant ICR female mice after 2–3 hr of recovery. On the due date, a C-section was performed to ascertain whether any of the tetraploid-complemented embryos

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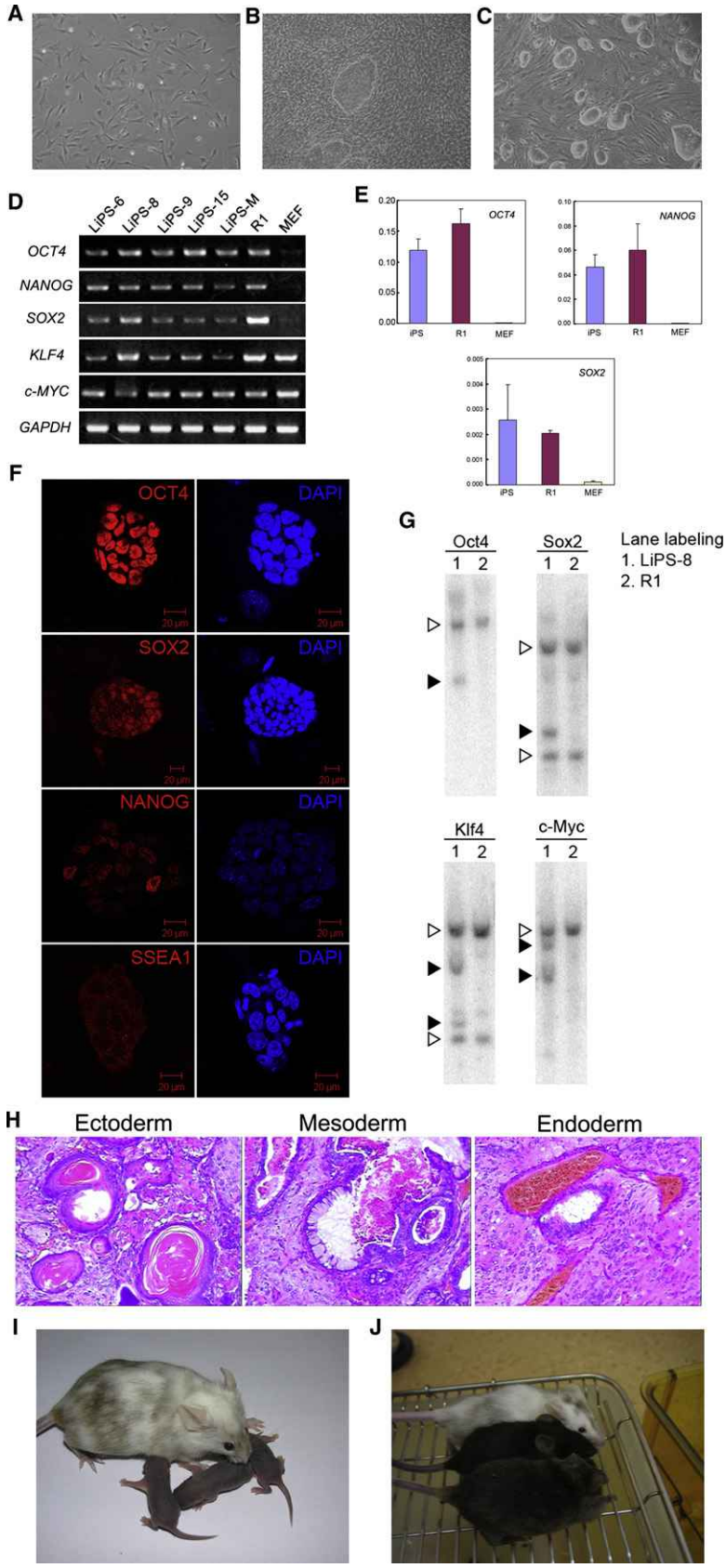

Figure 1. Generation of Inducible iPSCs and Characterization

(A) Morphology of MEF cells collected from ROSA26-M2rtTA transgenic mice.

(B) Colonies formed after infection and Dox induction.

(C) Morphology of iPSCs after propagation.

(D) RT-PCR revealed that inducible iPSCs expressed ESC marker genes including *OCT4*, *SOX2*, and *NANOG*.

(E) qPCR revealed that the expression level of ESC marker genes in iPSCs was similar to that of normal ESCs.

(F) Immunofluorescent staining results demonstrated that the iPSCs were positive for OCT4, SOX2, NANOG, and SSEA-1. Scale bars, 20 μm.

(G) Southern blot analysis showed that there were one or two integrations for each virus into the iPS genome.

(H) Teratomas formed 4 weeks after injection of iPSCs into SCID mice. Tissues of the three germ layers were detected by H&E staining.

(I) The chimeric mouse showed germline transmission capability.

(J) High chimerism (>90%) was found in the chimeric mice produced from a particular iPSC line (LiPS-8).

had developed into live animals. As shown in Figure 2A, we produced two full-term mice from the iPSC-complemented tetraploid blastocysts. One pup (Figure 2Ab) was overweight (2.72 g) and died within 1 hr following the C-section. The other pup (Figure 2Aa) was of normal weight (1.36 g) and survived for 3 days but died because the surrogate mother refused to feed it. To test whether the fully iPSC-derived mouse can survive to adulthood, we repeated this experiment with tetraploid blastocysts from ICR mice. After transplantation of 187 tetraploid-complemented blastocysts into the oviducts of seven pseudopregnant mice, we successfully produced two more full-term pups from these embryos (Figure 2A), and one pup survived into adulthood and appears to be healthy (Figure 2B). The efficiency of generating full-term mice from iPSC-complemented ICR tetraploid embryos was similar to the B6D2F2 background embryos (1.07% versus 1.00%, Figure 2F). We then performed SSLP and rtTA analysis of the organs collected from the three dead pups and the tail tip of the live mouse. The results clearly indicated that all the pups were derived from the iPSCs (mostly C57BL/6 background) and not from the tetraploid embryos (B6D2F2 or ICR background) (Figures 2C–2E). Moreover, we successfully cultured skin fibroblast cells from the pups that died shortly after the C-section. After adding Dox to

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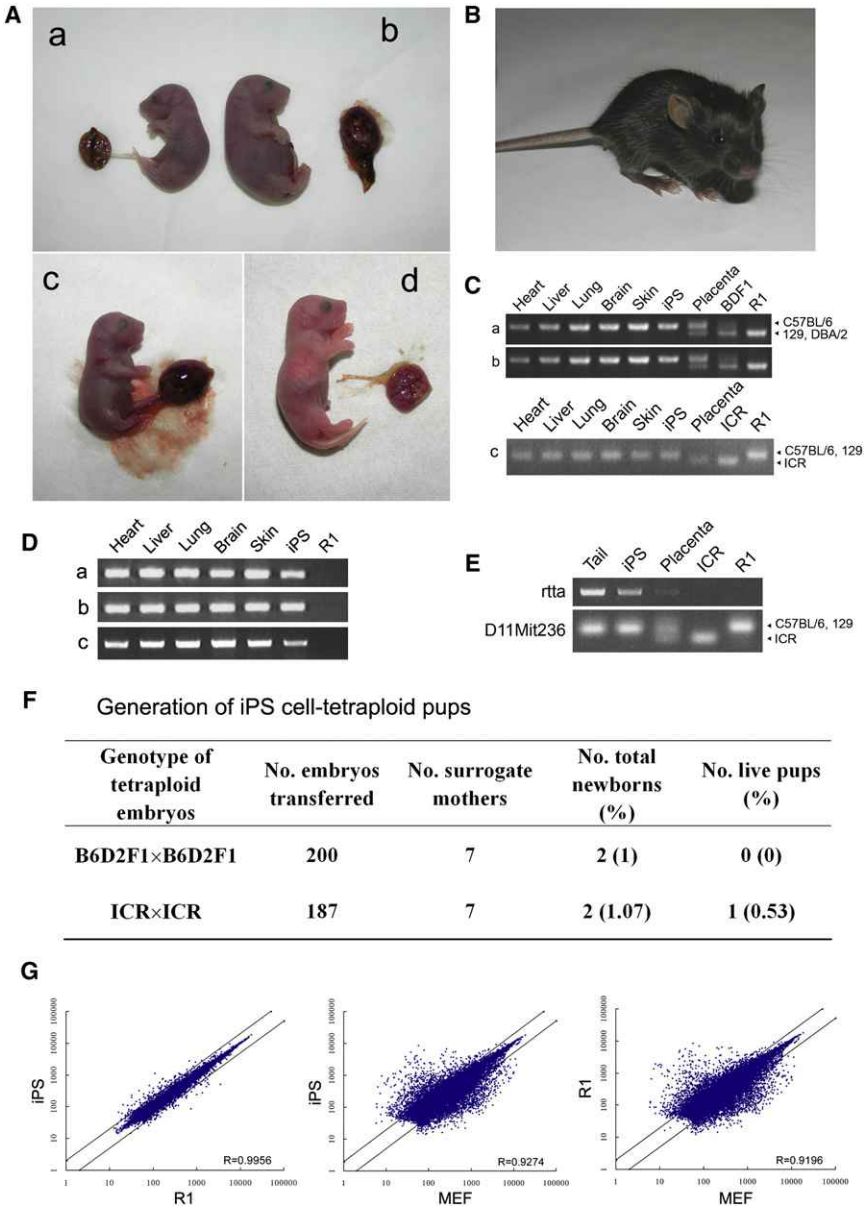

F Generation of iPS cell-tetraploid pups

Genotype of tetraploid embryos	No. embryos transferred	No. surrogate mothers	No. total newborns (%)	No. live pups (%)
B6D2F1×B6D2F1	200	7	2 (1)	0 (0)
ICR×ICR	187	7	2 (1.07)	1 (0.53)

Figure 2. Generation of iPS-Tetraploid Mice

(A) Four pups were produced autonomously from iPSCs complemented with tetraploid blastocysts collected from either B6D2F1 mice or ICR mice. One pup from iPS-B6D2F1 tetraploid complementation died 3 days after C-section because the surrogate mother refused feeding (Aa), one overweight pup from iPS-B6D2F1 tetraploid complementation died (Ab), one pup from iPS-ICR tetraploid complementation died shortly after C-section (Ac), and one pup from iPS-ICR tetraploid complementation survived (Ad).

(B) The pup (Ad) generated from iPS-ICR tetraploid complementation grows into an adult.

(C) SSLP analysis of organs of three dead tetraploid-complemented pups (Aa–Ac). D6Mit15 and D11Mit236 were used.

(D) Genomic PCR analyzed the transgene, rtTA, in the organs of three dead tetraploid-complemented pups.

(E) Genomic PCR and SSLP were performed to analyze the live adult mouse derived from iPS-tetraploid complementation. Transgene rtTA and D11Mit236 were analyzed, respectively.

(F) Efficiency of generation of iPSC-tetraploid-complemented pups. The efficiency is lower than the efficiency of using normal B6D2F1 ESCs (Table S1).

(G) Scatter plots comparing global gene expression patterns between iPSCs and ESCs (left), between iPSCs and MEFs (middle), and between R1 and MEFs (right), as determined by DNA microarray. "R" indicates Pearson correlation coefficient, and the lines indicate 2-fold changes in gene expression levels between the samples.

the medium, many ES-like colonies appeared (Figure S6), and the efficiency was substantially higher than for the first round of iPS induction. As the inducible iPSC line LiPS-8 is, to our knowledge, the first iPSC line that has been used successfully to generate mice by tetraploid complementation, we investigated the gene expression profile of this particular cell line as compared with the normal ESC lines, R1, and the parental MEF cells. The data clearly showed that the gene expression profile of this specific iPS line is very similar to the normal ESCs (Figure 2G). Meanwhile, bisulfite genomic DNA sequencing analysis of the Oct4 promoter showed that it is highly unmethylated in the inducible iPSC line LiPS-8, whereas it is highly methylated in the parental MEF cells (Figure S7).

In conclusion, we have successfully produced full-term mice from iPSC-complemented tetraploid blastocysts. Although these findings are an important proof of principle, it would be premature to make claims based on them about whether iPSCs in general are functionally equivalent to normal ESCs, as we have here only identified one iPSC line that possesses this capacity, and the process is also very inefficient. It is possible that this line has specific characteristics that allow tetraploid-complementation activity but are not shared by other iPSC lines. It will be interesting to evaluate whether iPSCs generated using fewer factors or different approaches are capable of producing tetraploid-complemented mice (Kim et al., 2008, 2009; Nakagawa et al., 2008) to assist with advancing overall understanding of the molecular mechanisms involved in iPSC generation. Moreover, partial reprogramming could potentially provide an explanation for the inefficient differentiation of iPSCs into certain cell lineages, and obtaining more fully reprogrammed iPSCs in humans might facilitate directed differentiation of iPSCs into ideal cell types.

ACCESSION NUMBERS

Microarray data can be assessed at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE17004.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References,

seven figures, and one table and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00335-X.

ACKNOWLEDGMENTS

We thank Professor Rudolf Jaenisch from the Whitehead Institute of the Massachusetts Institute of Technology for generously supplying the vectors for lentiviruses containing the cDNAs of four transcription factors, and Dr. Konrad Hochedlinger for personal communications. We are also grateful to the other colleagues in our laboratory for useful help during experiments and preparation of the manuscript. This project was supported by the Ministry of Science and Technology (grants 2008AA022311, 2006AA02A101, and 2009CB941100) and the National Science Foundation of China (grant 30670302).

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