Original article

SSEA-3 and 4 are not essential for the induction or properties of mouse iPS cells

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(Received December 11, 2019; Accepted December 21, 2019)

Abstract: Stage-specific embryonic antigens (SSEA-1, 3, and 4) are carbohydrate antigens that have been used as markers of embryonic stem (ES) cells. However, the roles of these antigens in the establishment and maintenance of stemness of ES and induced pluripotent stem (iPS) cells are still poorly understood. This study investigated the biological and functional significance of globo-series glycolipids such as SSEA-3 and 4 in mouse iPS cells induced from tail-tip fibroblasts (TTFs) of $\alpha 1,4$ Gal-T-knockout mice (lacking SSEA-3 and 4). These iPS cells were induced by retroviral transduction of four factors (Oct3/4, Sox2, Klf4, and c-Myc) into TTFs, and colonies were picked up. Morphologically, the colonies resembled ES cells and were positive for alkaline phosphatase and ES cell markers. Furthermore, *in vitro*-differentiated induction experiments after embryoid body formation revealed that some colonies derived from $\alpha 1,4$ Gal-T knockout mice were able to differentiate into three germ layers. Three germ layers were also observed in teratomas from iPS cells derived from $\alpha 1,4$ Gal-T-knockout mice. These results suggest that SSEA-3 and 4 are not essential, at least for the establishment and maintenance of stemness of mouse iPS cells.

Keywords; $\alpha 1,4$ Gal-T, iPS cells, SSEA-3, SSEA-4

Introduction

Carbohydrate chains play essential roles in disease onset such as inflammation, infection, and degeneration, as well as in the maintenance of homeostasis of organ systems [1-3]. They are also cancer antigens known to be involved in the malignant properties of various cancer cells [4,5]. For example, the disialyl gangliosides GD3 and GD2 promote cell proliferation and invasion by enhancing the phosphorylation of p130Cas, focal adhesion kinase (FAK), and paxillin via activation of Src family kinases in human melanoma and osteosarcoma cells [6-8]. Furthermore, carbohydrate chains are considered to be markers of embryonic stem (ES) cells and mesenchymal stem cells [9-13]. The stage-specific embryonic antigens (SSEA) 1, 3, and 4 are known to be expressed on ES cells, mesenchymal stem cells, and teratocarcinomas [9-12]. However, their functional significance is not well understood.

Induced pluripotent stem (iPS) cells, first developed by Yamanaka et al. [14], resemble ES cells in terms of morphology and properties such as self-renewal and pluripotency to differentiate into multiple cell lineages. The use of iPS cells has provided opportunities for exploring the pathogenesis of various diseases and the development of new therapeutic drugs [15-17].

In the present study, iPS cells were generated from tail-tip fibroblasts (TTFs) of $\alpha 1,4$ Gal-T-knockout mice, which lack SSEA-3 and 4 [18], and the mRNA levels of ES cell marker genes were examined in the resulting colonies to confirm that they were iPS cells. To determine the pluripotency of these iPS cells *in vitro*, floating cultivation was performed to derive embryoid bodies, which were then transferred to gelatin-coated tissue culture dishes and examined for expression of ectoderm, mesoderm,

and endoderm differentiation markers. Furthermore, to evaluate the pluripotency of the iPS cells *in vivo*, teratoma formation experiments were conducted.

Materials and Methods

Retroviral vectors, cells and mice

PMXs retroviral vectors containing cDNA of Oct3/4, Sox2, Klf4, or c-Myc [14] were provided by Addgene. SNL feeder cells were provided by Dr. Allan Bradley of the Sanger Institute. iPS-MEF-Ng-20D-17 [19] was obtained from RIKEN (Japan). $\alpha 1,4$ Gal-T^{-/-} mice were generated as described previously [18]. All protocols for animal experiments were approved by the animal experimental Committee of the Graduate School of Medicine in Nagoya University (approval number 21283 and 21363).

Cell culture

iPS cells were maintained in ES medium (DMEM containing 15% FBS (vol/vol), 1×10^{-4} M non-essential amino acids, 1×10^{-4} M 2-mercaptoethanol, 50 U/mL penicillin and 50 μ g/mL streptomycin) on feeder layers of mitomycin C-treated SNL cells. Plat-E packaging cells, which were also used to produce retroviruses, were maintained in DMEM containing 10% FBS, 50 U/mL penicillin and 50 μ g/mL streptomycin. SNL cells were maintained in DMEM containing 7% FBS, 50 U/mL penicillin and 50 μ g/mL streptomycin. Fibroblasts from mouse tail-tips (TTFs) were established as described previously [20] with some modifications. Briefly, the tail from a 7-week-old male $\alpha 1,4$ Gal-T^{-/-} mouse was peeled, minced into 1-cm pieces with scissors, placed on gelatin-coated 6-well plates, and incubated in MF-start medium (Toyobo, Osaka, Japan) for 5 days. Cells that migrated from the tail fragments were transferred to new dishes (passage 2) and maintained in DMEM containing 10% FBS. TTFs at passage 3 were used for iPS cell induction.

iPS cell induction

iPS cell induction was performed as described previously [20] with some modifications. Briefly, TTFs were isolated from 7-week-old male $\alpha 1,4$ Gal-T^{-/-} mice. On the day before transduction, Plat-E cells were seeded at 8×10^6 cells per 100-mm dish. Then, on the following day, PMXs-based retroviral vectors for Oct3/4, Sox2, Klf4, c-Myc, or GFP were introduced into Plat-E cells using FuGENE 6 transfection reagent (Roche, Mannheim, Germany) in accordance with the manufacturer's protocol. Twenty-seven microliters of FuGENE 6 transfection reagent was diluted in 300 μ L Opti-MEM I medium (Invitrogen, Carlsbad, CA, USA) and incubated for 5 min at room temperature. Nine micrograms of plasmid DNA was added to the mixture, which was then incubated for another 15 min at room temperature. After incubation, the DNA/FuGENE 6 mixture was added drop by drop onto Plat-E cells, which were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Twenty-four hours after transduction, the medium was replaced. TTFs were seeded at 8×10^5 cells per 100-mm dish on mitomycin C-treated SNL cells. After 24 h, virus-containing supernatants derived from these Plat-E cultures were filtered through a 0.45- μ m pore size cellulose acetate filter (Schleicher & Schuell, Keene, NH, USA) and supplemented with 4 μ g/mL polybrene. Target cells were incubated overnight in the virus/polybrene-containing supernatants. After infection, the medium for the cells was replaced with fresh medium. Four days after infection, the cells were reseeded at 5×10^3 - 10^5 per 100-mm dish on mitomycin C-treated SNL feeder in ES medium, and the medium was changed

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J-STAGE Advance Publication: July 17, 2020

Color figures can be viewed in the online issue at J-STAGE.

doi.org/10.2334/josnusd.19-0513

DN/JST.JSTAGE/josnusd/19-0513

Table 1 RT-PCR primers

Gene	forward primer	backward primer
Nanog	5'-AGGGTCTGCTACTGAGATGCTCTG-3'	5'-CAACCACTGGTTTTCTGCCACCG-3'
Oct3/4 (endo)	5'-TCTTTCCACCAGGCCCGGCTC-3'	5'-TGCGGGCGGACATGGGGAGATCC-3'
Sox2 (endo)	5'-TAGAGCTAGACTCCGGGCGATGA-3'	5'-TTGCCTTAAACAAGACCACGAAA-3'
Klf4 (endo)	5'-GCGAACTCACACAGGCGAGAAACC-3'	5'-TCGCTTCCTCTTCCCGACACA-3'
c-Myc (endo)	5'-TGACCTAACTCGAGGAGAGCTGGAATC-3'	5'-AAGTTTGAGGCAGTTAAATATGGCTGAAGC-3'
Gdf3	5'-GTTCAACCTGTGCCTCGCGTCTT-3'	5'-AGCGAGGCATGGAGAGAGCGGAGCAG-3'
Cripto	5'-ATGGACGCAACTGTGAACATGATGTCGCA-3'	5'-CTTTGAGGTCTGGTCCATCAGTGACCAT-3'
ERas	5'-ACTGCCCTCATCAGACTGCTACT-3'	5'-CACTGCCTTGACTCGGGTAGCTG-3'
Esg1	5'-GAAGTCTGGTTCCTTGGCAGGATG-3'	5'-ACTCGATACACTGGCCTAGC-3'
map2	5'-CATCGCCAGCCTCGGAACAAACAG-3'	5'-TGCGCAAAATGGAAGTGGAGGCAAC-3'
α Cardiac actin	5'-GCTTTGGTGTGTGACAATGG-3'	5'-ACAATGCCTGTGGTCTTCC-3'
mesp1	5'-CAGAACCTGACCAAGATCGAG-3'	5'-CTTCTGGGATGCTGTTTCT-3'
gata6	5'-ACCTTATGGCGTAGAAATGCTGAGGGTG-3'	5'-CTGAATACTTGAGGTCACTGTTCTCGGG-3'
Gapdh	5'-GGTGCTGAGTATGCTGTGGA-3'	5'-CCTTCCACAATGCCAAAGTT-3'

Fig. 1 Generation of iPS cells from TTFs derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice. (A) Synthetic pathway of globo-series glycolipids and the effects of targeted disruption of $\alpha 1,4\text{Gal-T}$. All globo-series glycolipids in the box were deleted in $\alpha 1,4\text{Gal-T}^{-/-}$ mice. (B) Morphology of iPS clones (left). iPS clones were stained with an alkaline phosphatase kit (Millipore, Bedford, MA) (right).

every other day. Two to three weeks after retroviral infection, colonies were selectively picked up.

Alkaline phosphatase staining

Alkaline phosphatase staining was performed using an Alkaline Phosphatase Detection Kit (Millipore, Bedford, MA, USA) in accordance with the manufacturer's protocol. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1.5 min at room temperature. The fixative was then aspirated and the cells were rinsed with 1 $\times$ rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20). Reagents for alkaline phosphatase staining were then added, and the cells were incubated in dark at room temperature for 15 min. The staining solution was then aspirated and the dishes were rinsed with 1 $\times$ rinse buffer before being covered with PBS to prevent drying.

RT-PCR for ES cell marker genes

After extraction of total RNA, cDNA was synthesized by reverse transcription using the random primer and M-MLV reverse transcriptase (Invitrogen). PCR was conducted with Taq polymerase (Takara, Kusatsu, Japan). Primer sequences are listed in Table 1 [20].

Fig. 2 The expression patterns of ES cell marker genes in iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice. RT-PCR was performed using RNA isolated from iPS clones derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice (D5, D10, D12, D17, D21, D22, D24, E1, E2, G3, G4, N1, N2, and N6). Of note, iPS-Ng-20D-17 generated by Yamanaka S. et al. [19] was used as a positive control for iPS cells.

In vitro differentiation of iPS cells

For embryoid body (EB) formation, iPS cells were harvested by trypsinization and transferred to 60-mm low-cell-binding dishes (Nunc, Roskilde, Denmark) in ES medium without LIF. After 3 or 5 days of floating culture, EBs were transferred to gelatin-coated tissue culture dishes and cultured for another 5 days. Total RNA derived from these differentiated cells was used for RT-PCR analysis.

Teratoma formation and histological analysis

iPS cells were resuspended at 5×10^6 cells/mL in DMEM, and 1×10^6 cells were injected subcutaneously into the dorsal flank of nude mice. After 4–6 weeks, tumors were surgically dissected from the mice, fixed with 10% formaldehyde in PBS, and embedded in paraffin. Sections were then cut and stained with hematoxylin and eosin.

Results

Generation of iPS cells from TTFs derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice

TTFs derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice were transduced with retroviruses containing Oct3/4, Sox2, Klf4, c-Myc, and GFP (Fig. 1A). Four days after infection, the cells were harvested by trypsinization and plated onto mitomycin C-treated SNL feeder at 5×10^3 – 10^5 per 100-mm dish in ES medium. Two to three weeks after retroviral infection, colonies were selectively picked up (Fig. 1B). It was observed ~ 10 ES cell-like colonies on the basis of morphology, and some clones (D10, D17, D22, G4, N2) were strongly positive for alkaline phosphatase (Fig. 1B). On the other hand, some colonies (N1 etc.) were negative or only weakly positive for alkaline phosphatase.

Fig. 3 Pluripotency of iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice *in vitro*. (A) After 3 (EB3) or 5 (EB5) days of floating culture, EBs (middle) were transferred to gelatin-coated tissue culture dishes and cultured for another 5 days (right). (B) RT-PCR was performed using RNA isolated from these differentiated cells. Map2 (a marker of ectoderm), α Cardiac actin (mesoderm), mesp1 (mesoderm), gata6 (endoderm) were used as individual differentiation markers.

Expression of mouse ES markers in iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice

RT-PCR was performed to examine whether ES cell marker genes were expressed in iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mouse TTFs. This revealed that many iPS clones generated in this study as well as iPS-MEF-Ng-20D-17 used as a positive control expressed undifferentiated ES cell-marker genes such as Nanog, Oct3/4 (endogenous), Sox2 (endogenous), growth and differentiation growth factor 3 (gdf3), Cripto, and Eras (Fig. 2).

Embryoid body-mediated differentiation of iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice

To determine the differentiation ability of iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice, floating cultivation was performed to obtain embryoid bodies (EBs). After 3 or 5 days of floating culture, EBs were transferred to gelatin-coated tissue culture dishes and cultured for another 5 days (Fig. 3A). RT-PCR was performed using RNA isolated from these differentiated cells. Microtubule-associated protein-2 (map2) (a marker of ectoderm), α Cardiac actin (mesoderm), mesp1 (mesoderm), and gata6 (endoderm) were used as differentiation markers. RT-PCR revealed that differentiated cells from three clones (G4, D17, and N2) expressed map2, α Cardiac actin, mesp1, and gata6 (Fig. 3B). These data demonstrated that the three clones were able to differentiate into three germ layers *in vitro*.

Teratoma formation from iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice

To determine the pluripotency of iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice *in vivo*, they were transplanted subcutaneously into the dorsal flank of nude mice. After 4–6 weeks, tumor formation was evident, and histological analysis (hematoxylin and eosin staining) revealed that two clones (D17 and N2) differentiated into all three germ layers, including neural tissue (Fig. 4Aa and Ba), epidermis (Fig. 4Ab and Bb), cartilage (Fig. 4Ac and Bc), adipose tissue (Fig. 4Ad and Bd), bone (Fig. 4Ad and Bd), trachea-like epithelium (Fig. 4Ae and Be), and gut-like epithelium (Fig. 4Af).

Discussion

This study confirmed that lack of SSEA-3 and 4 does not affect the establishment or maintenance of stemness of mouse iPS cells. It was possible to generate some colonies that resembled ES cells using TTFs from $\alpha 1,4\text{Gal-T}$ knockout mice. These colonies showed strong expression of alkaline phosphatase and ES cell marker genes such as Nanog, Oct3/4, Gdf3, and Cripto. Furthermore, *in vitro* differentiation experiments after embryoid body formation using the iPS cells obtained demonstrated that marker genes of ectoderm, mesoderm, and endoderm were expressed in the differentiated iPS cells. Histological analysis of teratoma formation also revealed that these iPS cells were able to differentiate into all three germ layers. Collectively, these results supported the pluripotency of iPS cells

Fig. 4 Various tissues generated in teratomas from iPS cells derived from $\alpha 1,4\text{Gal-T}^{-/-}$ mice. Clones were transplanted subcutaneously into nude mice. After 4–6 weeks, tumors were excised, sectioned, and stained with hematoxylin and eosin. (A) clone D17. (a) neural tissue, (b) epidermis, (c) cartilage and adipose tissue, (d) bone, (e) trachea-like epithelium, and (f) gut-like epithelium. (B) clone N2. (a) neural tissue, (b) epidermis, (c) cartilage, (d) bone, and (e) trachea-like epithelium. Bar = 50 μm

obtained from $\alpha 1,4\text{Gal-T}$ -knockout mice.

It has been reported that blocking of SSEA-3 and 4 using a glycosphingolipid biosynthesis inhibitor did not affect pluripotency maintenance in human ES cells [21]. To clarify the importance of SSEA-3 and 4 for establishment and maintenance of stemness of mouse iPS cells, iPS cells were generated from $\alpha 1,4\text{Gal-T}$ -knockout mice. The results revealed that teratoma formed from iPS cells defective in SSEA-3 and 4 contained derivatives of all three germ layers, such as neural tissue, epidermis, cartilage, bone, and trachea-like epithelium.

SSEA-3 and 4 are known to be expressed in a variety of cancer cells [22]. A recent report has indicated that SSEA4 is not only a cancer marker antigen but also plays important roles in the malignant properties of cancer cells [22,23]. For example, administration of anti-SSEA-4 mAb to mice attenuated the tumor volume of gliomas [22]. Furthermore, SSEA-4 directly binds to FK-506 binding protein 4 (FKBP4), which activates PDK1/Akt-dependent cell proliferation signaling in breast cancer cells [23,24].

Expression of SSEA profiles and stages differs between mouse and human embryonic cells. For instance, SSEA-1 is observed in the inner cell mass of mouse but not that of human [10,11], the latter expressing SSEA-3 and 4 instead. During mouse embryogenesis, SSEA-3 and 4 are expressed earlier than SSEA-1 [9,11]. In this study, knockout of SSEA-3 and 4 in mouse iPS cells did not affect the maintenance of stemness. Therefore, SSEA-1 rather than SSEA-3 and 4 may play essential roles in the maintenance of stemness in mouse ES cells.

iPS cells established from $\alpha 1,4\text{Gal-T}$ -knockout mice were able to differentiate into three germ layers and were capable of forming teratoma. However, the issue of whether or not iPS cells can differentiate into various specific cells remained unresolved, and therefore further analysis will be necessary. In summary, the present study has demonstrated that SSEA-3 and 4 do not play critical roles, at least in the establishment and maintenance of mouse iPS cells. Analyses of iPS cells derived from gene knockout mice such as those used in the present study might be useful for exploring the pathogenesis of oral diseases and the development of therapeutic drugs.

Acknowledgments

The authors thank Dr. K. Yamamori, Ms. T. Mizuno and Y. Nakayasu for technical assistance. This study was supported by Nagoya University Academic Promotion Fund (K.H.).

Conflict of interest

The authors have no conflict of interest to declare.

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