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Derivation of Induced Pluripotent Stem (iPS)-like Cells from Microminipig Somatic Cells by Sendai Viral Transduction of 4 HumanG, *OCT4*, *SOX2*, *KLF4*, and *c-MYC*

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Abstract

Research on the stem cell biology of the minipig is rapidly developing. Although research on mouse and human stem cells currently predominates over that in other species, data from these species have provided a good foundation for current and future porcine stem cell research. In addition, the increasing popularity of alternate-species models in the study of human diseases and disease mechanisms has spurred porcine stem cell research. As a source of pluripotent embryonic stem cells, the pig presents several challenges as compared with mice and humans. Nonetheless, porcine minipig embryonic germ cells have been recently produced and may prove particularly useful for *in vitro* and *in vivo* differentiation studies, in gene targeting, and in the creation of transgenic animals. Regarding research on somatic stem cells, transplantation studies of mesenchymal stem cells into porcine heart, cartilage, and bone have yielded very promising results. The microminipig is a small minipig that was established by Fuji Micra Inc. Here, we investigated whether induced pluripotent stem cells (iPSCs) could be derived from somatic cells of the microminipig by using Sendai viruses to introduce human pluripotency transcription factors. Although virally transduced fibroblasts derived from the microminipig generated ES-like colonies, when the cells were introduced into SCID mice, we could not detect the formation of teratomas containing ectodermal, mesodermal, and endodermal tissues. These findings suggest that further research is required to identify the signaling pathways and supplementary factors required for the induction of pluripotent microminipig iPSCs.

Keywords: Porcine; Microminipig; iPS; Stem cells

Introduction

The pig is a potentially useful model in biomedical research because of similarities in its organ size, immunology, and whole animal physiology to those of humans [1-4]. However, the use of pigs and minipigs for in *in vivo* studies is difficult due to their heavy weight. The microminipig was recently developed by Fuji Micra Inc. as a porcine model that is more suitable for life science research. A female minipig named "Catherin" (the microminipig "Eve") was bred by mating the Pot-bellied pig and another type of minipig. The microminipig is an extremely small pig, weighing 7.0 kg at 6 months of age, and is therefore suitable for *in vivo* experiments [4]. In addition, recent legislation by the European Parliament has broadly banned the use of great apes, such as chimpanzees, bonobos, gorillas, and orangutans for scientific testing. The Commission's draft law would also in principle restrict the use of other non-human primates (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). Therefore, the use of non-primate animals will be required hereafter, and the microminipig will be a candidate non-rodent animal for biomedical research studies.

Research on the stem cell biology of the pig is developing rapidly. In the field of regenerative medicine, the pig has gained increased attention given recent breakthroughs in *in vitro* cell models, including porcine iPSCs [5,6]. However, despite these breakthroughs, porcine cells in general are more difficult to culture compared with cells obtained from mice and humans. In addition, porcine iPSCs are unable to silence the inserted transgenes, and cannot self-renew when the transgenes are repressed, suggesting that they are incompletely reprogrammed [5,7-11].

Therefore, additional research on porcine stem cells is required to establish the conditions for generating porcine iPSCs. Here, we investigated whether microminipig iPSCs could be established using Sendai viruses to introduce human pluripotency transcription factors into the fibroblasts of these animals.

Materials and Methods

Microminipig skin tissues

Skin and dermal tissues were obtained from a 7-month-old male microminipig. The fresh dermal tissue was subjected to enzymatic digestion [12], and the dissociated cells were plated on 10-cm tissue culture dishes coated with 0.1% gelatin (Sigma) in a standard culture medium (Dulbecco's modified Eagle's medium [DMEM, Invitrogen] containing 7% fetal bovine serum [FBS], 2 mM glutamine [Sigma], and antibiotics [50 U/ml penicillin and 50μg/ml streptomycin, Sigma]). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 14 days, resulting in the establishment of a microminipig fibroblast cell line.

Cell culture

Mitomycin C-treated mouse embryonic fibroblast (MEF) feeder cells (ReproCELL) were plated in 0.1% gelatin-coated 10-cm culture dishes in the standard culture medium. iPS cells were generated as described below and maintained in Primate ES medium (ReproCELL), supplemented with 4 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems). Primate ES medium was purchased by ReproCell, and the contents of medium is kept an industrial secret. The iPS cells were grown in Primate ES cell medium supplemented with 4 ng/ml bFGF and 10 μM Y27632 (Wako), and the medium was changed every day. Y27632 is a cell-permeable, highly potent and selective inhibitor of Rho-associated coiled-coil forming kinase (ROCK). For passaging, the iPS cells were rinsed once with Hank's balanced salt solution (HBSS) (Invitrogen) and incubated in dissociation medium (ReproCELL) at 37 °C. All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

iPS cell generation

To generate iPS cells, CytoTune^R-iPS 1.0, a kit containing four Sendai viruses (SeV), encoding human OCT4, SOX2, KLF4, and C-MYC (DNAVEC) was used. 5 X 10^5 Microminipig fibroblast cells were plated in 1well of a 6 well plate. The microminipig fibroblast cells were incubated in SeV-containing medium for 24 h, followed by incubation with the standard medium (Dulbecco's modified Eagle's medium [DMEM, Invitrogen] containing 7% fetal bovine serum [FBS], 2 mM glutamine [Sigma], and antibiotics [50 U/ml penicillin and 50 μg/ml streptomycin, Sigma]), which was replaced every day. Seven days later, the cells were harvested by trypsinization, and 5×10^4 cells were placed on MEF feeder cells in a 10-cm dish in Primate ES cell medium supplemented with 4 ng/ml bFGF. Primate ES medium was purchased by ReproCell, and the contents of medium is kept an industrial secret. The medium was changed every day, and the cells were monitored daily for morphological changes.

Immunostaining and immunofluorescence microscopy

To analyze the expression of stem cell markers, the Human Embryonic Stem Cell Marker Antibody Panel was used (R&D Systems). The cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde for 20 min, permeabilized for 60 min with PBS containing 0.1% (v/v) Triton X-100, and then blocked for 3 h with PBS containing 20% donkey serum. The fixed samples were incubated with anti-human alkaline phosphatase monoclonal, anti-human NANOG polyclonal, anti-human OCT4 polyclonal, anti-human SSEA-1 monoclonal, and anti-human SSEA-4 monoclonal antibodies (all from R&D Systems) as indicated, and then washed three times with PBS containing 0.1% (v/v) Triton X-100, and probed with the appropriate secondary antibodies (Alexa 488-conjugated anti-goat IgG antibody or Alexa 488-conjugated anti-mouse IgG antibody [Molecular Probes]). Nucleic acids were stained with SYTOXR Orange Nucleic Acid Stain (Molecular Probes).

In vitro pluripotency assessment

Confluent iPS cells in a 6-cm dish were harvested by trypsinization and transferred to Poly (hydroxyethyl methacrylate-co-methyl methacrylate; HEMA-MMA)-coated 6-well dishes in Primate ES cell medium without bFGF. The medium was changed every other day, and the cells were maintained in floating culture for 8 d. The cells were then placed on 0.1% gelatin-coated 6-well dishes and incubated with ES medium for 8 d. After embryoid body formation, we confirmed the cells' ability to differentiate in vitro by examining the expression of differentiation markers by immunocytochemistry. The cells were washed twice with PBS, fixed with 4% (w/v) paraformaldehyde for 20 min, permeabilized for 60 min with PBS containing 0.1% (v/v) Triton X-100, and then blocked for 3 h with PBS containing 20% donkey serum. Anti-α-fetoprotein mouse IgG (R&D Systems), anti-α-smooth muscle actin mouse IgG (Dako), and anti-βIII-tubulin mouse IgG (Chemicon) were used to analyze the expression of endodermal, mesodermal, and ectodermal markers, respectively. The cells were then washed three times with PBS containing 0.1% (v/v) Triton X-100, followed by incubation with an Alexa 488-conjugated anti-mouse IgG antibody (Molecular Probes). Nucleic acids were stained with SYTOX^R Orange Nucleic Acid Stain (Molecular Probes).

In vivo pluripotency assessment

Approval for the animal studies was obtained from the Animal Committee of Toho University School of Medicine (registration certificate number 14-53-186). Confluent iPS cells in a 6-cm dish were harvested by trypsinization, collected in tubes, and centrifuged, and the pellets were suspended in ES medium supplemented with 10 μM Y27632. The cells were then injected into the testes of 8-week-old SCID mice (Charles River). Three months after injection, the mice were examined for teratoma formation.

Results

Generation of microminipig dermal tissue-derived iPS-like cells

The microminipig was very small, weighing 7.5 kg at 7 months of age (Figure 1a). The dermal tissue was processed, and the isolated cells were cultured and subjected to iPS induction by transduction with a set of SeV constructs (see Materials and Methods). Cells with an ES cell-like morphology were first visible 15-16 days after transduction. The resulting colonies were maintained under human ES cell culture conditions (Figure 1b). In this experiment, we obtained approximately 20-30 ES cell-like colonies from 5 × 10^4 cells (data not shown). The colonies were collected 21-30 days after transduction and transferred into a 24-well plate with MEF feeder cells in Primate ES cell medium containing bFGF and Y27632. The morphology of the microminipig-derived cells was similar to that of human ES cells. From these colonies, which represented multiple sub-cloned cell lines from one microminipig, we selected one cell line for further characterization.

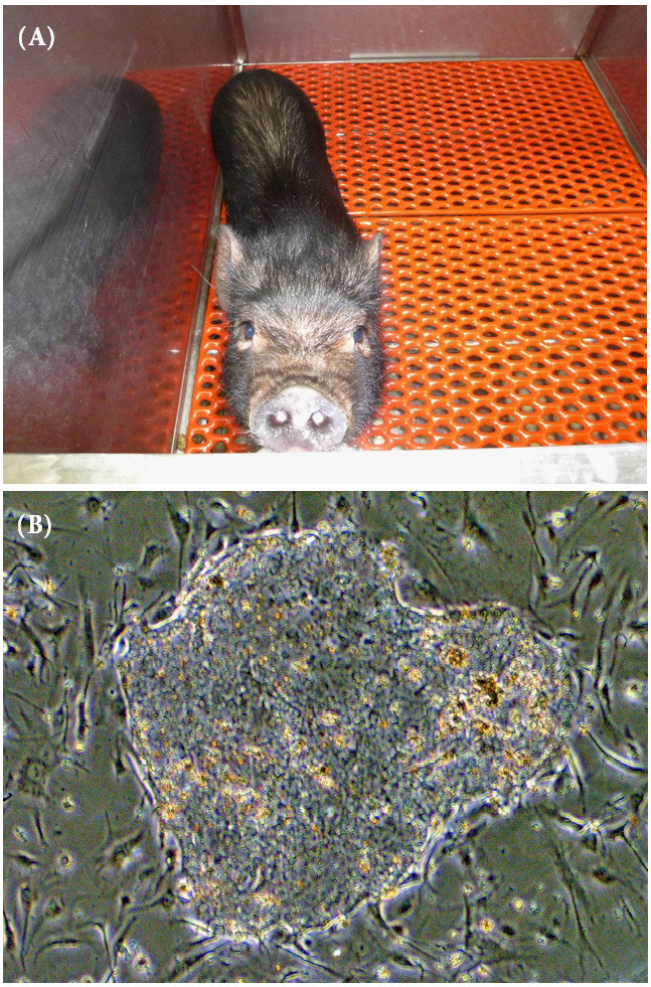

Figure 1: Generation of iPS-like cells derived from the dermal tissue of a microminipig. The isolated cells were transduced with a SeV construct expressing four transcription factors (*OCT4, SOX2, KLF4*, and *C-MYC*)
A) Photograph of a 7-month-old microminipig weighing 7.5 kg
B) Morphology of microminipig iPS-like cells on MEF feeder cells. The passage is second generation and time after transfection is 49 days. Bars = 60 μm

Microminipig-derived iPS-like cells express stem cell markers

We performed immunocytochemistry to examine the protein expression of ES cell markers in the microminipig-derived iPS cells. We showed that these cells expressed alkaline phosphatase (AP), NANOG, OCT4, and SSEA-4, but not SSEA-1 (Figure 2).

Microminipig-derived iPS-like cells are multipotent *in vitro*

To investigate the multipotency of the microminipig-derived iPS cells, we examined their ability to form embryoid bodies *in vitro*. After culturing the cells for 8 d under floating culture conditions, the presence of embryoid bodies was confirmed (Figure 3a). To examine the expression of markers for the three germ layers, we analyzed the plate-attached embryoid bodies by immunocytochemistry. We confirmed that the embryoid bodies expressed α-smooth muscle actin, α-fetoprotein, and β III-tubulin (Figure 3b).

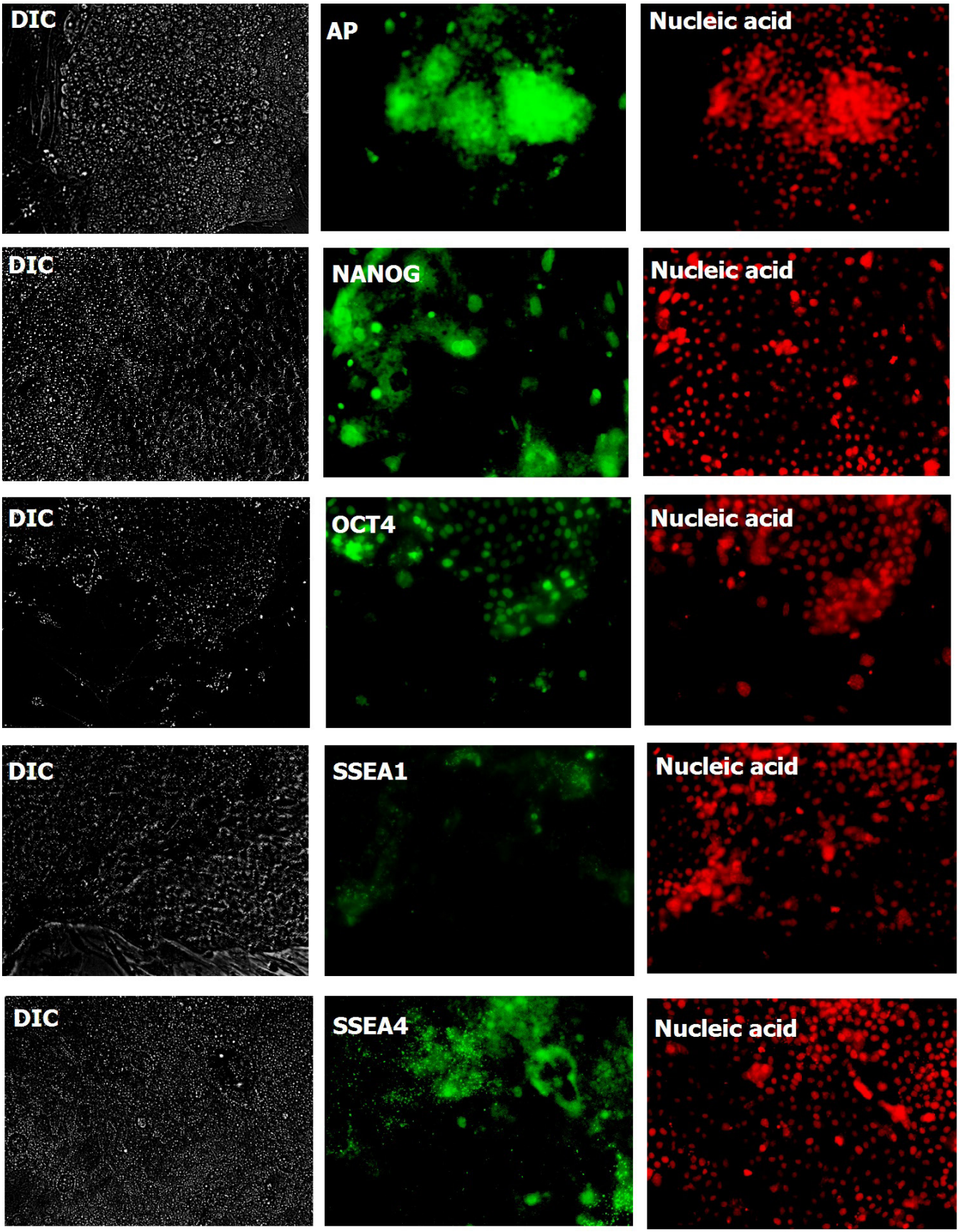

Figure 2: Immunostaining analysis of microminipig iPS-like cells. Nuclei were stained with SYTOXR Orange. Bars = 100 μm

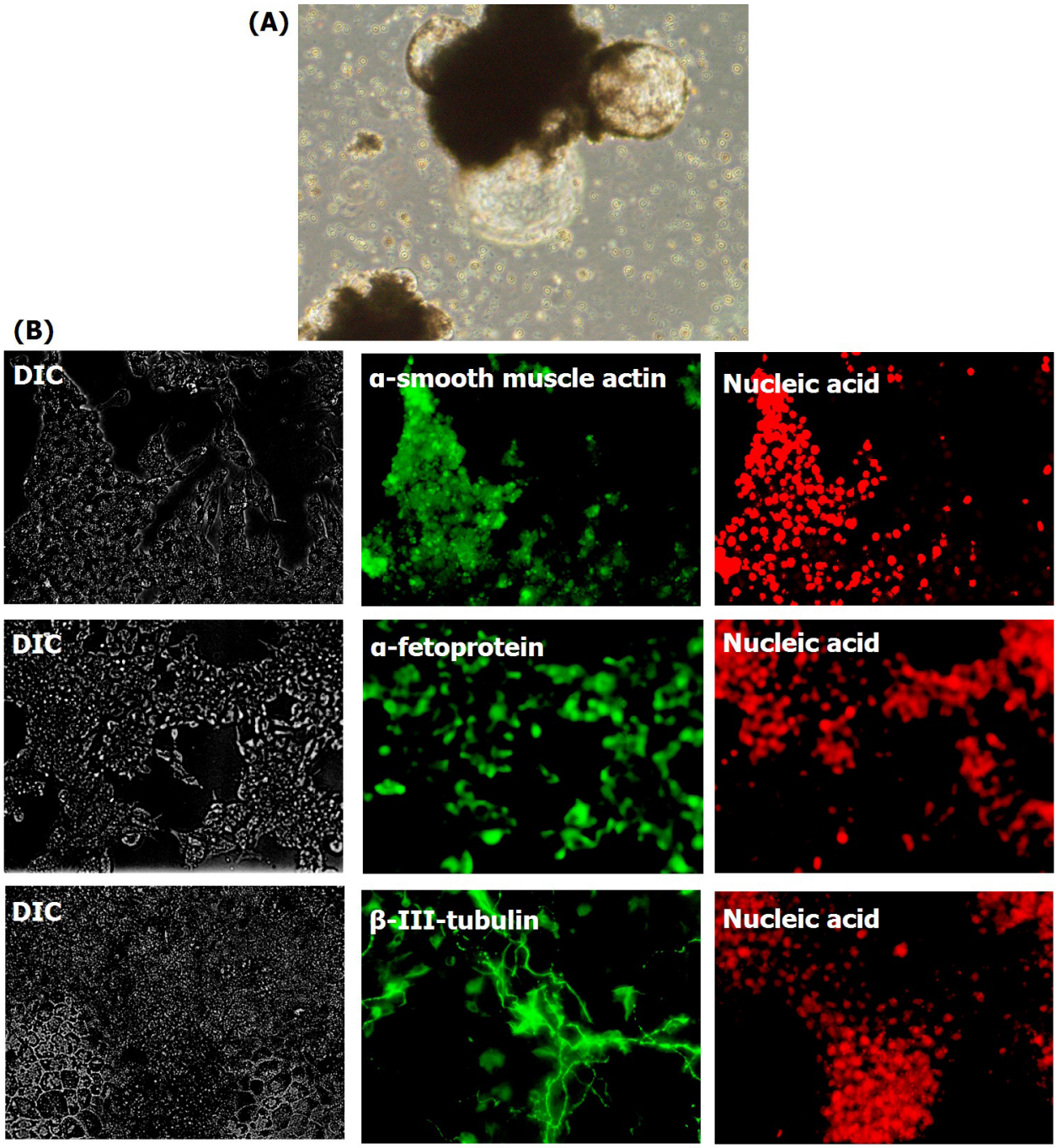

Figure 3: Embryoid body formation and *in vitro* differentiation of microminipig iPS-like cells
A) Embryoid bodies derived from the iPS cells *in vitro*. Bars = 30 μm
B) Immunostaining analysis of the embryoid bodies and depiction of the three germ layers. Nuclei were stained with SYTOX^{R} Orange. Bars = 100 μm

Microminipig-derived iPS-like cells are not multipotent *in vivo*

To examine the differentiation potential of the iPS cells *in vivo*, we transplanted the cells into the testes of SCID mice. We monitored the formation of tumors in the testes for three months after the injection. However, we did not observe teratoma formation in any of the mice (data not shown).

Discussion

Here, we investigated the possibility of generating iPS cells from the dermal tissue of a microminipig using Sendai viruses encoding human *OCT4, SOX2, KLF4*, and *C-MYC*. We found that the virally transduced microminipig-derived cells exhibited an ES-like morphology and demonstrated *in vitro* multipotency, but did not produce teratomas *in vivo*. We also showed that these cells expressed the ES cell markers, AP, OCT4, NANOG, and SSEA-4, but not SSEA-1. The expression of SSEA-1 in porcine iPSCs was previously reported by one group [5], whereas another group claimed that SSEA-1 expression was not observed in their porcine

iPSCs [13]. The inconsistent findings relating to SSEA-1 expression suggest that the different porcine iPS cell lines may have different properties. These differences could result from dynamic differences in the cultured porcine stem cell state related to the reprogramming factors or culture conditions [14]. Although we previously generated human iPSCs that produced teratomas *in vivo* [15] the microminipig-derived iPSCs generated in this study did not produce detectable teratomas when injected into the testes of SCID mice. This finding suggests that these cells may not be true self-renewing stem cells and that the conditions or the state of the cultured cells may have been suboptimal for generating iPSCs.

Pluripotent stem cell proliferation is maintained by complex cell signaling pathways, and the pathways involved in *in vitro* proliferation and self-renewal differ between mouse and human pluripotent stem cells. It is also known that various supplements are required to sustain pluripotent stem cells in culture. For example, basic FGF is required for human iPSCs and leukemia inhibitory factor (LIF) is required for mouse iPSCs. There are well-described differences between early porcine embryonic development and mouse and human embryonic development [16-18]. Given that the composition of the media currently used for the culture of porcine embryonic stem cells (ESCs) and iPSCs is based on that used for the culture of mouse or human ESCs and iPSCs, it is reasonable to assume that the media composition may not be optimal for the generation of porcine iPSCs. Further studies determining which signaling pathways are active in porcine pluripotent cells may be required to refine the media composition and/or to block the predisposition of these cells to form neural cells [19]. The addition of cell signaling inhibitors to cultured microminipig iPSCs could reveal which cell signaling pathways are crucial in regulating self-renewal and proliferation.

In conclusion, we were unable to generate microminipig iPSCs that exhibited both *in vitro* and *in vivo* pluripotency using a standard method that has been used to establish mouse and human iPSCs. To establish stable microminipig iPSCs, further research will be required to identify the signaling pathways and supplements involved in regulating self-renewal and pluripotency in these iPSCs.

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H.K. supervised the entire project.

Disclosures

Conflict of interest

Fumito Yamabe, Koichi Nagao, Koichi Nakajima, and Hideyuki Kobayashi declare that they have no conflict of interest.

Animal studies

All institutional and national guidelines for the care and use of laboratory animals were followed.

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