Original Article

Canine induced pluripotent stem cells efficiently differentiate into definitive endoderm in 3D cell culture conditions using high-dose activin A

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A R T I C L E   I N F O

Article history:
Received 30 July 2022
Received in revised form 9 September 2022
Accepted 8 October 2022

Keywords:
3D cell culture
Canine
Definitive endoderm
Endodermal differentiation
Induced pluripotent stem cell

A B S T R A C T

Introduction: Endoderm-derived organs support indispensable functions in the body. Pluripotent stem cells can generate endoderm-derived cells or tissues and have excellent therapeutic potential to replace the functions of endodermal tissues. However, there is no viable method to induce endodermal precursor cells, definitive endoderm (DE), from canine induced pluripotent stem cells (ciPSCs).

Methods: A ciPSC line was used in this study. In order to induce DE, ciPSCs were cultured with high dose activin A and fetal bovine serum. We considered the optimal differentiation period and starting cell density. Next, to reduce the remaining undifferentiated cells and improve the DE induction efficiency, DE was induced from 3D cell aggregates with knockout serum replacement instead of fetal bovine serum. Finally, hepatic and pancreatic induction were performed to investigate whether DE could differentiate into downstream lineages.

Results: After differentiation, some cells expressed the DE markers FOXA2 and SOX17. DE induction period and starting cell density were found to be important for efficient DE induction. However, some cells remained undifferentiated even after optimization of cell density and culture period. Cell differentiation under 3D culture conditions reduced undifferentiated cells and the replacement of fetal bovine serum with knockout serum replacement improved the DE induction efficiency. After hepatic and pancreatic induction, cells expressed some early hepatic and pancreatic markers.

Conclusions: A ciPSC line was successfully differentiated to DE efficiently using a high dose of activin A with knockout serum replacement under 3D cell culture conditions. We believe that this study will be fundamental to achieving the generation of canine endodermal tissues from ciPSCs. © 2022, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
conditions such as diabetes [5], cirrhosis [6], and inflammatory bowel disease [7]. Thus, an efficient method to obtain pancreatic, hepatic, and intestinal cells or tissues from ciPSCs could offer a new cell transplantation-based treatment for dogs with diseases in endoderm-derived organs.

Additionally, ciPSCs can also benefit human medicine by providing a model for validating the efficacy and safety of regenerative therapies. Although rodent animal models are usually used to examine the efficacy and safety, they do not reproduce in full human disease [8]. In contrary, canines share a variety of biochemical and physiological characteristics with humans, live longer, and are outbred. Because they are also exposed to external and environmental factors that contribute to diseases such as obesity, diabetes, and cancer, naturally occurring disease in dogs might be a valuable preclinical model for humans [9].

During embryonic development, endodermal tissues develop from the same origin, the definitive endoderm (DE) [10]. Human PSCs (hPSCs) and mouse PSCs differentiate into endoderm-derived tissues via DE induction, mimicking embryogenesis [11,12]. A high dose of activin A is widely used for the differentiation of DE cells from hPSCs [13,14]. In addition, some reports have suggested that 3D cell culture conditions and the replacement of fetal bovine serum (FBS) with knockout serum replacement (KSR) improved DE induction efficiency from hPSCs [15–17]. Because the gastrointestinal and respiratory tracts of dogs might also develop from the same endodermal progenitors [18], it should be possible to generate endoderm-derived tissues from ciPSC-derived DE. Considering the potential clinical applications, we previously reported the generation of ciPSCs from not only embryonic cells [19] but also adult cells including peripheral blood mononuclear cells (PBMCs) [20–22]. However, the optimal method and mechanism of DE induction from ciPSCs remains unknown.

We hypothesized that a high dose of activin A induces ciPSCs into endodermal lineages, and 3D cell culture and KSR supplementation improves DE induction efficiency as is the case with hPSCs. In order to obtain DE efficiently in this study, we studied the DE induction conditions based on a high dose of activin A using one ciPSC line.

2. Materials and methods

2.1. Cell culture

All experiments were performed using one ciPSC line, OPUiD05-A, which was previously established in our laboratory from canine PBMCs using a Sendai virus vector encoding the human KLF4, OCT3/4, SOX2, and C-MYC genes [22]. ciPSCs were maintained on a laminin-511 E8 fragment (iMatrix511; Nippi, Inc., Tokyo, Japan) using StemFit AK02N (StemFit; Ajinomoto, Tokyo, Japan). They were passaged mechanically using a glass Pasteur pipette. ciPSCs from passages 40 to 65 were used for differentiation studies.

2.2. DE induction from ciPSCs

ciPSCs were dissociated into single cells using TrypLE Select (Thermo Fisher Scientific, Waltham, MA, USA). They were seeded into 24-well cell culture dishes, coated with 1:30 dilution of Matrigel Matrix (Corning, Inc., Corning, New York, NY, USA), at a density of 2.5 × 10^4–8.0 × 10^4/cm² by adding 10 µM ROCK inhibitor (Nacalai Tesque, Kyoto, Japan) (day –2). The next day (day –1), we removed the ROCK inhibitor and cultured the ciPSCs for 24 h. When the ciPSCs were differentiated in 3D conditions as cell aggregates, Matrigel was added to ice-cold StemFit (1:30 dilution), ciPSC culture medium was changed using ice-cold StemFit containing Matrigel to put ciPSCs between Matrigel layers. On day 0, ciPSCs were washed one time with RPMI1640 (Nacalai Tesque) and ciPSCs were cultured in a DE induction medium at 37 °C and 5% CO₂. The DE induction medium was RPMI1640 containing 1% non-essential amino acids (NEAA; Nacalai Tesque), 1% GlutaMAX (Thermo Fisher Scientific), 1% B27 supplement (Thermo Fisher Scientific), and 100 ng/ml human activin A (Nacalai Tesque). In the series of cultures, 0% (day 0–1), 0.2% (day 1–2), and 2.0% (day 2–) FBS (Sigma-Aldrich, St. Louis, MO, USA) or same concentration of KSR (Thermo Fisher Scientific) were added to the DE induction medium, as previously reported [13].

2.3. Assessment of DE induction

DE differentiation was analyzed using immunocytochemistry and flow cytometry (FCM). For immunocytochemistry, cells were washed in phosphate-buffered saline (PBS) (–), fixed in 4% paraformaldehyde (Sigma-Aldrich) for 5 min, and permeabilized with 0.1% Tween 20 in PBS (–) for 5 min at around 25 °C. The cells were incubated with 10% bovine serum albumin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) for 30 min, followed by overnight incubation at 4 °C in the presence of primary antibodies against OCT3/4, SOX17, and FOXA2. The negative control cells were incubated in PBS (–) without primary antibodies. The next day, the cells were washed with PBS (–) and incubated for 1 h at around 25 °C with the secondary antibodies. The cells were washed with PBS (–), labeled with DNA using ProLong Gold Antifade Reagent 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific), and observed using confocal laser microscopy (FV3000; Olympus, Tokyo, Japan). For FCM, the cells were dissociated using 0.25% trypsin–EDTA. Cell pellets were resuspended in FACS buffer (PBS (–)) containing 2% FBS and 1 mg/ml sodium azide (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and labeled with CXCR4–PE and c-kit–FITC on ice for 30 min. Negative control cells were incubated with the isotype control. The cells were washed in FACS buffer and analyzed using FCM (CytoFLEX; Beckman Coulter, Brea, CA, USA). All the antibodies used in this study are listed in Supplementary Table 1.

2.4. Differentiation of DE into downstream endodermal lineages

The hepatic lineage was induced as described in a previous study with some modifications [23]. Briefly, DE cells were cultured in RPMI1640 with 1% NEAA, 1% GlutaMAX, 1% B27 supplement, 0.5% dimethyl sulfoxide (Sigma-Aldrich), 0.5 µM A83-01 (Reprocell, Yokohama, Japan), and 250 nM sodium butyrate (Fujifilm Wako Pure Chemical Corporation) for 10 days. The medium was changed every day. The pancreatic lineage was induced as described in a previous study, also with slight modifications [24]. Briefly, DE cells were cultured in RPMI1640 with 1% NEAA, 1% GlutaMAX, 1% B27 supplement, 50 ng/ml fibroblast growth factor 10 (FGF10; Peprotech, Rocky Hill, NJ, USA), 2 µM retinoic acid (Fujifilm Wako Pure Chemical Corporation), 10 µM SB431542 (Fujifilm Wako Pure Chemical Corporation), and 1 µM dorsomorphin (Tocris, Ellisville, MO, USA) for 14 days. The medium was changed every day.

After differentiation, quantitative reverse transcription polymerase chain reaction (qRT–PCR) was performed. For qRT–PCR, total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RT was performed using random primers and ReverTra Ace (Toyobo, Osaka, Japan). Quantitative PCR was carried out in triplicate using the Plus One System (Thermo Fisher Scientific) with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) according to the manufacturer’s instructions. The cDNA of liver and pancreas from a 12-year-old male beagle was preserved in our laboratory and used as the positive controls. The animal experiment was approved by the Institutional Animal Experimental Committee of Osaka Prefecture University (Permission number: 21–67). All the primers used are listed in Supplementary Table 2.
2.5. Statistical analysis

The experiments for FCM were performed using one well per condition. The qRT-PCR was performed in triplicate, using one well per condition. These experiments were repeated three times. Data are expressed as the mean ± standard deviation. Statistical significance was determined using the Tukey–Kramer multiple comparison procedure or the student’s t-test using Statcel software (Statcel 3; OMS Ltd., Tokyo, Japan). A p value < 0.05 was considered statistically significant.

3. Results

3.1. ciPSCs differentiated into DE expressing the same marker pattern as human DE

When ciPSCs were seeded at the density of $1.0 \times 10^4$, $2.0 \times 10^4$, $4.0 \times 10^4$, or $8.0 \times 10^4$/cm$^2$ on day 2 and cultured in DE induction medium from day 0. The differentiation schema is illustrated in Fig. 1A. After three days of differentiation, the cells changed from PSC-like to cobble-like morphologies only when ciPSCs were seeded...
at a density of $1.0 \times 10^4$/cm$^2$ (Fig. 1B). Under other conditions, the cells reached confluence before differentiation (day 0) and their morphologies did not change after differentiation (Fig. 1B). Using FCM analysis, we evaluated the ratio of other DE markers CXCR4/c-kit double-positive cells as the DE induction efficiency; it was revealed that the efficiency was the highest at $1.0 \times 10^4$/cm$^2$ (9.89 ± 1.66%, 1.22 ± 1.57%, 2.44 ± 1.44%, 5.13 ± 2.40% at $1.0 \times 10^4$/cm$^2$, $2.0 \times 10^4$/cm$^2$, $4.0 \times 10^4$/cm$^2$, $8.0 \times 10^4$/cm$^2$, respectively) (Fig. 1C). Further, we assessed the expression states of other DE markers (FOXA2 and SOX17) and an undifferentiated marker (OCT3/4) by immunocytochemistry before and after differentiation. On day 0, immunocytochemistry revealed that the majority of cells expressed OCT3/4 and very few cells expressed FOXA2 and SOX17 over the entire cell density (Supplementary Fig. 1). On day 3, as expected, only when ciPSCs were seeded at $1.0 \times 10^4$/cm$^2$, some cells that were negative for OCT3/4 expressed the DE marker FOXA2. Immunolabeling also showed that some cells expressed another DE marker, SOX17 (Fig. 1D and Supplementary Fig. 2).

### 3.2. Determining an appropriate DE induction period and starting cell density

To calculate the DE induction period, after ciPSCs were seeded at a density of $1.0 \times 10^4$/cm$^2$, FCM was performed every day from day 0 (Fig. 2A). Daily FCM analysis revealed that the DE differentiation efficiency was highest on day 4 (Fig. 2B). Next, we considered the initial cell density to induce DE. After ciPSCs were seeded at a density of $2.5 \times 10^3$, $5.0 \times 10^3$, or $1.0 \times 10^4$/cm$^2$, DE was induced for four days from day 0 (Fig. 3A). After DE differentiation, fewer ciPSCs were seeded and fewer PSC-like cells were observed (Fig. 3B). FCM analysis revealed that DE induction efficiency was highest when ciPSCs were seeded at $5.0 \times 10^3$/cm$^2$ (Fig. 3C). Under conditions of $5.0 \times 10^3$/cm$^2$ seeding and four days differentiation, the DE differentiation efficiency was 41.26 ± 6.25%. However, immunocytochemistry revealed that OCT3/4-positive undifferentiated cells were present in differentiated DE cells (Fig. 3D).

### 3.3. Improvement of DE induction from ciPSCs

After DE induction under the conditions described above (2D conditions), undifferentiated cells remained. Therefore, we attempted to induce DE under 3D conditions to reduce the number of remaining undifferentiated cells. This schema is illustrated in Fig. 4A. After ciPSCs were seeded at $5.0 \times 10^3$/cm$^2$ and cultured in Matrigel, ciPSCs formed 3D cell aggregates (Fig. 4B). After four days of DE induction, the number of PSC-like cells decreased in the 3D condition (Fig. 4B). Immunocytochemistry also showed that OCT3/4-positive and SOX17-negative undifferentiated cells also decreased under 3D conditions (Fig. 4C). By contrast, the number of OCT3/4 and SOX17 double-negative cells increased rather than OCT3/4-negative and SOX17-positive cells (Fig. 4C).

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**Fig. 2.** Consideration of the appropriate DE induction period. (A) The schema for the consideration of the DE induction period. From day 0, DE induction efficiencies were determined every day (B) The result of FCM every day. The bar graph below showed the comparison of DE induction efficiency. There is a statistically significant difference ($p < 0.05$) between the different codes. $n = 3$. 

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Finally, FBS was replaced with KSR during four days of induction (0% day 0; 0%, day 1; 0.2%, and day 2; 2.0%) under 3D conditions. After four days of differentiation, no significant morphological differences were observed (Fig. 4D). However, KSR significantly improved DE differentiation efficiency compared to FBS, from 43.55 ± 3.14% to 64.32 ± 8.12% (Fig. 4E).

3.4. ciPSC-derived DE differentiated hepatic and pancreatic lineage

DE is the precursor of endodermal tissues such as the liver and pancreas. Therefore, we investigated whether ciPSC-derived DE has the potential to differentiate into these lineages. After hepatic lineage induction, HNF4A and AFP were upregulated compared to those in ciPSCs (Fig. 5A). HNF4A was also upregulated in the DE stage, and the expression level did not change between DE and hepatic lineage-induced cells. However, the expression of the mature hepatic marker ALB did not increase in differentiated cells compared with that in ciPSCs (Fig. 5A). After pancreatic differentiation, the early pancreatic marker PDX1 was upregulated compared to ciPSCs and DE, albeit with lower expression levels compared to adult pancreatic tissue (Fig. 5B). The mature pancreatic marker, INS, was not detected in the differentiated cells (Fig. 5B). During hepatic and pancreatic differentiation, some cells differentiated into beating cardiomyocytes (Supplementary Video 1, after hepatic differentiation, and Supplementary Video 2, after pancreatic differentiation).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.reth.2022.10.002.

4. Discussion

In this study, we sought the efficient methods to differentiate ciPSCs into DE cells as endodermal precursors. After ciPSCs were differentiated into DE for three days, the cells seeded at a low density
expressed the DE markers FOXA2, SOX17, CXCR4, and c-kit. In contrast, ciPSCs seeded at a high density did not differentiate and maintained their undifferentiated state. This coincides with a previous report that hPSCs seeded at a low density differentiated into DE [25]. They also reported that hPSCs seeded at a high density maintained their undifferentiated state even after DE induction, which corresponded with our results. However, some groups reported that DE was induced efficiently when hPSCs were seeded at a high density [24,26], which might be due to the properties of each hPSC line. Therefore, in dogs, it is necessary to consider the initial seeding cell density when DE is induced in other ciPSC lines.

During differentiation on Matrigel, DE induction efficiency was the highest at $5.0 \times 10^3$/cm$^2$ seeding density and four days induction. However, even after differentiation under these conditions, some undifferentiated cells were observed with immunocytochemistry. It has also been reported that undifferentiated cells remain after DE induction in hPSCs [27]. The inclusion of activin A in the DE induction medium activates activin/nodal signaling and phosphorylates SMAD2/3 [28]. Highly activated activin/nodal signaling results in DE differentiation because SMAD2/3 directly binds to endodermal lineage-specifiers such as SOX17 and FOXA2 [29]. In contrast, phosphorylated SMAD2/3 in hPSCs under low
concentrations of activin A enters the nucleus and directly binds to the promoter regions of pluripotency-associated genes, such as NANOG and OCT3/4, resulting in the maintenance of their undifferentiated states [30]. Therefore, activin A has two distinct functions, maintenance of the undifferentiated state in hPSCs and induction of the endoderm from the pluripotent state. Meanwhile, insulin and insulin-like growth factor (IGF), they are included in FBS, activate PI3K signaling and inhibit activin/nodal signaling by crosstalk [31], resulting in the remaining of undifferentiated cells after DE induction from hPSCs. In canine PSCs, although activin A reportedly phosphorylates SMAD2/3 [32], the function and crosstalk of activin/nodal and PI3K signaling remain unknown. The understanding of these signaling functions and interactions will facilitate the more efficient differentiation of ciPSCs into DE.

DE induction from 3D cell aggregates resulted in a reduction of undifferentiated cells and an increase of OCT3/4 and SOX17 double-negative cells. We speculated that these cells represent those that had just escaped from the pluripotent state and were heading toward transformation to the DE state. During hPSC aggregate culture, cells in the center region of aggregates showed a reduction in their proliferation ability, accompanied by enhanced apoptosis or necrosis. This can be explained by the limited oxygen and nutrient availability in the center region as the size of aggregate increased, in particular at a size of over 400 μm [33]. In our study, the size of ciPSC aggregates at day 0 was approximately 50 μm and the aggregates attached to the bottom of dish, proliferated, and finally differentiated to DE in two dimensions. Therefore, under our experimental conditions, ciPSC aggregates might have been only minimally impacted by stress conditions that could induce apoptosis or necrosis. Finally, during differentiation under 3D conditions, the replacement of FBS with KSR improved the DE induction efficiency. The detailed mechanisms of our results remained unclear. Because FBS and Matrigel contain undefined factors, it requires attention to discuss about their effects. During DE induction from hPSCs, cell differentiation was also improved under 3D conditions because cell survival, cell growth, and cell-cell contact were promoted [16]. Furthermore, a sandwich culture using Matrigel promoted the epithelial-to-mesenchymal transition in hPSCs, resulting in improved DE induction efficiency [15]. Various growth factors included in Matrigel had the possibility to improve DE induction efficiency from ciPSCs. On the other hand, while FBS contains both insulin and IGF, KSR contains only insulin [17]. Although DE induction was not performed under 2D conditions with KSR, these studies raised the hypothesis that 3D cell cultures

![Fig. 5. Differentiation of hepatic or pancreatic lineages from canine DE. (A) The relative mRNA expression levels for the hepatic lineage markers, HNF4A and AFP, and the mature hepatic marker, ALB. mRNA expression levels were normalized by β-ACTIN as the housekeeping gene. **p < 0.01; n = 3 (B) The relative mRNA expression levels for the pancreatic progenitor marker, PDX1, and the mature pancreatic marker, INS. mRNA expression levels were normalized by β-ACTIN as the housekeeping gene. **p < 0.01; n = 3.](image-url)
promote cell growth, cell-cell contact, and epithelial-to-mesenchymal transition in ciPSCs and KSR activated PI3K signaling to a lesser extent than FBS, resulting in an efficient DE induction.

Although the hepatic lineage-induced cells expressed HNF4A and AFP, the pancreatic lineage-induced cells expressed the pancreatic progenitor marker PDX1, albeit at a low level. Furthermore, some cells differentiated into beating cardiomyocytes following hepatic and pancreatic differentiation. During embryonic development, the cardiac mesoderm reportedly induces the liver program and suppresses the pancreatic program through fibroblast growth factor secretion [34]. This may explain why the pancreatic lineage marker was not remarkably upregulated. Because PSCs pass through the mesendoderm stage, which is in a bipotent state, into DE and mesodermal lineages [2], the development of the cardiac lineage from ciPSCs indicated that some cells differentiated toward the mesoderm at the DE stage. Additionally, during DE induction, molecules modulating bone morphogenetic protein signal or Wnt/β-catenin signal were reported to direct hiPSCs into three subtypes of DE based on their differentiation potentials [35]. In our study, canine DE was induced without other signaling modulators; although it is unknown that subtypes of canine DE are exist, we might need to consider them to induce endodermal lineages.

Additionally, the expression level of mature hepatocyte, ALB was not increased in hepatic lineage-induced cells in contrast to the upregulated expression of HNF4A and AFP. HNF4A is the regulator gene for the direct reprogramming of canine mesenchymal stem cells to hepatocytes [36] and AFP is expressed in canine hepatic progenitor cells [37]. Although other canine hepatic progenitor or hepatocyte markers such as CK7 or Hep Par1 [38] should be assessed to confirm the differentiation status of hepatic lineage-induced cells, our results indicate that hepatic lineage-induced cells did not reach a mature phenotype. In humans and mice, appropriate stepwise culture conditions are necessary to obtain adequate mature endodermal cells such as hepatocytes or pancreatic cells [39,40]. These studies might provide suggestion to induce mature endodermal cells from ciPSCs via DE.

Because there are no reports on the identification of canine DE, suitable DE markers for canines are unknown. Therefore, in this study, we employed the DE markers that are used in humans [41]. Our data indicate that canine DE, defined using human DE markers, can differentiate into pancreatic and hepatic lineages assessed by only mRNA expression levels. Although protein expression should be assessed to confirm the cell differentiation, our results suggested that FOXA2, SOX17, CXXR4, and c-kit are appropriate canine DE markers. Although we showed that ciPSCs derived from PBMCs could differentiated into DE cells efficiently, the limitation in this study was that DE differentiation was performed using only one ciPSC line. Human iPSC lines reportedly had the large variation in their differentiation capacity to specific lineages due to the epigenetic memory from somatic cells [42], the aberrations in DNA methylation during cell reprogramming [43], and the genetic differences among donors [44]. In addition, because Matrigel is extracted from murine tumor and its component is not defined [45], the cells cultured in Matrigel are not suitable for transplantation. In order to generate the endodermal tissues/cells from ciPSCs and apply them for veterinary regenerative medicine, further study must be needed to replace Matrigel with chemically defined extracellular matrix and evaluate the DE induction protocol could apply to other ciPSC lines.

5. Conclusions

We are the first to report that ciPSCs seeded at an appropriate density can efficiently differentiate into DE under 3D conditions using a high dose of activin A and a low dose of KSR. ciPSC-derived DE could differentiate into downstream lineages, albeit in immature differentiation states. To apply iPSC-derived endodermal cells for regenerative medicine, DE must be induced from iPSCs with high purity and efficiency. Further studies are therefore needed to establish a reliable method for generating transplantable cells from various ciPSC lines. However, considering the successful generation of canine endodermal cells from ciPSCs, our study offers fundamental information and differentiation strategies for the efficient induction of DE from ciPSCs.

Declaration of competing interest

The authors declare no conflicts of interest directly relevant to the content of this article.

Acknowledgment

This work was supported by JSPS KAKENHI (Grant numbers JP18K19273, JP18H02349, and JP19J22851). We would like to thank Editage (www.editage.com) for the English language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.10.002.

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