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Methods and reagents

Improved methods for the differentiation of hypothalamic vasopressin neurons using mouse induced pluripotent stem cells

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ABSTRACT

High differentiation efficiency is one of the most important factors in developing an *in vitro* model from pluripotent stem cells. In this report, we improved the handling technique applied to mouse-induced pluripotent stem (iPS) cells, resulting in better differentiation into hypothalamic vasopressin (AVP) neurons. We modified the culture procedure to make the maintenance of iPS cells in an undifferentiated state much easier. Three-dimensional floating culture was demonstrated to be effective for mouse iPS cells. We also improved the differentiation method with regards to embryology, resulting in a greater number of bigger colonies of AVP neurons differentiating from mouse iPS cells. Fgf8, which was not used in the original differentiation method, increased iPS differentiation into AVP neurons. These refinements will be useful as a valuable tool for the modeling of degenerative disease in AVP neurons *in vitro* using disease-specific iPS cells in future studies.

1. Introduction

The hypothalamus is essential for the homeostatic regulation of vital bodily functions. The study of functional hypothalamic cells is complicated by the technical difficulties involved in obtaining and maintaining sufficient numbers of cells. One solution is the usage of pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, which represent an unlimited source of patient-derived cells, and, in theory, can give rise to all cell types. Therefore, they can be used in regenerative medicine, basic research for developmental biology, and disease modeling.

Organ formation during embryogenesis consists of complex processes but can be modeled *in vitro*. Our colleagues previously established a three-dimensional culture method for ES cells called serum-free culture of embryoid body-like aggregates with quick re-aggregation (SFEBq) (Eiraku et al., 2008; Watanabe et al., 2005). This floating culture technique has revealed intrinsic programs that drive locally autonomous modes of organogenesis and homeostasis. Using SFEBq, we and our colleagues have developed differentiation culture methods for hypothalamic neurons such as vasopressin-positive neurons from mouse and human ES cells (Ogawa et al., 2018; Wataya et al., 2008), and have utilized these lines as *in vitro* models for analyses (Takeuchi et al., 2016). These hypothalamic induction methods are based on the recapitulation of embryogenesis, particularly the principal that the hypothalamus originates from the most rostral part of the neural plate in the embryo. Strict removal of exogenous patterning factors during early differentiation steps in culture is important for the generation of rostral hypothalamic-like progenitors. We designated this culture medium as growth factor-free, chemically defined medium (gfCDM). Our culture methods (Ogawa et al., 2018; Wataya et al., 2008) are the only techniques to have achieved vasopressin (AVP) secretion from induced cells, indicating that our methods can induce well-differentiated and functional AVP neurons. Therefore, they are suitable for utilization in analyses as a hypothalamic model.

In addition to its functionality, another important advantage of this hypothalamic model is the differentiation efficiency of the induction culture. Our methods have achieved relatively higher efficiency for AVP neuron differentiation as compared with other reports (Merkle et al., 2015; Wang et al., 2015). However, technical improvements for higher efficiency have always been required to ensure the induced cells can be easily used as an *in vitro* model.

Here in this report, we demonstrate improved induction of

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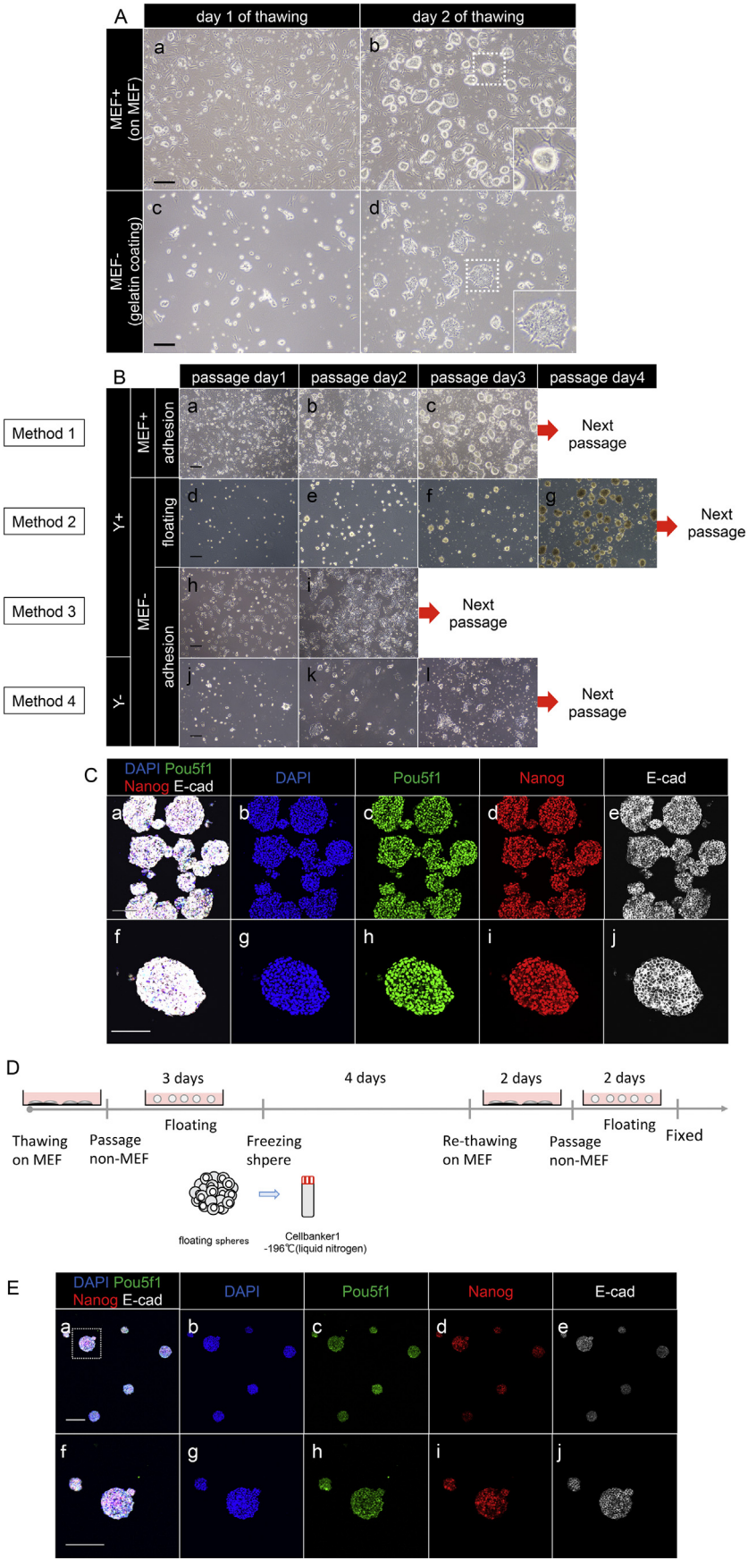

Fig. 1. Improvement in cell maintenance procedures. Mouse iPS colonies with MEFs (A.a., A.b.) and without MEFs (A.c., A.d.) during the thawing process. Scale bars, 200 μm. Mouse iPS colonies after the first passage with Method 1 (B.a.-B.c.; adhesion culture after passaging with MEF coating containing Y27632), Method 2 (B.d.-B.g.; floating culture after passage containing Y27632), Method 3 (B.h., B.i.; adhesion culture after passage without MEF coat containing Y27632), and conventional adhesion culture Method 4 (B.j.-B.l.; adhesion culture after passaging with neither MEF coat nor ROCK inhibitors). Scale bars, 200 μm. Expression of undifferentiated markers in Method 2 aggregates (C.a.-C.j.). Scale bars, 100 μm. Protcol of freezing & thawing experiment of the spheres (D). Expression of undifferentiated markers in freezing & thawing experiment aggregates (E.a.-E.j.). Scale bars, 100 μm.

hypothalamic AVP neurons from mouse iPS cells in which the modified factors are concordant with embryology.

2. Materials and methods

2.1. Mouse iPS cell line generation

To produce a mouse iPS cell line, fibroblast cells from C57BL/6J mice fetuses (E12.5) were reprogrammed through the transfection of retroviral vectors carrying four Yamanaka factors (Takahashi and Yamanaka, 2006). Mixed aliquots containing retroviral vectors (pMXs-Oct3/4, pMXs-Sox2, pMXs-Klf4, pMXs-cMyc) were added to fibroblasts from C57BL/6J mice in culture medium containing high glucose D-MEM (Wako, JPN) supplemented with 10% fetal bovine serum (FBS; GE Healthcare, USA) and 1 μg/mL puromycin (ant-pr-5; InvivoGen, USA). Approximately 7 days later, colonies were picked for expansion into individual iPS cell lines, transferred onto mouse embryonic fibroblast (MEF) feeder cells treated with mitomycin C (MMC) to prevent proliferation, and cultured with high glucose D-MEM supplemented with 15% KnockOut Serum Replacement (KSR) (Gibco, USA), 4% FBS (Cell Culture Bioscience, Japan), 1 × MEM non-essential amino acids (Wako, JPN), 1 × EmbryoMax Nucleosides (Millipore, USA), 55 μM 2-mercaptoethanol (Gibco), 1000 U/mL leukemia inhibitory factor (Millipore, USA), and 0.5 × penicillin-streptomycin solution (Wako). Established mouse iPS cell lines were confirmed to be alkaline phosphatase-positive and to have a normal karyotype.

2.2. Thawing of mouse iPS cells

Mitomycin C-treated MEF feeder cells were prepared on 0.1% gelatin-coated dishes (0.6×10^6 cells/6 cm dish). Mouse iPS cells frozen in liquid nitrogen were thawed in a water bath at 37 °C and then immediately washed in maintenance medium containing Glasgow's MEM (Gibco) supplemented with 15% (vol/vol) KSR (Gibco), 1% FBS (Sigma, USA), 1 mM pyruvate (Sigma), 0.1 mM non-essential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Wako), and 2000 U/mL LIF before being distributed over MEF-coated dishes in maintenance medium with 10 μM Y27632, and culturing at 37 °C in 5% CO_2. The following day, the medium was changed to regular maintenance medium without Y27632.

2.3. First passage of mouse iPS cells in floating culture

Mouse iPS cells on MEF-coated dishes were washed with PBS and treated with 500 μL trypsin-EDTA for 2 min at 37 °C in 5% CO_2. Detached mouse iPS cells were dissociated into single cells, washed, and distributed into 10 mL of maintenance medium containing 10 μM Y27632 on 10 cm non-coated cell culture dishes (IWAKI, Japan) without any coating. Floating mouse iPS cells were cultured at 37 °C in 5% CO_2.

2.4. Mouse iPS cell maintenance in floating culture after second passaging

Four days after the first passage, dissociated mouse iPS cells had formed floating spheres. These floating cells were collected, washed with PBS, and treated with 500 μL trypsin-EDTA for 4 min at 37 °C in 5% CO_2. Dissociated mouse iPS cells were counted and 1.0×10^6 cells were distributed into 10 mL maintenance medium on 10 cm dishes. This passaging procedure was repeated every 4 days.

2.5. Freezing and throwing the sphere

The mouse iPS was thawed and passaged using Method 2.2-3, then formed into floating spheres. In order to check whether the spheres are capable to survive the process of freezing and thawing, the spheres were frozen with CELLBANKER1 (Zenoaq, Japan, 2500 μL/10 cm dish), and stored in liquid nitrogen. Four days later, they were thawed and plated on MEF. They were passaged with Method 2.3, and the formed floating spheres were evaluated by immunohistochemistry (Fig. 1D, E).

2.6. In vitro differentiation of mouse iPS cells into hypothalamic AVP neurons

Maintained mouse iPS cells were dissociated as described above. A total of 5.5×10^5 cells were suspended in 11 mL of gfCDM (1:1 Iscove's modified Dulbecco's medium (Gibco)/Ham's F-12 (Gibco, USA), 1 × chemically defined lipid concentrate (Gibco), 438 μM monothioglycerol (Sigma) and apo-Transferrin-free purified BSA (Sigma) (Johansson and Wiles, 1995; Wataya et al., 2008) with 10 μM Y27632. Then 100 μL suspension containing 5.0×10^3 cells was distributed into each well of a 96-well round-bottom low-cell adhesion plate (Thermo, USA). Cells autonomically and quickly aggregated in culture at 37 °C in 5% CO_2. On day 7, 100 ng/mL mouse Fgf8b (R&D, USA) and 5 μg/mL heparin (NIPRO, Japan) were added to the gfCDM differentiation medium. On day 10, half of the medium was replaced with DFNB (DMEM/F12; Sigma) supplemented with 3.85 g/L glucose (Sigma), 1.2 g/Lsodium hydrogencarbonate (Sigma), 50 U/mL penicillin, 50 μg/mL streptomycin (Gibco), N2 (Gibco), B27 (Gibco), and 10 ng/mL CNTF (R&D Systems, USA)) with 100 ng/mL Fgf8b and 5 μg/ml heparin. On day 13, iPS cell aggregates were transferred on Transwell culture plates (Corning, NY, USA) in which the bottom wells were each filled with 1.5 mL DFNB, 100 ng/mL Fgf8b, and 5 μg/mL heparin. Hereafter, medium was changed every other day with 1.5 mL DFNB, 100 ng/mL Fgf8b, and 5 μg/mL heparin. After day 26, the frequency of the whole medium change was increased daily.

2.7. Differentiation markers

2.7.1. Immunohistochemistry

Immunohistochemistry of frozen sections was performed using the following primary antibodies: mouse anti-Pou5f1 (Oct3/4) (1:100; BD Biosciences, RRID: AB_398736), rabbit anti-nanog (1:500; Cosmo Bio, RRID: AB_1962694), rat anti-E-cadherin (1:100; Takara Bio, Cat#M108), guinea pig anti-Otp (1:2000; Takara Bio, Cat#MS1535GS), goat anti-Pou3f2 (Brn2) (1:500; Santa-Cruz, RRID: AB_2167385), mouse anti-neurophysin II (NPII) (1:100; Millipore, Cat#MABN845), goat anti-copeptin (1:100; Santa Cruz, RRID: AB_2061966), guinea pig anti-AVP (1:2000; Peninsula, RRID: AB_518680), mouse anti-OXT (1:100; Millipore, RRID: AB_2157626), goat anti-AgRP (1:250; Neuromics, RRID: AB_2687600), rabbit anti-NPY (1:1000; abcam, RRID: AB_1566510), and rabbit anti-TRH (1:1000; Sigma, RRID: AB_10669525). Representative staining for AVP, neurophysin II, and copeptin are shown in Supplementary Fig. 1.

2.7.2. FACS sorting

Cells were counted on a FACS Vantage SE (BD) and the data were analyzed with FACS Diva software (BD). MEFs were labeled by the PKH26 red fluorescence dye according to the product information (MINI26, Sigma, USA). Cells were dissociated into single cells using Trypsin-EDAT, DNase I, and Y-27632 treatment and filtered through a Cell Strainer (BD Biosciences, USA), and analyzed at room temperature. PKH26+ cells (indicating MEFs) and PKH26- cells (indicating mouse iPS cells) were gated by referring to scattered plots of the undifferentiated mouse iPS cells.

2.7.3. Quantitative RT-PCR

Total RNA was purified using the RNAeasy kit (Qiagen, NLD) after treatment with DNase (Qiagen). Quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies, USA) and the MX3000P system (Agilent Technologies, USA). Data were normalized compared to GAPDH expression. The primers used were shown in Supplementary Fig. 2.

2.7.4. Vasopressin release analysis

For vasopressin release analysis, aggregates cultured on Transwell plates for 30–40 days were subjected to analysis. Ten aggregates per filter were incubated with 1000 μl of artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 3 mM KCl, 26 mM NaHCO3, 2 mM CaCl2, 1 mM MgSO4, 1.25 mM KH_2PO_4, and 10 mM D-glucose, pH 7.4) for 10 min at 37 °C, followed by stimulation with high K+ aCSF (100 mM KCl) for an additional 10 min. Each incubated solution was individually frozen and its AVP content was analyzed with an RIA kit (Yamasa, Japan), which is used clinically in Japan. The quantification limit of Yamasa's AVP kit is 0.4 pg/mL and the detection limit is 0.25 pg/mL.

3. Results

3.1. Improvement in maintenance

Generally, maintenance procedures for mouse ES/iPS cells are sophisticated, and gelatin or Matrigel have been frequently used instead of MEFs because of their simplicity. However, the effect of MEFs as a feeder cell line was more powerful than these approaches, at least in this study. We used MEFs (mitomycin C-treated), and not gelatin, during the thawing period because it is the most critical aspect of the protocol for their survival. As a result, many healthy colonies that showed characteristic round swollen shapes formed on MEF-coated dishes (Fig. 1A.a., A.b.), compared with gelatin-coated dishes (Fig. 1A.c., A.d.).

However, MEF contamination is undesirable for the use of ES/iPS cell-derived differentiated tissue as an *in vitro* model. Therefore, we did not use MEFs after the first passage and instead developed a novel passage procedure using suspended cell culture (Fig. 1B.a.-l.), comparing it with conventional adhesion culture using 0.1% gelatin coating (Wataya et al., 2008). With conventional adhesion culture, we observed considerable numbers of differentiated cells, which appeared to be neural and glial differentiating cells, between the healthy colonies of undifferentiated cells (Fig. 1B.j.-l; Method 4). With the novel floating culture approach, many undifferentiated cells occupied the dominant part (Fig. 1B.g.). A small number of differentiated cells were attached to the surface of culture dishes (Fig. 1B.e.-g.), preventing contamination of these differentiated cells into the next passage. Immunohistochemical staining was shown to be better suited for maintaining the undifferentiated stage in the floating culture in Method 2 (Fig. 1C.a-j). The undifferentiated stage was maintained even after the freezing and thawing procedure (Fig. 1E.a.-j.).

Furthermore, contaminating MEFs during the early passages seemed to adhere to and be trapped on the surface of culture dishes in the suspended culture, which was helpful for mouse iPS cell purification through several passages. Actually, cell sort analysis confirmed that there was no MEF contamination in the floating mouse iPS spheres after 2 passages, using MEF labeling culture (Suppl Fig. 3A.-E.).

3.2. Improvement in differentiation

Our differentiation protocol was based on the method of Wataya et al. (Wataya et al., 2008), the key point of which is the recapitulation of embryogenesis, especially the early stage, with the strict removal of exogenous patterning factors. Initial addition of ROCK inhibitor, Y-27632, promoted better growth of differentiating iPS aggregates (Method A/Y– *vs.* Method A/Y+, Fig. 2A., Fig. 3.).

Additionally, in this study, we focused on optimizing the later stage of differentiation. As a result, we found that treatment with Fgf and heparin (Method B, Fig. 3), which binds directly to Fgf receptors to accelerate Fgf binding, was effective. Treatment with Fgf8b and heparin increased the expression of Otp and Pou3f2, which are precursors of AVP (Fig. 2B.a., B.b., B.e., B.f., B.i, B.j.), and also increased the positive rate of AVP neuron differentiation (Fig. 2B.c., B.d., B.g., B.h., B.k, B.l., D, Suppl Fig. 4). As shown in Fig. 2B, sporadic and scattered AVP-positive cells were apparent with the conventional method (Fig. 2B.c, B.g.). Treatment with Fgf resulted in the accumulation of more AVP-positive cells (Fig. 2B.k., E.). AVP secretion by KCl stimulation was greater than that by Fgf-treated aggregates (Fig. 2C). Additional immunohistochemistry and PCR analyses showed that the differentiated AVP neurons and other hypothalamic neurons were similar to the previous reports (Suppl Figs. 2, 5).

4. Discussion and conclusions

ES and iPS cells can be utilized in regenerative medicine, developmental basic research, and disease modeling, and thus establishment of differentiation methods is important. Several reports have demonstrated the differentiation of hypothalamic neurons from pluripotent stem cells (Lund et al., 2016; Merkle et al., 2015; Ogawa et al., 2018; Wang et al., 2015; Wataya et al., 2008). Among them, our methods using three-dimensional culture (Ogawa et al., 2018; Wataya et al., 2008) have produced AVP-secreting neurons from mouse and human ES cells.

For the construction of an *in vitro* model using ES/iPS cells, high efficiency and good differentiation of the culture are important. Our team studies AVP neurons and their diseases (Arima et al., 2016; Azuma et al., 2014; Hagiwara et al., 2019, 2014; Hayashi et al., 2009; Morishita et al., 2011; Tochiya et al., 2018), and have undertaken many trials to improve hypothalamic differentiation. In this report, we present two important factors for the improved efficiency of AVP neuron differentiation, as follows.

First, the maintenance of an undifferentiated state is more efficient in the improved approach than our conventional method. Combination of MEF culture and floating sphere culture to reduce differentiation signals resulted in better maintenance and proliferation of undifferentiated mouse iPS cells. LIF was identified as essential for the maintenance of undifferentiated mouse pluripotent stem cells (Niwa et al., 2009), but we sometimes find that LIF treatment is not sufficient to maintain their undifferentiated state. It seems to depend, at least in part, on the characteristics of individual cell lines (Simeone et al., 1994; Ying et al., 2008). The mouse iPS cell line used in this current report seemed relatively difficult to keep healthy in its undifferentiated state. Previous reports have shown that MEF secretes activin A and TGF beta1 (Eiselleova et al., 2008). Other reports have indicated that the endogenously activated autocrine loops of activin-Nodal-TGFβ signaling promote ES cell self-renewal (Ogawa et al., 2007). We chose MEF to assist cell growth and maintenance just after thawing. Thus, temporal usage of MEFs or maintenance in floating spheres could be effective for such cell lines. Those spheres were able to maintain the undifferentiated state even after the freezing and thawing procedure, which may make the way easier for maintenance and mass culture.

The second factor is the improvement made in the differentiation steps. Wataya et al. previously developed a chemically defined medium that contained minimized positional information (Wataya et al., 2008) because they focused on recapitulating the environment from which the hypothalamus originates; the most rostral part of neural plate during early embryogenesis. In this report, we focused on the later stages of hypothalamic differentiation, which have not yet been considered in mouse ES/iPS differentiation culture.

In the Wataya's report (Wataya et al., 2008), Rax was used as a marker of hypothalamic progenitor cells. In general, Rax is useful for the recognition of differentiated ventral hypothalamic neuronal areas (Lu et al., 2013). However, based on recent *in vivo* reports, Rax expression may not be related directly to AVP differentiation, but only to Otp (Orquera et al., 2016), which means that Rax seems to be partly involved in the AVP differentiation process. Three transcription factors, Otp (Caqueret et al., 2006; Del Giacco et al., 2008; Simeone et al., 1994; Wang and Lufkin, 2000), Sim1 (Caqueret et al., 2006; Duplan et al., 2009; Michaud et al., 1998), and Pou3f2 (Caqueret et al., 2006; Nakai et al., 1995), were reported to be important and directly connected in

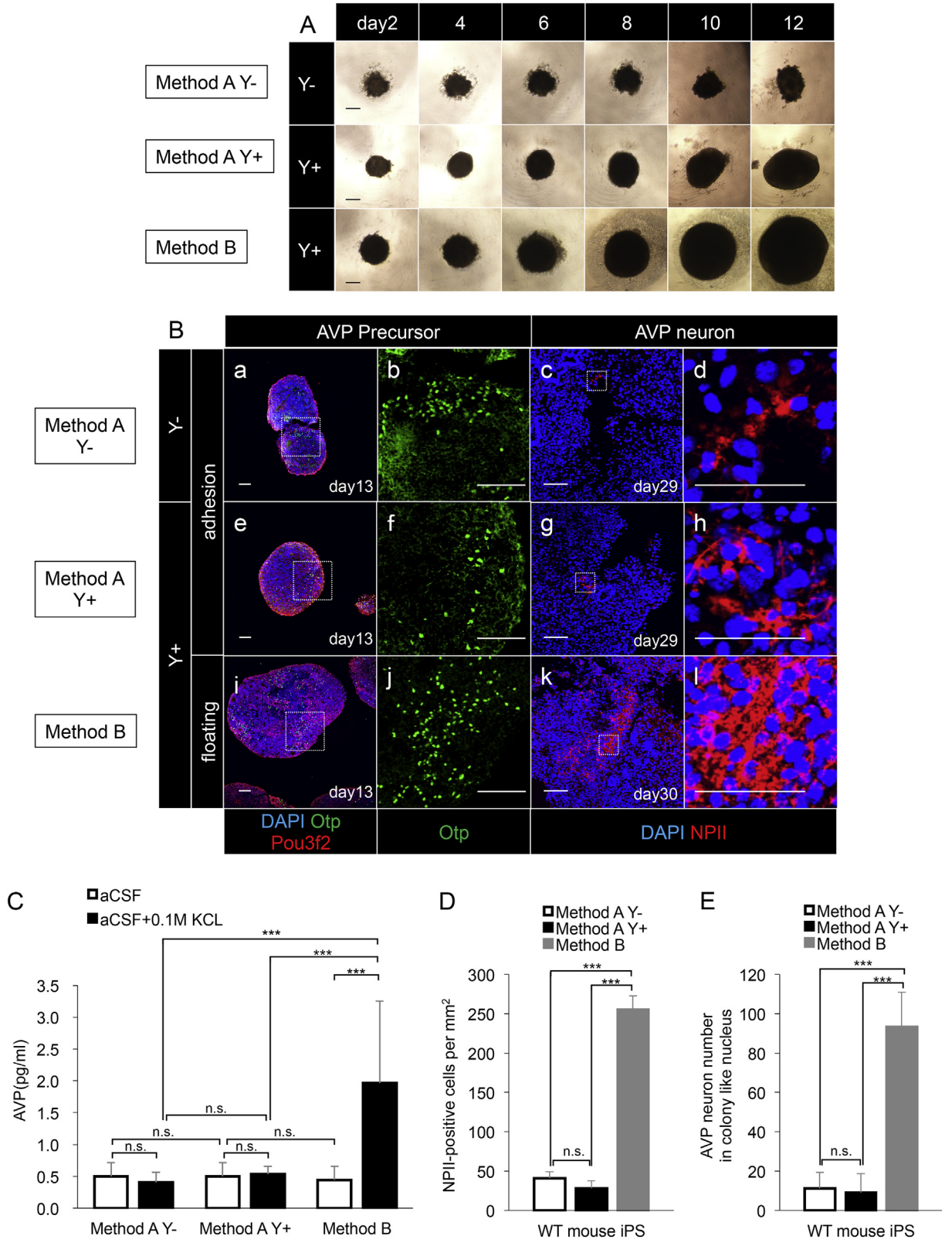

Fig. 2. Improvement in differentiation procedures. (A) Proliferation of iPS cell aggregates. Method A (see also Fig. 3) was performed with iPS cells maintained using Method 1 (Fig. 1.B.). Method B (see also Fig. 3) was performed using iPS cells maintained with Method 2 (Fig. 1.B.). Scale bars, 200 μm. (B) Differentiation into AVP neurons. Otp and Pou3f2 expression as precursors of AVP neurons (B.a., B.b., B.e., B.f., B.i, B.j.). NPII expression as a mature marker for AVP neurons (B.c., B.d., B.g., B.h., B.k, B.l.). Scale bars, 100 μm. (C) AVP secretion into the medium before and after KCl depolarlization. (D) Number of AVP neurons per 1 mm^2. € Number of AVP neurons per one colony. $*P < .05$, $**P < .01$, $***P < .001$.

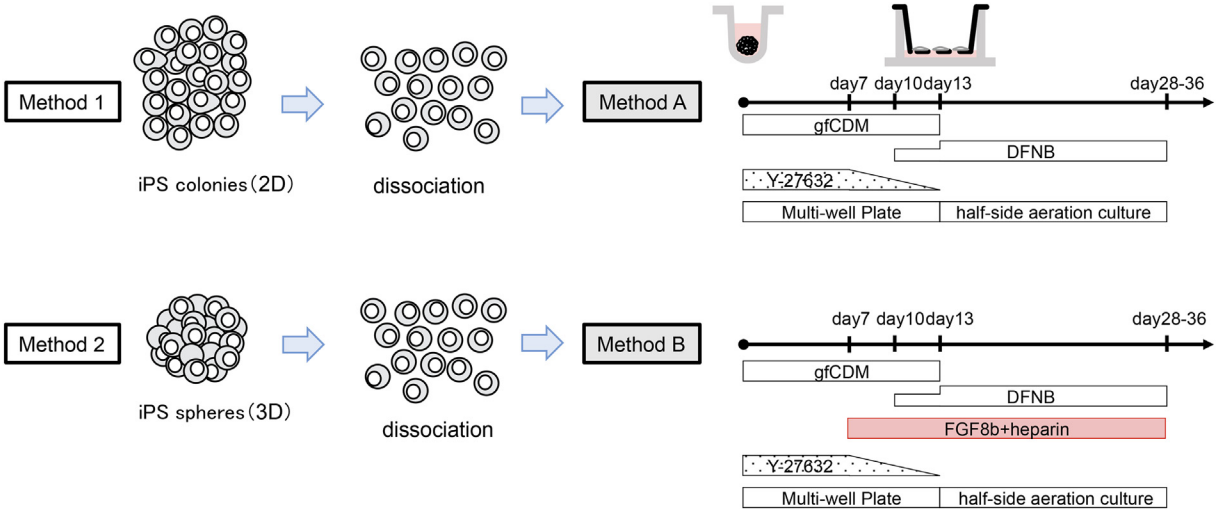

Fig. 3. Schema of differentiation procedure. Method A (Fig. 2) was performed with iPS cells maintained using Method 1 (Fig. 1.B.). Method B (Fig. 2), which was the best way to promote AVP-neuronal differentiation in this report, was performed using iPS cells maintained with Method 2 (Fig. 1.B.).

the AVP differentiation tree (Alvarez-Bolado, 2019; Bedont et al., 2015; Caqueret et al., 2005; Qin et al., 2018; Xie and Dorsky, 2017). Fgf8 is reportedly necessary to increase Otp expression in the supraoptic nucleus (SON) (Tsai et al., 2011). Moreover, Fgf8-hypomorphic mice showed a reduction in AVP (McCabe et al., 2011; Tsai et al., 2011). We integrated Fgf8b treatment and optimized its timing and concentration, resulting in increased numbers of Otp-positive cells and AVP neurons. The expression patterns of Fgf8 and FGFR1 have reported to be conserved in humans (McCabe et al., 2011), with Fgf8 signaling being important for hypothalamo-pituitary axis development (McCabe et al., 2011). Therefore, we expect it to be applicable to the culture of human iPS cells in future studies.

We have previously used induced AVP neurons from mouse ES cells as an *in vitro* model to examine their secretory mechanism (Takeuchi et al., 2016). The major rate-determining step was revealed to be the differentiation efficiency of AVP neurons from pluripotent stem cells. Through the improvements in differentiation reported here, induced AVP neurons could be used more conveniently as a suitable model. In future studies, we will use disease-specific iPS cells to establish models of diseases such as familial neurogenic diabetes insipidus, an incurable disease caused by gene mutations. Through pathological studies using these disease models, novel treatments may be developed in the clinic. The improvements achieved in this study will contribute to future disease modeling research using disease-specific iPS cell lines.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101572.

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