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LETTER TO THE EDITOR

Mice generated from tetraploid complementation competent iPS cells show similar developmental features as those from ES cells but are prone to tumorigenesis

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Dear Editor,

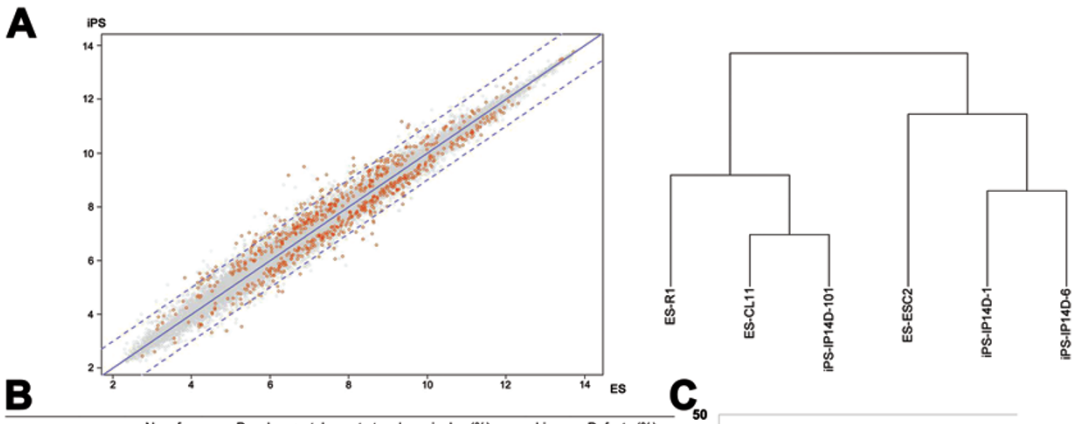
Ever since the creation of induced pluripotent cells (iPSCs) from adult somatic cells by the ectopic expression of defined transcription factors [1, 2], whether iPS cells are equivalent to embryonic stem cells (ESCs) in function and safety aspects has been a major concern regarding their potential applications. Previously, we and others have demonstrated that fully reprogrammed iPSCs were capable of producing full-term mice via the tetraploid complementation method [3-5], yet a thorough postnatal development evaluation of iPS mice is still lacking.

To characterize whether mice derived from iPSCs are equivalent to those from ESCs, we first examined the mRNA expression profiles of three ES cell lines and three iPS cell lines derived from mouse embryonic fibroblasts (MEFs) using the four Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc). We have previously shown that the external expression of the Yamanaka factors was successfully turned off in these cell lines, and they were all capable of producing healthy mice through the tetraploid complementation assay (4N-iPSCs) [3]. The morphology, karyotype, molecular markers, embryoid bodies and teratoma formation abilities of these iPS and ES cell lines were indistinguishable from each other (Supplementary information, Table S1). Hierarchical clustering of mRNA expression profiles detected by Affymetrix gene expression microarrays resulted in a mixed grouping of the iPS and ES cell lines (Figure 1A). Only 14 genes were identified to be differentially expressed between the iPS and ES cell lines using 2-fold expression change and Student's *t*-test *P*-value < 0.05 as cutoffs (Supplementary information, Table S2). The 14 genes had no functional bias and no reported roles in regulating stem cell property maintenance or differentiation.

We next analyzed the embryos derived from 4n-iPS cells and ES cells by dissecting pregnant mice at embryonic days (E) 13.5, 16.5 and 19.5 (P0), respectively. Although the survival rates of iPSC- and ESC-derived embryos were both very low, slightly higher survival rates were observed among iPSC-derived embryos at all examined time points (Figure 1B). Comparable arrest rates (dominated at E6.5 to E8.5) and same phenotypes between the iPSC- and ESC-derived embryos were observed, including resorbing decidua, resorbed embryo proper with well-formed placenta, and arrested embryos with abdomen closure failure and interstitial bleeding (Supplementary information, Figure S1). After delivery, 17 pups derived from 4N-iPS cells and 12 from ES cells had very low respiration rate and weak breath (Figure 1B), and died within half an hour, most likely due to pulmonary insufficiency caused by respiratory failure (RF). Open eyelid (OE) defect with congenital cataracts was also found among over one-fourth of the total number of iPSC- and ESC-derived pups at P0 (Figure 1B). No difference between the iPS and ES groups was observed in terms of these defects.

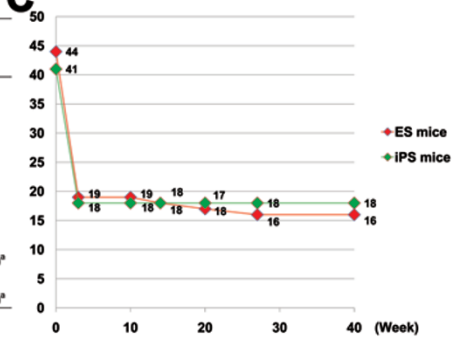
We next evaluated the postnatal growth and health conditions of the iPS pups. The six experimental cell lines produced 41 iPS and 44 ES newborn mice, of which 18 (43.9%) iPS mice and 19 (43.2%) ES mice survived to weaning (3 weeks) and grew healthily to puberty (10 weeks); 18 iPS mice and 16 ES mice were still alive at 38-40 weeks (Figure 1C). The survived animals were all fertile, and the iPS and ES groups exhibited no significant differences in progeny numbers or gender ratios (data not shown). Morphological inspection of the major internal organs of iPS, ES and control mice ($n = 5$ for each group) at age 10-12 weeks revealed no visible abnormalities in any individual (Supplementary information, Figure S2), indicating normal postnatal organ and tissue development in the iPS mice.

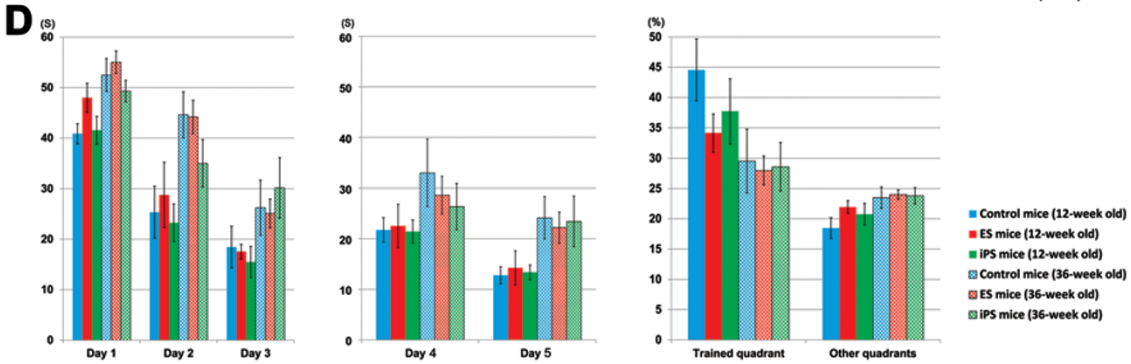
To study the intelligence and memory ability of iPS mice, 7 control mice, 7 iPS mice and 6 ES mice of age 12 weeks were tested in 5 consecutive days using the Morris water maze test. Comparable performance and significant improvement over days were observed for all tested mice ($P < 0.01$ for comparisons between any two

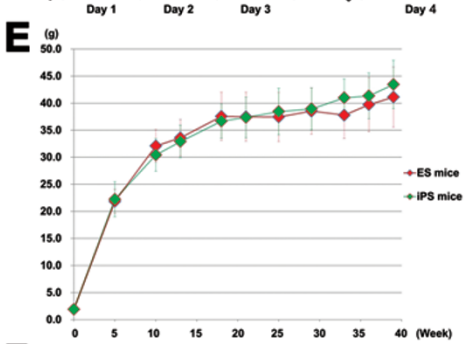


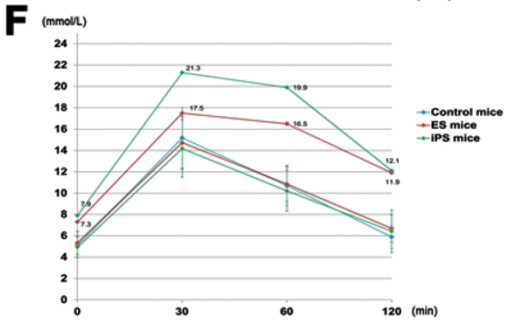
B

Dissection time	Cell origin	No. of Embryo transferred	Developmental arrest at embryonic day (%) E6.5-E8.5	E9.5-E12.5	E13.5-E15.5	E16.5-E18.5	Live pups (%)	Defects (%) RF	OE
E13.5	ES	367	240 (65.4)[a]	33 (9.0)[a]			21 (5.7)[a]		
	iPS	343	230 (67.1)[a]	29 (8.5)[a]			21 (6.1)[a]		
E16.5	ES	383	300 (78.3)[a]	31 (8.1)[a]	4 (1.0)[a]		13 (3.4)[a]		
	iPS	335	253 (75.5)[a]	32 (9.6)[a]	6 (1.8)[a]		13 (3.9)[a]		
E19.5 (P0)	ES	1732	1381 (79.7)[a]	45 (2.6)[a]	3 (0.2)[a]	0	47 (2.7)[a]	12 (25.5)[a]	14 (29.8)[a]
	iPS	1582	1234 (78.0)[a]	33 (2.1)[a]	4 (0.3)[a]	1	60 (3.8)[a]	17 (28.3)[a]	16 (26.7)[a]









G

Mouse	Age (Weeks)	Tumorigenesis (%)	Tumor Type	Tumor grade	Primary tumor tissue	Metastatic tumor tissue
Control mice	38-40	0/7[a]	---	---	---	---
ES F0 mice	38-40	0/7[a]	---	---	---	---
iPS F0 mice	38-40	3/13 (23.1%)[b]	0405-2#: Papillary adenocarcinoma	Tumor grade I	Pancreas	---
			0405-4#: Papillary adenocarcinoma	Tumor grade I	Pancreas	---
			583#: Osteosarcoma	Tumor grade I	Rib	Lung
iPS F1 mice	38-40	3/23 (13.0%)[b]	0117-1#: Papillary adenocarcinoma	Tumor grade I	Lung	---
			0117-5#: Malignant tumor	Tumor grade III	Pancreas	---
			0201-9#: Malignant tumor	Tumor grade III	✧ Neck, pancreas, kidney, spleen	

Figure 1 Molecular, embryonic and postnatal developmental comparison of iPS and ES cell lines, and their derived mice. **(A)** Gene expression profiles obtained from three tetraploid complementary iPS and three ES cell lines. The blue dashed lines represent the threshold for 2-fold expression change. Genes with expression differences of *P*-values < 0.05 by *t*-test are shown as orange circles, others are shown as grey circles. Global gene expression patterns of ES and iPS cell lines were indistinguishable by hierarchical clustering analysis. Clusters were determined using the hclust package of R. **(B)** Statistics of development and defects among iPS and ES cell-derived embryos. a values with the same superscript letters in the same column at the same embryonic day have no statistical difference ($P > 0.05$ by chi-square test). **(C)** The postnatal survival rates of iPS and ES mice. **(D)** The average time taken by iPS, ES and control mice to find the hidden platform in acquisition trials and reversal trials during 5 consecutive days using the Morris water maze test, and the proportion of searching time that the iPS, ES and control mice spent in the trained and untrained quadrants. Error bars represent the standard error of the mean. **(E)** The weight increment of iPS and ES mice, as measured every 3 weeks over 40 weeks. **(F)** Blood glucose levels in aged iPS, ES and control mice were measured by IPGTT. 13 iPS mice, 17 ES mice and 17 control mice showed no significant differences at any of the four tested time points. All readings for 1 iPS mouse and 1 ES mouse were significantly higher than those for other mice. Error bars represent the standard error of the mean. **(G)** Statistics of tumorigenesis in iPS, ES and control mice. a and b: Values with different superscript letters in the same column have statistically significant difference ($P < 0.01$ by chi-square test). ✧ Malignant tumors were found in these tissues with unclassified tumor type and origin.

groups) in both the acquisition (day 1 to day 3) and reversal (day 4 and day 5) trials of the hidden platform test as well as the probe test (Figure 1D). Similar results were also obtained when testing with 36-week-old mice ($n^{iPS} = 7$; $n^{ES} = 15$; $n^{control} = 7$, Figure 1D), revealing no difference among both the young and old iPS, ES and control mice in learning ability and spatial memory.

The iPS mice exhibited identical weight-gain rate as the ES mice during the examined life span (Figure 1E). However, hematological analysis of 38-week-old iPS, ES and control mice ($n = 7$ for each group) revealed significantly higher concentrations of both the high-density lipoprotein (HDL) and low-density lipoprotein (LDL) in iPS and ES mice than in control ones (one-way ANOVA test, Table S3). However, such difference may not cause physiological defects as the consistent increment of HDL and LDL concentrations resulted in comparable levels of HDL/LDL ratio, which is considered as a better indicator of heart disease, among the three groups.

When determining the glucose tolerance capacity by intraperitoneal glucose tolerance test (IPGTT), both the 9-month-old iPS and ES groups had one abnormal mouse with higher fasting plasma glucose values (FPG, 7.3 and 7.9 mmol/l, respectively) and 2-h postchallenge plasma glucose values (PG, 11.9 and 12.1 mmol/l, respectively) when compared with the human clinical threshold for diabetes (7.0 and 11.1 mmol/l for FPG and PG value, respectively), whereas the corresponding values of all other mice were below the standard (Figure 1F). Whether the iPS and ES mice have a higher risk for abnormal glucose metabolism needs to be further investigated with larger sample groups.

Although all surviving iPS and ES mice looked healthy through 38 weeks, structural changes in organs or tissues may have already occurred before external physiological or behavioral alterations were apparent. To address this question, 38-week-old iPS mice ($n = 13$) were killed and examined carefully on all viscera by histology analysis. Two cases of pancreatic tumor and one case of bone tumor among the 13 F0 generation iPS mice were identified (Figure 1G), as characterized by cell morphology and specific tumor markers (Supplementary information, Figure S3), whereas the ES and control mice were all tumor free ($n = 7$). In addition, 3 out of 23 iPS F1 mice, generated from iPS F0 male mice mated with healthy CD-1 females, had tumors in various tissues (Figure 1G and Supplementary information, Figure S3). To investigate whether the development of tumor in iPS mice is related to the cell induction method, the endogenous and transgenic expression levels of the four iPS induction factors were examined. Elevated expression of transgenic c-Myc, Klf4 and Oct4 was detected in both tumorous and normal tissues of the iPS mice when compared with those of ES and control ones (Supplementary information, Figure S4), and the expression of transgenic c-Myc was statistically higher in tumorous tissues than in normal tissues among iPS mice ($P < 0.01$), indicating that the overexpression of transgenic c-Myc may contribute to the tumor formation in F0 iPS mice (Supplementary information, Figure S4).

Taken together, the developmental comparsion between iPS and ES mice presented in this work suggested that animals derived from fully reprogrammed iPS cells are highly similar to those from ES cells, but caution regarding tumorigenic risk should be taken for iPS cells generated by exogenous oncogenes.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)