LETTER

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Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells

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The advantages of using induced pluripotent stem cells (iPSCs) instead of embryonic stem (ES) cells in regenerative medicine centre around circumventing concerns about the ethics of using ES cells and the likelihood of immune rejection of ES-cell-derived tissues[1,2]. However, partial reprogramming and genetic instabilities in iPSCs[3–6] could elicit immune responses in transplant recipients even when iPSC-derived differentiated cells are transplanted. iPSCs are first differentiated into specific types of cells *in vitro* for subsequent transplantation. Although model transplantation experiments have been conducted using various iPSC-derived differentiated tissues[7–10] and immune rejections have not been observed, careful investigation of the immunogenicity of iPSC-derived tissue is becoming increasingly critical, especially as this has not been the focus of most studies done so far. A recent study reported immunogenicity of iPSC- but not ES-cell-derived teratomas[11] and implicated several causative genes. Nevertheless, some controversy has arisen regarding these findings[12]. Here we examine the immunogenicity of differentiated skin and bone marrow tissues derived from mouse iPSCs. To ensure optimal comparison of iPSCs and ES cells, we established ten integration-free iPSC and seven ES-cell lines using an inbred mouse strain, C57BL/6. We observed no differences in the rate of success of transplantation when skin and bone marrow cells derived from iPSCs were compared with ES-cell-derived tissues. Moreover, we observed limited or no immune responses, including T-cell infiltration, for tissues derived from either iPSCs or ES cells, and no increase in the expression of the immunogenicity-causing *Zg16* and *Hormad1* genes in regressing skin and teratoma tissues. Our findings suggest limited immunogenicity of transplanted cells differentiated from iPSCs and ES cells.

There are three major concerns about the adequacy of the design and conclusions of a previous study that reported immunogenicity of iPSC- but not ES-cell-derived teratomas[11]. The first is that the immunogenicity of iPSCs was assessed by teratoma formation. Consistent with the focus on tumour immunity rather than transplant immunity, the causative gene identified in the regressing teratomas was tumour related[13]. Second, only a single ES-cell clone was included in the comparison of iPSC- and ES-cell-related immunogenicity. Third, the developmental ability of the iPSC and ES-cell lines used may have been compromised. Given that partial genome reprogramming can elicit immune responses and cause considerable variation among iPSC clones, the developmental abilities of iPSC and ES-cell clones should always be tested before such analyses. To evaluate better the immunogenicity of iPSCs, we conducted transplantation experiments involving terminally differentiated cells derived from iPSCs. Our experiments involved multiple ES-cell and iPSC lines (Supplementary Fig. 1a) that were established from an inbred mouse strain, C57BL/6, and were confirmed to have strong developmental capacities. We generated iPSCs by expressing plasmid-borne *Oct4* (also known as *Pou5f1*), *Sox2*, *Klf4* and *Myc* genes[14], and then selected clones that were free from genome-integration events (Supplementary Fig. 1b)[15], as iPSCs with integrated transgenes elicit marked immune responses[11]. Similar doubling times were measured for all the cell lines (Supplementary Fig. 1c). Germline transmission capability was confirmed for eight of ten iPSC clones and for six of seven ES-cell clones (Supplementary Table 1).

We used seven iPSC and five ES-cell lines in teratoma formation tests to obtain more conclusive results (Supplementary Fig. 2a). The tests in C57BL/6 wild-type mice were replicated a total of ten times for each of the twelve clones. We observed teratomas containing three germ layers[15] at a high incidence rate for both types of pluripotent stem cells, with no statistical difference between iPSCs and ES cells (Fig. 1a). Next, we used immunohistochemical staining to investigate the infiltration of the teratomas by T cells (Supplementary Fig. 2b). We did not observe any T cells in most sections, although fewer than two T cells were detected in some sections. Although slight differences were observed among the cell lines, the profiles between iPSCs and ES cells were quite similar overall (Fig. 1b), and no statistically significant difference between iPSC- and ES-cell-derived teratomas was detectable (Fig. 1c).

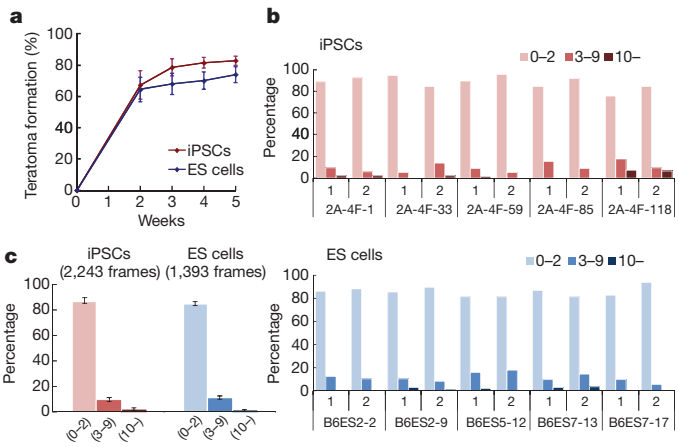

Figure 1 | Teratoma formation by iPSCs and ES cells. **a**, Time course of the appearance of teratomas after injection. Error bars show standard error of the mean (s.e.m.). iPSCs, $n = 7$; ES cells, $n = 5$. **b**, Frequency of T cells in each teratoma. Ten teratomas from iPSCs (red) and ten from ES cells (blue), two for each of five clones of both cell types, were stained with anti-CD3 antibody. More than 50 frames (each 0.382 mm × 0.507 mm) were randomly chosen for each teratoma. The numbers of T cells detected were categorized into three groups: 0–2, 3–9 or ≥10 T cells per frame. **c**, Summary of the data shown in **b** and the total number of frames investigated. Error bars show s.e.m. ($n = 10$).

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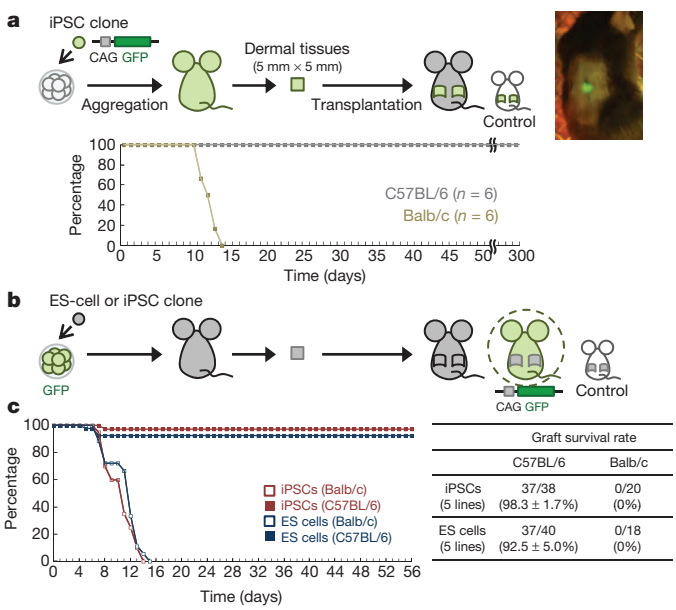
Focusing on teratoma formation is not suitable for assessing the immunogenic responses of relevance to regenerative medicine. Given that teratomas are a type of tumour, it is not surprising that they elicit immunological reactions in transplant recipients. Indeed, in a further teratoma formation test using severe combined immunodeficiency (SCID) mice[16] although, again, no difference was observed between iPSCs and ES cells, a similar frequency but a slight increase in size compared with wild-type mice was shown, indicating that certain immune systems respond to the teratomas derived from these two types of pluripotent stem cells (Supplementary Fig. 3). Hence, although we did not detect any significant T-cell infiltrations in various teratomas derived from either iPSCs or ES cells in our assay, we cannot rule out the possibility that substantial immune responses can be elicited under certain situations during teratoma formation in both types of pluripotent stem cells[11,17]. On the other hand, considering that iPSCs themselves would not be implanted, and that the stem cells would first be converted into specific types of differentiated cells before transplantation, the immunogenicity of terminally differentiated cells derived from iPSCs is critically relevant to the long-term prospects of their use in regenerative medicine. We therefore prepared dermal and bone marrow tissues from the highly chimaeric mice developed from iPSCs or ES cells for subsequent transplantation experiments. These tissues were selected on the basis of the central roles they have in the immune system.

For each type of terminally differentiated cell, we initially set up our assay system by using a green fluorescent protein (GFP)-labelled iPSC clone, because it allowed us to monitor the donor cells after transplantation, even in a single-cell manner. We used a genome-integration-free iPSC clone, 2A-3F-EGFPtg-4, which was established from a C57BL/6-Tg(CAG-EGFP) mouse[18] with *Oct4*, *Sox2* and *Klf4* genes; using Trichostatin A, we demonstrated that this iPSC line could achieve pluripotency[19]. On the basis of the investigation using the 2A-3F-EGFPtg-4 clone, we examined various non-labelled lines of iPSCs and ES cells.

We first attempted to establish a transplantation assay system for epidermal tissue based on a previously reported transplantation system[20]. We aggregated the GFP-positive iPSC clone 2A-3F-EGFPtg-4 with GFP-negative embryos to develop GFP-positive highly chimaeric mice. Skin samples from the tails of the chimaeric mice were prepared, and then transplanted onto the backs of syngeneic GFP-negative C57BL/6 mice and allogeneic Balb/c mice (Fig. 2a). All of the six GFP-positive transplanted grafts generated could be sustained over a 10-month period. In contrast, in the control experiment, the transplanted grafts on the Balb/c mice remained physically attached to the backs of recipient mice for over a week but not as long as 2 weeks after transplantation (Fig. 2a). This system thus provides a rapid and highly efficient way of assaying the immunogenicity of epithelial tissues.



	Graft survival rate	
	C57BL/6	Balb/c
iPSCs (5 lines)	37/38 (98.3 ± 1.7%)	0/20 (0%)
ES cells (5 lines)	37/40 (92.5 ± 5.0%)	0/18 (0%)

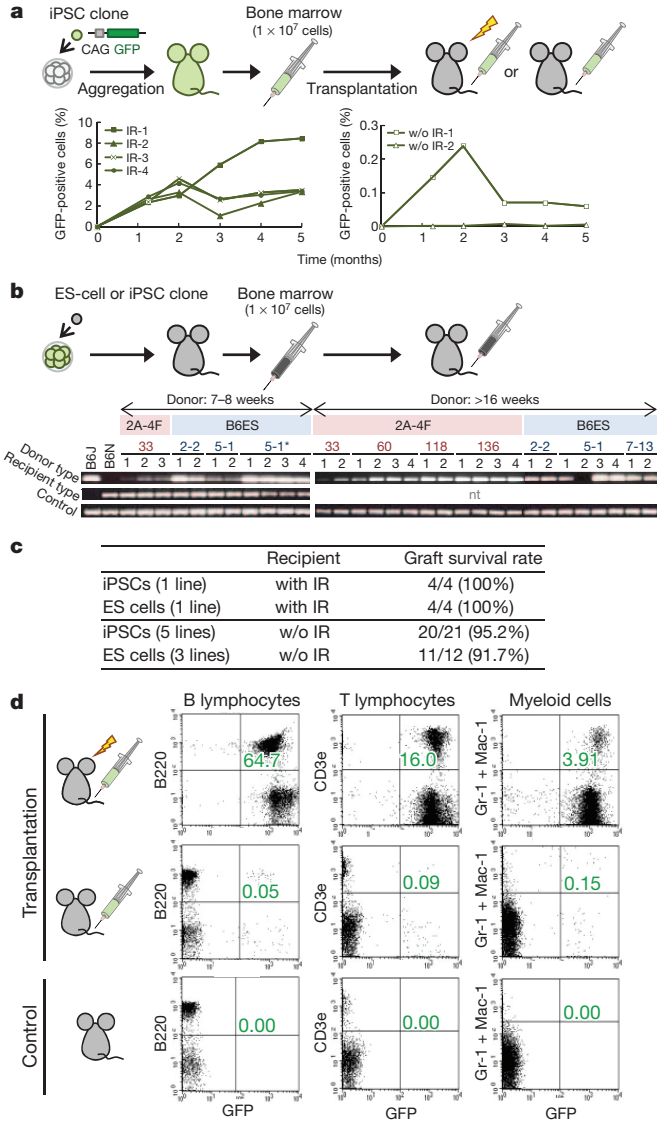
Figure 2 | Skin transplantation. **a**, Schematic diagram of the method for skin transplantation using a GFP-positive iPSC clone and time course of graft survival. Right, a mouse transplanted with GFP-positive skin at one month post transplantation. **b**, Schema of the skin transplantation assay for GFP-negative iPSCs and ES cells. **c**, Summary of the time course for graft survival.

Using this same assay system, we subsequently examined four additional iPSC clones (2A-4F-1, -33, -60 and -100) and five ES-cell clones (B6ES2-2, B6ES2-9, B6ES5-1, B6ES7-13 and B6ES7-15). Three mouse strains—C57BL/6, C57BL/6-Tg(CAG-EGFP) and Balb/c—were used as recipients (Supplementary Fig. 4a). Because all of these cell lines are GFP negative, each clone was aggregated with GFP-positive embryos to ensure that the skin tissues of the chimaeric mice used for subsequent transplantation tests did not contain cells other than those derived from either an iPSC or an ES cell (Fig. 2b). Another motivation for using GFP-positive embryos for the aggregation step was to confer tolerance to the GFP molecules in the recipient mice used to analyse immune responses, such as T-cell infiltration (Fig. 2b, broken circle), as GFP molecules can elicit immune responses[21]. Between 4 and 12 replicate transplantations were conducted for each clone, and graft survival was measured 2 months after transplantation. Graft survival rates were 98.3 ± 1.7% and 92.5 ± 5.0% for iPSCs and ES cells, respectively (Fig. 2c and Supplementary Fig. 4). In contrast, no long-term survival was noted for grafts derived from Balb/c mice. These data suggest that iPSCs are not demonstrably more immunogenic than ES cells.

To analyse immunogenicity further, we used confocal microscopy to investigate the invasion of the grafts by GFP-positive cells produced by the recipient C57BL/6-Tg(CAG-EGFP) mice. In the control experiment, which involved transplantation of Balb/c tail skin, a large number of GFP-positive cells were observed within the grafts (Supplementary Fig. 5a). Moreover, as expected, subsequent histochemical staining with anti-CD3 antibody revealed the presence of CD3-positive cells within the GFP-positive population (Supplementary Fig. 6a). As a result, very few GFP-positive cells were detected around or within the transplanted tissues in any iPSC- or ES-cell-derived tissue sections 2 months after transplantation (Supplementary Figs 5b, c and 6b). However, we note the possibility that our assay system may have missed some lymphocytes with low GFP expression levels. Nevertheless, if such T cells existed, the number that invaded the graft must have been quite small, because flow cytometry analysis did not detect GFP-negative T cells in the whole blood of C57BL/6-Tg(CAG-EGFP) recipients (Supplementary Fig. 7c).

The first step in the assessment of the immunogenicity of bone marrow tissues involved mimicking the bone marrow transplantation treatment. We transplanted bone marrow from mice derived from the GFP-positive iPSC clone 2A-3F-EGFPtg-4 into C57BL/6 wild-type mice that had been irradiated with a lethal (9.5 gray (Gy)) dose of X-rays (Fig. 3a). Flow cytometry analysis demonstrated the presence of GFP-positive cells 5 months later (Fig. 3a, left, and Supplementary Fig. 7a). We also detected B lymphocytes, T lymphocytes and granulocytes in the GFP-positive cell population 4 months after transplantation (Fig. 3d, top) in each of the four mice that received transplants. This indicates that both short- and long-term haematopoietic stem cells were fixed and maintained. No major phenotypical or behavioural abnormalities were observed in any of these mice more than 5 months after transplantation, although some skin ulcers were observed in one recipient, IR-1 (Fig. 3a, left). As anticipated, bone marrow derived from the ES-cell line B6ES5-1 also reconstituted the bone marrow of each of four recipient mice after they received a lethal dose of irradiation (Fig. 3b, c).

It is noteworthy that although almost all B lymphocytes, T lymphocytes and granulocytes were replaced with iPSC-derived cells (Fig. 3d, top), recipient-derived cells still remained in all transplanted mice



	Recipient	Graft survival rate
iPSCs (1 line)	with IR	4/4 (100%)
ES cells (1 line)	with IR	4/4 (100%)
iPSCs (5 lines)	w/o IR	20/21 (95.2%)
ES cells (3 lines)	w/o IR	11/12 (91.7%)

Figure 3 | Bone marrow transplantation. **a**, Schematic diagram of the bone marrow transplantation experiments using 2A-3F-EGFPtg-4 iPSCs and the survival of donor bone marrow cells. Time-course analysis of the survival of GFP-positive cells in the irradiated (left) and non-irradiated recipients (right) is shown. The percentage of GFP-positive cells to whole blood cells was measured by flow cytometry over 5 months (Supplementary Fig. 7a). IR-1, -2, and so on, indicate irradiated recipient 1, 2, and so on. w/o, without. **b**, Schematic diagram of the bone marrow transplantation experiments using non-labelled iPSCs and non-labelled ES cells, and of PCR assessment of the engraftment of donor-derived bone marrow cells. The existence of donor-derived bone marrow cells over 4 months was investigated by PCR using genomic DNA prepared from peripheral blood cells harvested at 4 months after transplantation. Asterisk indicates the use of irradiated recipients. **c**, Summary of the incidence of graft survival. **d**, Long-term reconstitution of bone marrow by 2A-3F-EGFPtg-4 iPSC-derived bone marrow cells. Peripheral blood cells at 4 months after transplantation were analysed using flow cytometry. The results of irradiation recipient IR-4 (top) and recipient without (w/o) irradiation IR-1 (middle), which are described in **a**, are shown as a representative case. The C57BL/6 mouse is shown as a control (bottom).

(Supplementary Fig. 7b). Importantly, the coexistence of donor- and recipient-derived bone marrow cells in the recipient mice more than 5 months from the time of transplantation is strongly suggestive of a limited immunogenicity of iPSC-derived bone marrow cells. This is because substantial immune responses must occur during the fixation and maintenance of iPSC-derived cells if the bone marrow cells derived from iPSCs are to show even a low level of immunogenicity. Thus, these experiments indicate that iPSC-derived bone marrow cells have the same potential as ES-cell-derived bone marrow cells to reconstitute the bone marrow system, and indicate the low immunogenicity of the bone marrow cells derived from iPSCs and ES cells. These results with X-ray irradiation are consistent with those of a previous study that showed similar repopulation by iPSC- and ES-cell-derived haematopoietic progenitors[7].

To assess whether low levels of immunogenicity had been overlooked, we next conducted transplantations under more severe conditions. This involved transplanting iPSC-derived bone marrow cells into wild-type C57BL/6 mice without any prior irradiation. Because the immune activity of the bone marrow in wild-type recipient mice must be normal, this enables better assessment of transplantation immunity. We injected 1×10^7 bone marrow cells from chimaeric mice developed using the iPSC line 2A-3F-EGFPtg-4 into the tail veins of recipient wild-type mice, and analysed peripheral blood samples at 1-month intervals for 5 months. Flow cytometry and semi-quantitative polymerase chain reaction (PCR) analysis indicated that donor cells derived from iPSCs were sustained over 5 months even after transplantation into non-irradiated mice (Fig. 3a, right, and Supplementary Fig. 7a). This indicates successful engraftment of both short- and long-term haematopoietic stem cells. In a comparison with the control, non-transplanted C57BL/6 bone marrow using flow cytometry, B lymphocytes, T lymphocytes and granulocytes derived from the donor cells were detected, although the number of GFP-positive cells was small (Fig. 3d). We also examined bone marrow cells prepared 6 months after transplantation to confirm the presence of GFP-positive lymphocytes and granulocytes (Supplementary Fig. 7d).

For the various non-labelled iPSCs and ES cells, similar bone marrow transplantations using non-irradiated recipient mice were performed (Fig. 3b, c and Supplementary Fig. 9a). Four iPSC and three ES-cell clones were also examined, and an assessment was done at 4 months after transplantation using genomic PCR, which enables discrimination between the genome of the donor and that of the recipient (Supplementary Fig. 8). In addition, we prepared bone marrow from mice of various ages (5–54 weeks). The results showed that 20 of 21 challenges involving bone marrow derived from any one of the five iPSC lines, and 11 of 12 challenges involving bone marrow derived from any one of the three ES-cell lines, were successful. Notably, the bone marrows of 1-year old chimaeric mice generated from either one of the two iPSC lines, 2A-4F-118 and 2A-4F-136, or from an ES-cell line, B6ES7-13, were successfully engrafted (Supplementary Fig. 9a). Moreover, semi-quantitative PCR showed donor-origin cells in B-lymphocyte, T-lymphocyte and granulocyte fractions prepared from mice developed using the non-labelled pluripotent stem cell lines 2A-4F-60 and B6ES5-1 (Supplementary Fig. 9b). These data indicate that iPSC-derived haematopoietic stem cells can be engrafted and sustained in recipients even under conditions of normal immunocompetence. Although seven-to-twelve months have now passed since the time of implantation, we have not observed abnormalities in any of the recipient mice.

The *Zg16* and *Hormad1* genes have been causally linked to the immunogenicity of iPSC-derived teratomas, and both genes are expressed at elevated levels in regressing teratomas[11]. We also observed regression in both transplanted skin grafts and teratomas in these experiments, although no statistically significant difference was apparent between iPSC- and ES-cell-derived tissues (Supplementary Figs 10 and 11). Investigation of the expression of *Zg16* and *Hormad1* indicated no expression of *Zg16* in non-shrinking or shrinking grafts in dermal tissues, and a low level of expression of these genes in teratomas. A low level (less than 100-fold lower expression than that seen in the testis) of *Hormad1* expression was also observed in both transplanted grafts and teratomas. However, neither gene was expressed at an elevated level in the shrinking tissues. The expression profiles were quite similar between iPSC and ES-cell derivatives (Fig. 4a, b). Thus, the expression profiles of both genes were similar

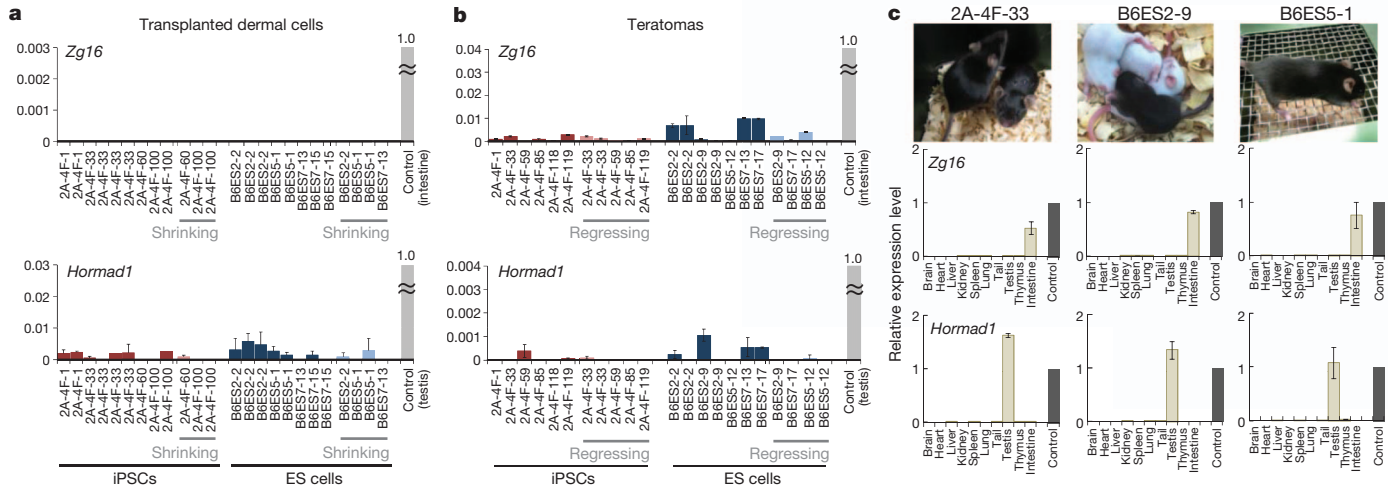


Figure 4 | Expression of *Zg16* and *Hormad1* genes in various tissues generated from iPSCs and ES cells. **a**, Expression of the *Zg16* and *Hormad1* genes in grafted skin 2 months after transplantation. **b**, Expression of the *Zg16* and *Hormad1* genes in the teratomas at 4 or 5 weeks after injection (Supplementary Fig. 11b). **c**, Expression of the *Zg16* and *Hormad1* genes in various tissues of iPSC tetraploid complementation chimaeric mice. Ten types of tissues were prepared from adult, 2–8-month old, tetraploid-complementation chimaeric mice derived from iPSC clone 2A-4F-33 or from the ES-cell clones B6ES2-9 and B6ES5-1, and then subjected to semi-quantitative RNA analysis. The intestines and the testes of C57BL/6 wild-type mice were used as a control for *Zg16* and *Hormad1* gene expression, respectively. Error bars show standard deviation ($n = 2$).

in both non-regressing and regressing teratomas, and we could not confirm previous findings[11] that *Hormad1* is upregulated in regressing teratomas derived from the iPSC clone 2A-4F-33.

Finally, we examined whether *Zg16* and *Hormad1* are expressed at higher levels in the various differentiated tissues derived from iPSCs relative to differentiated cells derived from ES cells. Ten tissue types were prepared from the adult mice generated by tetraploid complementation[22–24] with the iPSC line 2A-4F-33 and the ES-cell lines B6ES2-9 and B6ES5-1 for semi-quantitative expression analyses of the *Zg16* and *Hormad1* genes. The expression profiles of *Zg16* and *Hormad1* were indistinguishable between the three tissue panels derived from 2A-4F-33, B6ES2-9 and B6ES5-1 (Fig. 4c).

An important finding of this study is that the immunogenicity of iPSC-derived tissues is indistinguishable from that of ES-cell-derived tissues. Although all of the assays we performed demonstrated that the terminally differentiated cells developed from iPSCs elicit limited immune responses, it is not possible to exclude formally the possibility of immunogenicity, given the limitations of the experimental approaches at our disposal. Whether the immunogenicity of iPSCs is higher than that of ES cells therefore remains an important question. Addressing this issue effectively will require the analysis of substantial numbers of high-quality ES-cell lines, rather than relying on only a single ES-cell line as in a previous study[11]. We have successfully established seven ES-cell lines derived from C57BL/6 mice and confirmed germline transmission for six of these (Supplementary Table 1). Our results using these ES cells contrast with previous data because the immunogenicities of iPSC- and ES-cell-derived tissues were indistinguishable when scored using all three immunogenicity assays tested—teratoma formation, skin transplantation and bone marrow transplantation. None of our data show meaningful differences between iPSCs and ES cells.

Our data may indicate that the differences between specific clones of iPSCs and ES cells are more crucial than the difference between iPSCs and ES cells *per se*. Indeed, we observed a variation among iPSC clones in another experiment involving three iPSC clones from which chimaeric mice exhibited low chimaerism and no germline transmission (Supplementary Fig. 12). We performed teratoma formation tests in each case followed by T-cell infiltration analysis. Intriguingly, several lines exhibited significant T-cell infiltration. Furthermore, considerable variations in T-cell infiltration were observed among teratomas generated even from identical cell lines, although the frequency was similar not only to that among the three iPSC clones examined, but also to that of the lines of which fully developmental ability was confirmed. Our data may thus also indicate that the teratomas generated from the iPSCs for which developmental ability is not complete are prone to eliciting immune responses, and that this issue is crucial for the medical use of iPSCs. Because testing such as chimaera formation is not possible for human iPSCs, these findings underscore the need for careful discussion and longitudinal investigations of interclonal differences in iPSC and ES-cell lines[25,26], and the need to develop culture conditions that will achieve a pluripotent state[27–29]. This is in addition to broader generalizations regarding the immunogenicities of the two cell types.

In this study, we used terminally differentiated cells of adult mice, which had been generated from either iPSCs or ES cells as donor cells, as our aim was to determine whether iPSC-derived differentiated cells are intrinsically immunogenic. In other words, we attempted to clarify whether iPSC-derived differentiated cells can be used as donor cells in the same way as ES cells when the complete *in vitro* differentiation of these stem cells became possible. In this regard it must be noted that our study does not directly contribute to resolving the possible immunogenicity of *in vitro* differentiated cells. Further investigations are needed to assess this issue and hence the actual clinical use of these pluripotent cells. Notably, we observed significant levels of T-cell infiltration when we used *in vitro* derived cells, cardiomyocytes, as donors (Supplementary Fig. 13).

We have demonstrated that the limited immunogenicity of iPSC-derived differentiated cells is indistinguishable from that of comparable ES-cell-derived cells. However, the possibility remains that even terminally differentiated cells derived from iPSCs might elicit some immune responses. For example, the genetic aberrations that have been demonstrated recently in iPSCs could account for their immunogenicity[5,6]. However, considering the advantages of iPSCs compared with ES cells, such as the ease of direct establishment from patients without the same complex ethical considerations, the discussion surrounding the relative merits of using autologous iPSCs and autologous ES cells seems to be now less relevant. This might be further resolved by a direct comparison between autologous iPSCs and allologous and major histocompatibility complex (MHC)-matched ES cells[12].

METHODS SUMMARY

C57BL/6J Jms Slc and C57BL/6-Tg(CAG-EGFP)[18] mice were used for iPSC and ES-cell generation. iPSCs were generated with pCX-OKS-2A and pCX-c-Myc

plasmids (Addgene) as described previously[14,15]. We performed PCR analysis to confirm the absence of plasmid integration[14] and established ES-cell lines as described previously[30]. Established iPSC and ES-cell lines were evaluated on the basis of their morphologies, stem cell markers, neuploidy and pluripotency (Supplementary Fig. 1 and Supplementary Table 1). Skin and bone marrow were prepared from fully chimaeric adult male mice developed from either iPSCs or ES cells. The C57BL/6 and C57BL/6-Tg(CAG-EGFP) mouse strains were used as recipients.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions R.A. and M.A. designed the experiments, analysed the data and wrote the manuscript. R.A. and Y.H. established the iPSC and ES-cell lines. M.U., S.A., M.N. and Y.H. performed teratoma formation assays and transplantation assays. M. Sunayama and M. Sugiura performed molecular biological analyses. M.U., H.I., A.S. and A.N. performed immunohistochemical analyses.

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METHODS

Mice. C57BL/6J Jms Slc mice were used for iPSC and ES cell generation, and C57BL/6J Jms Slc, C57BL/6-Tg(CAG-EGFP)[18] and Balb/c mice were used for transplantation assays (Japan SLC). Only males were used for cell-line establishment and transplantation experiments. Animal experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines.

iPSC and ES cell generation. iPSCs were generated with pCX-OKS-2A and pCX-c-Myc plasmids (Addgene) as described previously[14,15]. Briefly, mouse embryonic fibroblasts (MEFs) were isolated from embryonic day (E)13.5 embryos of male C57BL/6 mice, and grown in DMEM supplemented with 10% FCS. The cells were transfected with the plasmids on days 1, 3, 5 and 7. Colonies were picked on day 15 after tranfection. We carried out PCR analysis to confirm the absence of plasmid vector integration[14]. Established iPSC lines were evaluated on the basis of their morphologies, stem cell markers and pluripotency. Neuploidy was also investigated. We established ES cell lines as described previously[30]. Briefly, E3.5 embryos were collected and plated individually onto mitomycin-C (Sigma-Aldrich) treated feeder MEFs. Established ES cell lines were evaluated on the basis of their morphologies, stem cell markers, neuploidy and pluripotency (Supplementary Fig. 1 and Supplementary Table 1).

Teratoma formation and T-cell immunostaining. Teratomas were produced by injecting cells (3×10^6 cells per injection) subcutaneously into the flanks of the mice. Four or five weeks after injection, the tumours were surgically dissected, fixed and embedded in paraffin. Tumour sections were immunostained with anti-CD3 antibody MCA-1477 (AbD serotec) as described previously[31].

Statistics. Normally distributed data are expressed as the means ± s.e.m., and the differences were tested using an unpaired *t*-test. *P* values < 0.05 were considered to be significant.

Immunocytochemistry of iPSC colonies. For immunocytochemical staining, anti-Nanog (1:50, ReproCELL), anti-Oct3/4 h-134 (1:100, Santa Cruz Biotechnology) and anti Sox-2 Y-17 (1:100, Santa Cruz Biotechnology) antibodies were used.

Generation of chimaeric mice from iPSCs and ES cells. Chimaeric mice were produced via the aggregation method, using 8-cell embryos or tetraploid embryo complementation[30].

Skin transplantation. Adult male chimaeric mice were used to prepare donor dermal tissues. Skin grafting was performed as described previously[32]. We used the tail as donor tissue. Because mouse tail tissue is resistant to ischaemia it can be transplanted efficiently without any technical troubles, and sensitive assays using tails have been developed for long-term (>1 month) investigations[33,34]. Briefly, donor skin (5×5 mm) was grafted onto the back of recipient mice, and then covered with a bandage. The bandage was removed after 7 days, followed by analysis each day.

Bone marrow transplantation. Donor bone marrows were harvested from the femurs and tibias of fully chimaeric adult male mice (5–54 weeks old) derived from either iPSCs or ES cells. We used 7–8-week-old C57BL/6 or C57BL/6-Tg(CAG-EGFP) male mice as recipients with a single tail vein injection of the bone marrow cells. The animals were killed and the tibia and femurs were clipped into small pieces with scissors after removing the muscle and connective tissues. Bone chips were thoroughly rinsed with complete medium (DMEM supplemented with 5% FCS, 2 mM L-glutamine and antibiotics) in a 50-ml tube. The washed solution containing bone marrow cells was then transferred into a 50-ml tube after filtration through a 40-μm nylon mesh filter, and centrifuged at 600*g* for 10 min. The supernatant was removed and 1 ml of 0.2% NaCl was added to the pellet and gently mixed for 1 min on ice. After addition of a further 1 ml of 1.6% NaCl, the mixture was centrifuged at 600*g* for 10 min and the pellet was resuspended in complete medium. Cells were counted and 10^7 cells in 0.4 ml of PBS were injected into the lateral tail veins using a 26-gauge needle for all animals, except for recipient without irradiation IR-2, for which 0.7×10^7 cells were injected.

Flow cytometric analysis. We used a capillary tube to collect 50 μl of whole blood from the venous plexus of eye socket of the recipient mice. A 5-μl aliquot of the blood was diluted with PBS without haemolysis and used for the measurement of GFP-positive cells using flow cytometry, performed with an Epics XL instrument (Beckman Coulter). To detect B lymphocytes, T lymphocytes and granulocytes derived from donor bone marrow, the remaining collected whole blood was haemolysed using Versalyse (Beckman-Coulter) to stain the cell-surface markers B220 (CD45R)-APC (clone; RA3-6B2, code no. 17-0452; eBioscience), CD3e-PE (clone; 145-2C11, code no. 12-0031; e-Bioscience), Gr-1 (Ly-6G)-PE (clone; RB6-8C5, code no. 12-5931; e-Bioscience) and Mac-1(CD11b)-PE (clone; M1/70, code no. 101208; Biolegend). Four-colour flow cytometric analysis was performed using FACSCalibur, and the data were analysed using CellQuest software (BD Biosciences). In addition, using FACSaria (BD Bioscience), we sorted B220-positive, CD3e-PE-positive and Gr-1/Mac-1-positive fractions from the bone marrow preparations for semi-quantitative measurement of donor-origin cells.

Genotyping. Allele-specific PCR was performed to discriminate between donor and recipient tissues in the bone marrow transplantation experiments. Sequences of the primers used to characterize *Nnt* loci are listed in Supplementary Fig. 8[35]. Genomic DNAs were prepared using the DNeasy Blood & Tissue Kit (Qiagen). PCR reactions were performed using Titanium Taq DNA polymerase (Clontech), and semi-quantitative PCR was performed using SYBR Premix EX Taq (Takara Bio) with the StepOnePlus Real-Time PCR System (Applied Biosystems).

Semi-quantitative expression analysis. Teratomas and tissues were homogenized using a Mixer mill MM300 (Qiagen), and total RNA fractions were prepared using the RNeasy Mini kit (Qiagen) in accordance with the manufacturer's protocol. All RT–PCR analyses were performed using the QuantiTect SYBR Green RT–PCR Kit (Qiagen). The data were normalized by comparison with *Gapdh* gene expression. The primers used were 5′-CATCACCGCCTTCCGTAT-3′ and 5′-CGTTGAAACTTGTGCCTG A-3′ for *Zg16*, and 5′-GCTGACACCAAG AAAGCAAG-3′ and 5′-TTGGGACTTCTCCCACATTT-3′ for *Hormad1*.

Immunogenicity test for cardiomyocytes. Embryoid bodies were prepared with Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 15% FBS, 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (1,000 cells per 200 μl per well) using 96-well low cell-adhesion plates (Lipidure Coat; NOF Corp.)[36], then transferred to 0.3% gelatin-coated 12-well dishes (BD Falcon) in αMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 0.1% ascorbic acid, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin[37,38]. Beating cardiomyocytes were implanted subcutaneously into C57BL/6-Tg(CAG-EGFP) recipient mice. Tissue sections were immunostained with anti-CD3 antibody (MCA-1477, AbD serotec).

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