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Variation in the safety of induced pluripotent stem cell lines

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We evaluated the teratoma-forming propensity of secondary neurospheres (SNS) generated from 36 mouse induced pluripotent stem (iPS) cell lines derived in 11 different ways. Teratoma-formation of SNS from embryonic fibroblast–derived iPS cells was similar to that of SNS from embryonic stem (ES) cells. In contrast, SNS from iPS cells derived from different adult tissues varied substantially in their teratoma-forming propensity, which correlated with the persistence of undifferentiated cells.

Many iPS cell clones have been generated using different transcription-factor combinations, tissues and selection methods, and some of these variables appear to influence teratoma formation after differentiation. Although mouse iPS cells produced with the transcription factors Oct3/4, Sox2, Klf4 and c-Myc[1] can contribute to germline-competent adult chimeras[2,3], these chimeras and their progeny often develop tumors owing to reactivation of the *c-Myc* transgene[2]. More recently, iPS cells were generated without *c-Myc* retroviruses, albeit with lower efficiency[4,5]. Chimeric mice derived from *c-Myc*$^-$ iPS cells showed substantially less tumor formation[4]. Chimera-competent iPS cells have been isolated by drug selection for the expression of pluripotency-associated genes such as *Nanog* and *Oct3/4* (also known as *Pouf51*; refs. 2,3) or without drug selection[6]. Moreover, iPS cells have also been derived from various tissues, including embryonic fibroblasts[2] and adult tail-tip fibroblasts (TTFs)[4], hepatocytes[7], gastric epithelial cells[7], pancreatic cells[8], neural stem cells[9–11] and B lymphocytes[12] in the mouse, and skin fibroblasts[13–15], keratinocytes[16] and peripheral blood cells[17] in the human. Considering all of the differences in reprogramming methods reported to date, the safety and therapeutic implications of these differences should be thoroughly evaluated before iPS cells are used in cell therapies[18].

In this study, we examined the effects of the *c-Myc* (also known as *Myc*) transgene, the tissue of origin and drug selection. We generated SNS from various mouse iPS cell clones and examined their neural differentiation capacity and teratoma-forming propensity after transplantation into the brains of nonobese/severe combined immunodeficient (NOD/SCID) mice. We used 36 iPS cell clones, characterized by (i) origin, that is, mouse embryonic fibroblast (MEF), TTF, hepatocyte or gastric epithelial cell; (ii) presence or absence of *c-Myc* retroviral transduction; and (iii) presence or absence of drug selection for *Nanog* or *Fbxo15* expression. The profiles and results of all the clones analyzed in this study are summarized in **Supplementary Tables 1** and **2**. PCR primers and antibodies used are summarized in **Supplementary Tables 3** and **4**. As controls, we used three ES cell clones: RF8 (ref. 19); a subclone (1A2) of RF8 carrying the *Nanog*-EGFP reporter[2]; and EB3 carrying the *Oct3/4* blasticidin-resistance reporter gene[20,21].

We generated neurospheres containing neural stem and progenitor cells from these iPS cells and ES cells using a published method[21] (**Supplementary Methods**). After embryoid body formation, most of the iPS clones and ES cells formed primary neurospheres in the presence of fibroblast growth factor 2; the primary neurospheres were then dissociated to form SNS (**Fig. 1a**). Three hepatocyte (Hep)-iPS clones and one gastric epithelial cell (Stm)-iPS cell clone failed to form neurospheres (**Supplementary Table 2**). Both iPS cell– and ES cell–derived SNS effectively differentiated into tri-lineage neural cells, that is, neurons, astrocytes and oligodendrocytes, *in vitro* (**Fig. 1b**) or *in vivo* (**Supplementary Fig. 1**).

To further assess the differentiation potential, we next examined how many undifferentiated cells persisted in SNS. We used MEF-iPS, TTF-iPS, Hep-iPS and ES cells containing the *Nanog*-EGFP reporter to detect undifferentiated cells by flow cytometry. The iPS cells were generated with or without the *c-Myc* retrovirus, and with or without drug selection for *Nanog* expression[2,4]. We found that SNS derived from MEF-iPS cells contained few *Nanog*-EGFP$^+$ cells regardless of the presence of the *c-Myc* retrovirus or the drug selection (0–0.38%). This result is similar to that of SNS derived from ES cells. By contrast, SNS from TTF-iPS cells had significantly ($P < 0.057$) higher amounts (0.025–20.1%) of *Nanog*-EGFP$^+$ undifferentiated cells than did those from MEF-iPS cells. SNS from Hep-iPS cells also contained higher amounts (0.034–12.0%) of *Nanog*-EGFP$^+$ undifferentiated cells (**Fig. 1c** and **Supplementary Fig. 2**). We found no significant effects of either the presence of *c-Myc* retrovirus (**Supplementary Fig. 3a**) or the drug selection (**Supplementary Fig. 3b**) on the proportion of *Nanog*-EGFP$^+$ cells in SNS.

To characterize the SNS *in vivo*, we transplanted them into the striata of NOD/SCID mice and examined tumor formation for up to 45 weeks (**Fig. 2a**, **Supplementary Fig. 4** and **Supplementary Methods**). We dissected the brains of animals that died or that became weak and were euthanized after transplantation. The remaining healthy mice were euthanized and dissected periodically 4–45 weeks after transplantation. In 34 mice transplanted with SNS derived from the three ES cell clones, three mice died or became weak because of tumors. On dissection, 31 mice showed no tumors and one mouse had a small tumor. Similar results were obtained with MEF-iPS cells. In 100 mice implanted with SNS from the 12 MEF-iPS cell clones, 9 mice died or became weak within 19 weeks after transplantation. In eight of these mice, we observed tumors. On dissection of the remaining mice, we observed that 66 mice did not have tumors, whereas 25 mice had tumors of various sizes.

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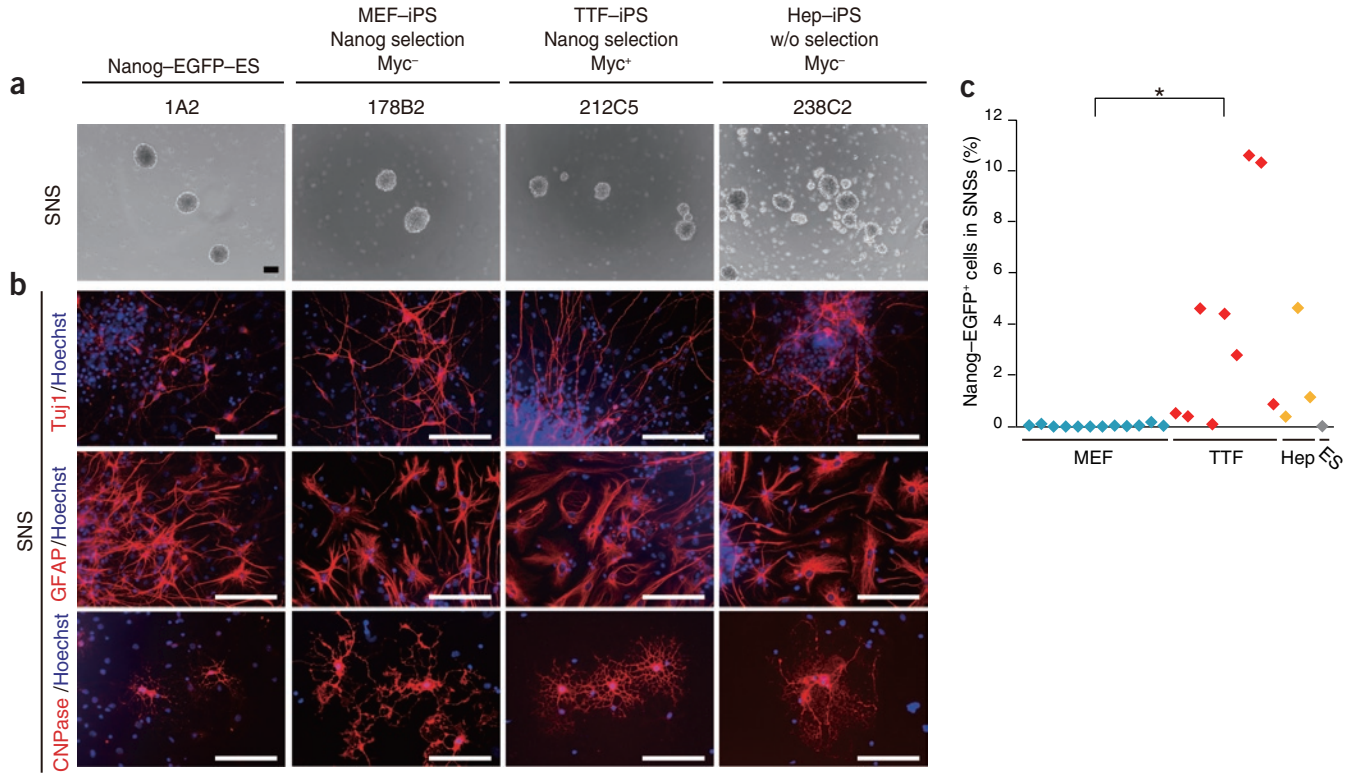

Figure 1 SNS formation from mouse iPS cells. (**a**) SNS derived from ES (1A2), MEF-iPS (178B2), TTF-iPS (212C5) and Hep-iPS cells (238C2). Scale bar, 200 μm. (**b**) Immunocytochemical analyses of cells differentiated from SNS for Tuj1 (neurons), GFAP (astrocytes) and CNPase (oligodendrocytes). Scale bars, 100 μm. (**c**) Comparison of the content of *Nanog*-EGFP⁺ cells in SNS derived from ES cells, MEF-iPS cells, TTF-iPS cells and Hep-iPS cells.

By contrast, in 55 mice transplanted with SNS from 11 TTF-iPS cell clones, 46 mice died or became weak within 9 weeks after transplantation because of tumors. In the remaining nine healthy mice, no tumors were observed. Of 36 mice transplanted with seven Hep-iPS cell clones, 13 died or became weak within 17 weeks after transplantation. Ten of these mice developed tumors. In the remaining 23 healthy mice, no tumors were found on dissection. In addition, we transplanted two Stm-iPS cell clones into eight mice. No tumors were observed in these mice when they were dissected 16 weeks after transplantation.

We performed histological analyses of one tumor for each iPS cell clone whose SNS gave rise to tumors (**Supplementary Tables 1** and **2**). We found that all tumors examined contained large portions of undifferentiated cells, with small areas of differentiated cells such as striated muscle, duct-forming epithelial cells, keratinized epithelial cells, cartilage and neural cells (**Supplementary Fig. 5**). Thus, these tumors were considered to be teratomas or, if they contained large portions of undifferentiated cells, teratocarcinomas. In sectioned brains without tumors, we confirmed survival of grafted cells.

Statistical analyses showed that SNS from TTF-iPS cells produced significantly ($P < 0.01$) larger teratomas than did those from the other iPS cells or ES cells (**Fig. 2b**). In addition, SNS from TTF-iPS cells and Hep-iPS cells resulted in significantly ($P < 0.01$) higher incidence of weakness and death (**Fig. 2c**). We found no significant effects of either the presence of *c-Myc* retrovirus (**Supplementary Fig. 6a**) or the drug selection (**Supplementary Fig. 6b**) on the sizes of tumors or the incidence of weakness and death. By contrast, there was a significant ($P < 0.01$) correlation between teratoma diameter and the content of *Nanog*-EGFP⁺ cells in SNS (**Fig. 2d**).

We have previously reported that tumors observed in iPS cell–derived chimeric mice were attributable to the reactivation of the *c-Myc* retrovirus[2]. In contrast, in the present study, use of the *c-Myc* retrovirus did not affect the teratoma-forming propensity of SNS (**Supplementary Fig. 6a**). Furthermore, we did not detect reactivation of *c-Myc* or other transgenes in SNS or teratomas (**Supplementary Fig. 7**). The mechanisms underlying the different teratoma-forming propensities of iPS cells remain to be determined.

The data presented here indicate that the teratoma-forming propensities of SNS vary significantly depending on the iPS cells' tissue of origin. SNS from TTF-iPS cells showed the highest propensity, whereas those from MEF-iPS cells and Stm-iPS cells showed the lowest, being comparable to those from ES cells. SNS from Hep-iPS cells showed an intermediate propensity. We note that teratoma formation by derivatives of iPS cells may be affected by the methods used for reprogramming and differentiation, the site of transplantation, the host background and other factors. For example, reprogramming methods that do not involve genomic integration have been reported to generate human iPS cells less susceptible to insertional mutagenesis[22,23]. All of the variables affecting safety must be rigorously evaluated before cell therapies based on iPS cells advance to the clinic.

Note: Supplementary information is available on the Nature Biotechnology website.

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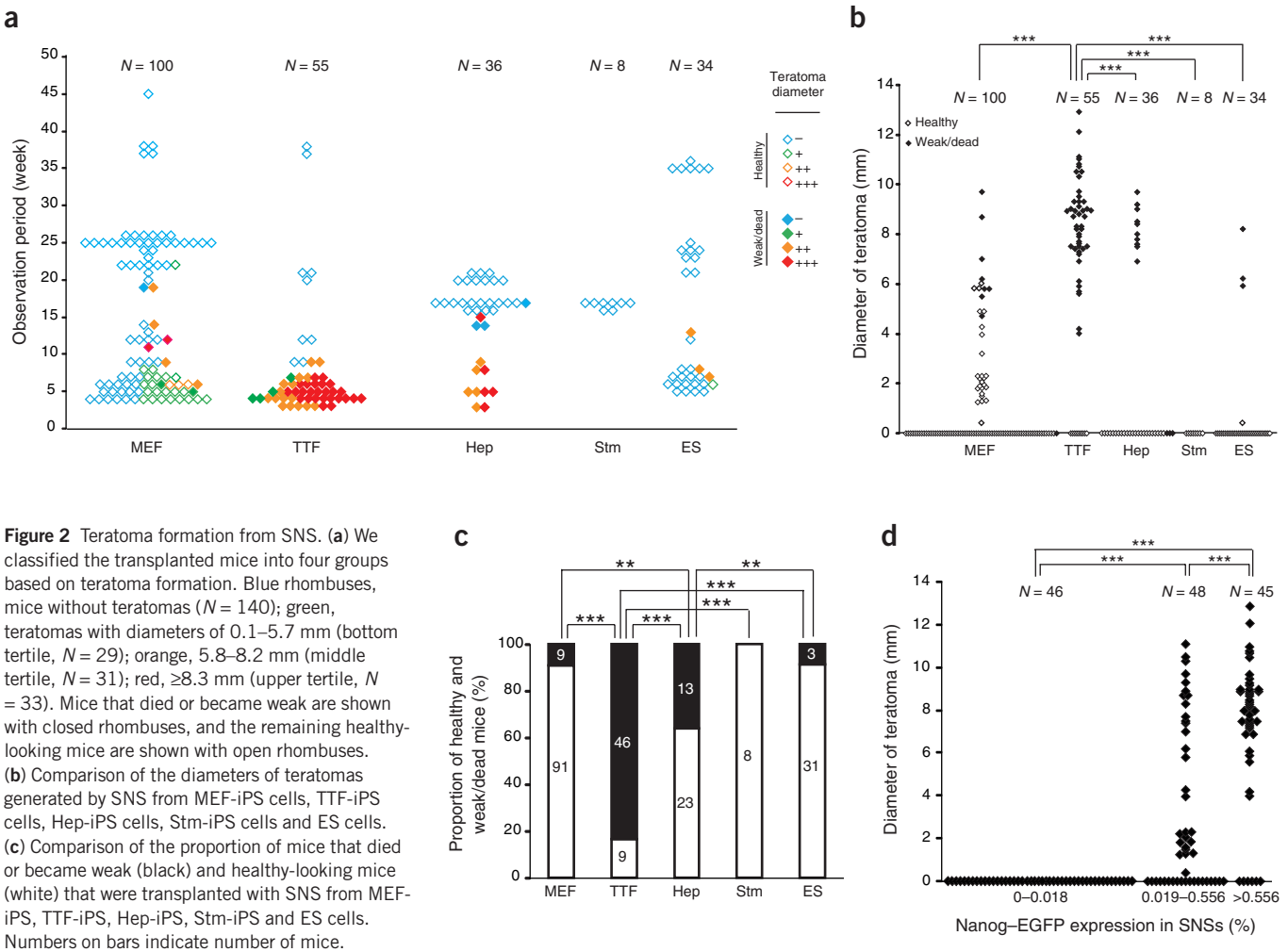

Figure 2 Teratoma formation from SNS. (**a**) We classified the transplanted mice into four groups based on teratoma formation. Blue rhombuses, mice without teratomas ($N = 140$); green, teratomas with diameters of 0.1–5.7 mm (bottom tertile, $N = 29$); orange, 5.8–8.2 mm (middle tertile, $N = 31$); red, ≥8.3 mm (upper tertile, $N = 33$). Mice that died or became weak are shown with closed rhombuses, and the remaining healthy-looking mice are shown with open rhombuses. (**b**) Comparison of the diameters of teratomas generated by SNS from MEF-iPS cells, TTF-iPS cells, Hep-iPS cells, Stm-iPS cells and ES cells. (**c**) Comparison of the proportion of mice that died or became weak (black) and healthy-looking mice (white) that were transplanted with SNS from MEF-iPS, TTF-iPS, Hep-iPS, Stm-iPS and ES cells. Numbers on bars indicate number of mice. (**d**) Correlation between the content of *Nanog*-EGFP$^+$ cells in iPS cell–derived SNS and the diameters of teratomas produced by the SNS. Mice transplanted with the SNS were grouped according to the percentage of *Nanog*-EGFP$^+$ cells (0–0.018%, bottom tertile (46 mice); 0.019–0.556%, middle tertile (48 mice); >0.556%, upper tertile (45 mice)). All procedures were approved by the ethics committee of Kyoto University and Keio University, which were in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health).

AUTHOR CONTRIBUTIONS

K.M. conducted most of the experiments. Y.O., T.A., A.O. and M.N. supported experiments and analyses. K. Takahashi, T.A., K.O., M.K., K. Tanabe and M.O. prepared undifferentiated iPS cells. E.I. and K. Takahashi assisted with the pathological analyses. D.O. assisted in some of the stereotactic transplantations. S.Y. and H.O. supervised the entire project. K.M. and S.Y. wrote the manuscript.

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