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REVIEW

Application of pluripotent stem cells for treatment of human neuroendocrine disorders

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Abstract

The neuroendocrine system is composed of many types of functional cells. Matured cells are generally irreversible to progenitor cells and it is difficult to obtain enough from our body. Therefore, studying specific subtypes of human neuroendocrine cells in vitro has not been feasible. One of the solutions is pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. These are unlimited sources and, in theory, are able to give rise to all cell types of our body. Therefore, we can use them for regenerative medicine, developmental basic research and disease modeling. Based on this idea, differentiation methods have been studied for years. Recent studies have successfully induced hypothalamic-like progenitors from mouse and human ES/iPS cells. The induced hypothalamic-like progenitors generated hypothalamic neurons, for instance, vasopressin neurons. Induction to adenohypophysis was also reported in the manner of self-formation by three-dimensional floating cultures. Rathke's pouch-like structures, i.e., pituitary anlage, were self-organized in accordance with pituitary development in embryo. Pituitary hormone-producing cells were subsequently differentiated. The induced corticotrophs secreted adrenocorticotropic hormone in response to corticotropin-releasing hormone. When engrafted in vivo, these cells rescued systemic glucocorticoid levels in hypopituitary mice. These culture methods were characterized by replication of stepwise embryonic differentiation. It is based on the idea of mimicking the molecular environment of embryogenesis. Thanks to these improvements, these days, we can generate hormone-secreting neuroendocrine cells from pluripotent stem cells. The next problems that need to be solved are improving differentiation efficiency even further and structuring networks.

Keywords Hypothalamus · Pituitary · Embryonic stem cells · Differentiation

Introduction

The hypothalamus and adenohypophysis are essential for the regulation of vital functions such as growth, metabolism, sexual development, the immune system and stress response. Their dysfunction causes systemic symptoms (Schneider et al. 2007; Willems and Vankelecom 2014) and continues permanently.

Recently, somatic stem cells have been recognized as a major source for tissue maintenance and regeneration. In the adenohypophysis, the existence of somatic stem cells has been reported (Chen et al. 2005). Subsequent studies have discussed their functions during early postnatal pituitary maturation (Fauquier et al. 2001; Kikuchi et al. 2007; Chen et al. 2009; Gremeaux et al. 2012; Mollard et al. 2012), after pituitary damage (Luque et al. 2011; Fu et al. 2012; Langlais et al. 2013) and in pituitary tumorigenesis (Gaston-Massuet et al. 2011; Andoniadou et al. 2012; Garcia-Lavandeira et al. 2012; Li et al. 2012a, b). Additionally, stem cells have been reported to be present in the hypothalamus (Li et al. 2012a, b; Lee et al. 2012; McNay et al. 2012). However, the hypothalamus and pituitary in adulthood do not turn over very actively (Alvarez-Buylla and Lim 2004; Rando 2006; Slack 2008; Wabik and Jones 2015), with the function of stem cells in them remaining enigmatic.

In addition to somatic stem cells, studies have focused on embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. These pluripotent stem cells exhibit self-renewal properties and pluripotent differentiation abilities. Therefore, they have attracted attention as a cell source for tissues in clinical applications.

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In regenerative medicine, neuroendocrine diseases that lose hormone-secretion ability are suitable targets. For instance, central diabetes insipidus seems suitable. In another application, disease-specific iPS cells could be available as disease models when they are differentiated into mature tissues. Incurable diseases caused by gene mutations, such as familial neurogenic diabetes insipidus, are the candidates. Through pathological studies using these disease models, novel treatment may be realized clinically.

Demand of hypothalamus and adenohypophysis regenerative medicine

The hypothalamus and adenohypophysis are located in adjacent regions, connected via a portal vein. Pituitary cells respond to rapid and short-acting signals from the hypothalamus. They coordinate as the center for the endocrine systems. In the case of their dysfunction, patients suffer from various systemic symptoms. Current treatment consists of hormone replacement therapy but various factors can complicate proper dosage in some cases. Drug administration cannot precisely mimic the circadian or stress-induced changes of hormone requirements. For example, we reported that some patients with central diabetes insipidus show unstable serum Na levels, resulting in a poor prognosis (Arima et al. 2014). This instability seems to be caused by the lack of positive and negative control systems, which is characteristic of hormone-producing cells, in conventional hormone-replacement therapy. As for hypopituitarism, adrenal crisis has been reported to occur in a substantial proportion of hypopituitary patients and adrenal crisis-associated mortality is not negligible, even in educated patients (Hahner et al. 2015). In other reports, adrenocorticotropic hormone (ACTH)-dependent adrenal insufficiency, as well as high-dose hydrocortisone treatment, serves as a predictor for acromegaly-associated mortality (Sherlock et al. 2009; Ben-Shlomo 2010). Furthermore, patients with pituitary disorder have higher risks of diabetes mellitus, hypertension, hyperlipidemia, depression and anxiety (Stewart et al. 2016). Taken together, there are many prospects for hypothalamus-pituitary regenerative medicine.

Mouse embryonic stem cells as a pioneer of human model

There are two reasons for the use of mouse ES cells as a first step, rather than human pluripotent stem cells, in our studies. One reason is the short developmental period; the duration of mouse fetal development is about 20 days, which is much shorter than the 300 days of human development. Therefore, mouse ES cells seem suitable for establishing de novo differentiation methods in preparation for numerous trial-and-error processes. Another reason is the similarity between mouse and human cells. The fundamental processes of mouse ES cells appear to be applicable to human ES cells. For example, the retinal differentiation method from human ES cells (Nakano et al. 2012) was established based on a previous report using mouse ES cells (Eiraku et al. 2011). Although the two inducing culture methods considerably differ, their key principles are similar.

Three-dimensional culture method for embryoid body

Organ formation during embryogenesis consists of complicated processes that involve various local interactions between different tissues or cells. Despite this complexity, organogenesis can be modeled in vitro. Our colleagues established a three-dimensional culture method for ES cells called "serum-free culture of embryoid body-like aggregates with quick re-aggregation (SFEBq)" (Watanabe et al. 2005; Eiraku et al. 2008). Maintained ES cells are dissociated to single cells in trypsin. Cells are autonomically and quickly aggregated when they are disseminated into low-cell adhesion well plates in differentiation medium (Fig. 1a).

This culture method is appropriate for induction of various ectodermal derivatives from ES cells. In SFEBq cultures, the ES cell aggregates exhibit self-organization (Sasai et al. 2012) and spontaneous formation of a highly ordered structure or patterning. This floating culture has revealed intrinsic programs that drive locally autonomous modes of organogenesis and homeostasis. Using the SFEBq method, mesencephalic dopamine neurons (Kawasaki et al. 2002; Morizane et al. 2006), cortex neurons (Eiraku et al. 2008; Danjo et al. 2011; Kadoshima et al. 2013), the optic cup (Eiraku et al. 2011; Ikeda et al. 2005; Osakada et al. 2008), cerebellar neurons (Muguruma et al. 2010) and hippocampal neurons (Sakaguchi et al. 2015) have been generated from mouse and human ES cells.

Hypothalamic neurons induced from mouse ES cells

Using SFEBq culture, hypothalamic neurons, such as vasopressin-positive neurons, have been induced from mouse ES cells (Muguruma et al. 2010). Differentiation occurs efficiently when the ES cell aggregates are cultured in growth factor-free, chemically defined medium (gfCDM). Strict removal of exogenous patterning factors during early differentiation steps induces efficient generation of rostral hypothalamic-like progenitors (Rax(+)/Six3(+)/Vax1(+); these combinations are characteristic for hypothalamic

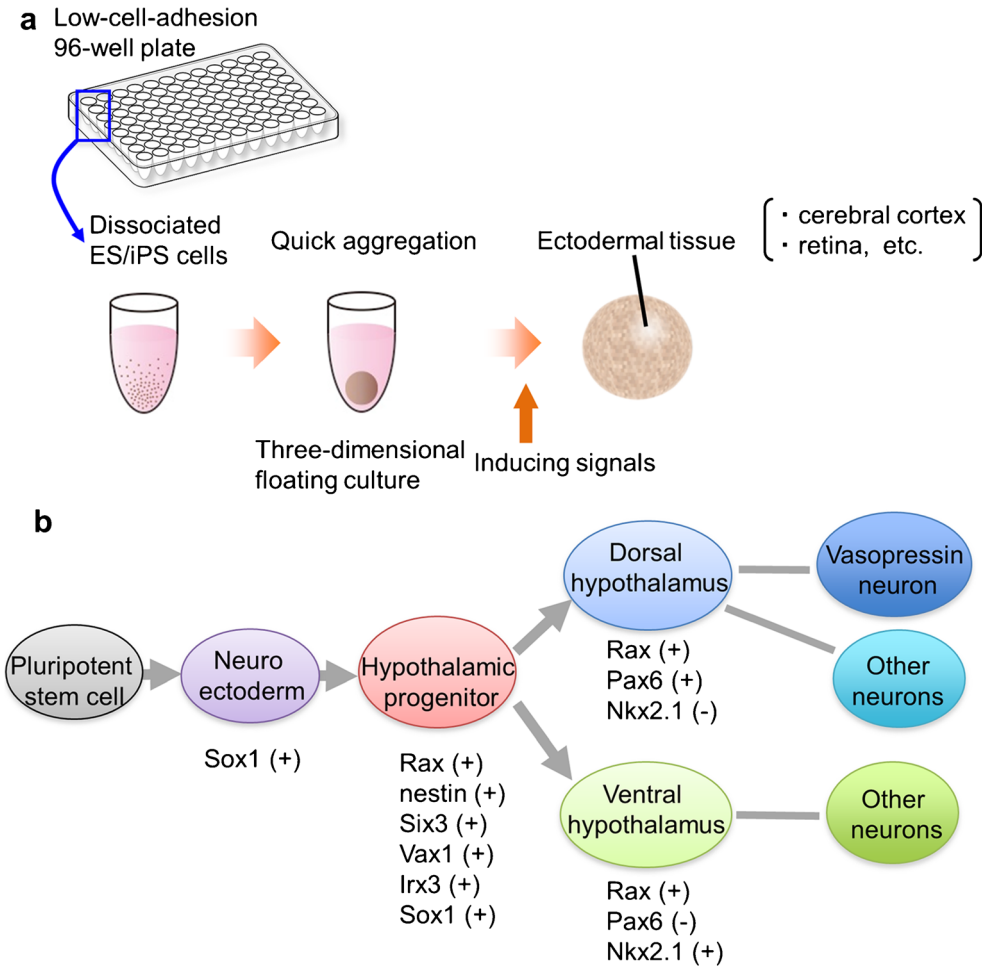

Fig. 1 **a** Schema of SFEBq method. Dissociated ES/iPS cells are distributed into the low-cell-adhesive well plate. Cells are quickly aggregated. Using inducing signals in the culture medium, the aggregate differentiates into aimed ectodermal tissue. **b** Schema of hypothalamic differentiation

precursors) in mouse ES cell aggregates (Wataya et al. 2008). The principal of minimized patterning factors seems important. For example, even the presence of exogenous insulin, which is commonly used in cell culture, strongly inhibits differentiation via the Akt-dependent pathway, indicating that the default fate of mouse ES cells is the rostral hypothalamus.

The ES cell-derived hypothalamic progenitors generate Otp(+)/Brn2(+) neuronal precursors (characteristic of rostral-dorsal hypothalamic neurons) and subsequent vasopressin neurons that release vasopressin upon stimulation (Wataya et al. 2008). Additionally, differentiation markers of rostral-"ventral" hypothalamic precursors and neurons have been induced from ES cell-derived Rax^{+} progenitors by treatment with Sonic Hedgehog (SHH).

ES cell differentiation into hypothalamic progenitors in SFEBq/gfCDM culture is summarized in (Fig. 1b). When cultured in the chemically defined medium containing no additional growth factors such as insulin, Wnt, Nodal, Fgfs, bone morphogenetic protein (BMP) and RA, ES cells frequently differentiate into Sox1+ naive neuroectodermal cells that are fated to become rostral hypothalamic progenitors (Rax+/nestin+/Six3+/Vax1+/Irx3+). Without SHH treatment, these progenitors have the characteristics of dorsal hypothalamic progenitors (Pax6+/Nkx2.1+), while SHH treatment promotes ventral hypothalamic differentiation (Pax6+/Nkx2.1+). ES cell-derived dorsal hypothalamic progenitors can generate vasopressin neurons, presumably via Otp+/Brn2+ intermediate precursors, as embryonic progenitors do. ES cell-derived ventral hypothalamic progenitors (SHH-treated) give rise to neurons characteristic of the ventral hypothalamus (e.g., SF1+ glutamatergic neurons in the VMH, A12 dopaminergic neurons and AgRP/NPY neurons in the arcuate nucleus).

Glial cells, such as astrocytes and oligodendrocytes, were also observed because they share the same origin as neurons. However, no microglia appeared because of the lack of mesodermal cells in this differentiation method.

Hypothalamic-pituitary development

We next tried to establish an in vitro differentiation method for the anterior pituitary. The key of the SFEBq method is replicating the embryonic differentiation environment. Therefore, the developmental biology of the pituitary is important.

Adenohypophysis, which corresponds to the anterior pituitary gland, contains several types of endocrine cells that secrete factors including adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL), thyroid stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The posterior pituitary gland consists of axons and terminals of hypothalamic neurons, i.e., vasopressin and oxytocin neurons.

During early development, the adenohypophysis anlage originates as a placode in the non-neural ectoderm adjacent to the anterior neural plate (Fig. 2 a). The hypothalamic anlage is situated in the top of the anterior neural plate. Both the adenohypophysis placode and the hypothalamic anlage interact with each other. Accordingly, the thickened placode invaginates (Fig. 2 b) and subsequently detaches from the oral ectoderm (Fig. 2 b') to form a hollowed vesicle termed "Rathke's pouch" (Zhu et al. 2007) (Fig. 2 b"). The molecular nature of this local inductive interaction during this initial phase of pituitary formation has been intensively investigated. Among them, fibroblast growth factor (FGF), BMP and SHH signals appear to be involved as important factors (Takuma et al. 1998; Brinkmeier et al. 2007). Rathke's pouch expresses Lhx3. Lhx3 pituitary progenitors become committed to several hormone-type-specific lineages (Fig. 2 c). Among them, the ACTH-producing corticotroph lineage requires the transcription factor Tbx19. Another lineage without Tbx19 expression gives rise to Pit1+ intermediate precursors, which subsequently differentiate into GH-, PRL- and TSH-producing cell lineages. The third lineage differentiates LH- and FSH-producing cells.

Two-layer formation in vitro as the first step of adenohypophysis differentiation

Rathke's pouch is known to form as a result of interactions between the hypothalamus and the neighboring oral ectoderm (Sakaguchi et al. 2015). To recapitulate these embryonic pituitary developmental processes, we co-induced these two tissues simultaneously within one ES cell aggregate in vitro (Suga et al. 2011).

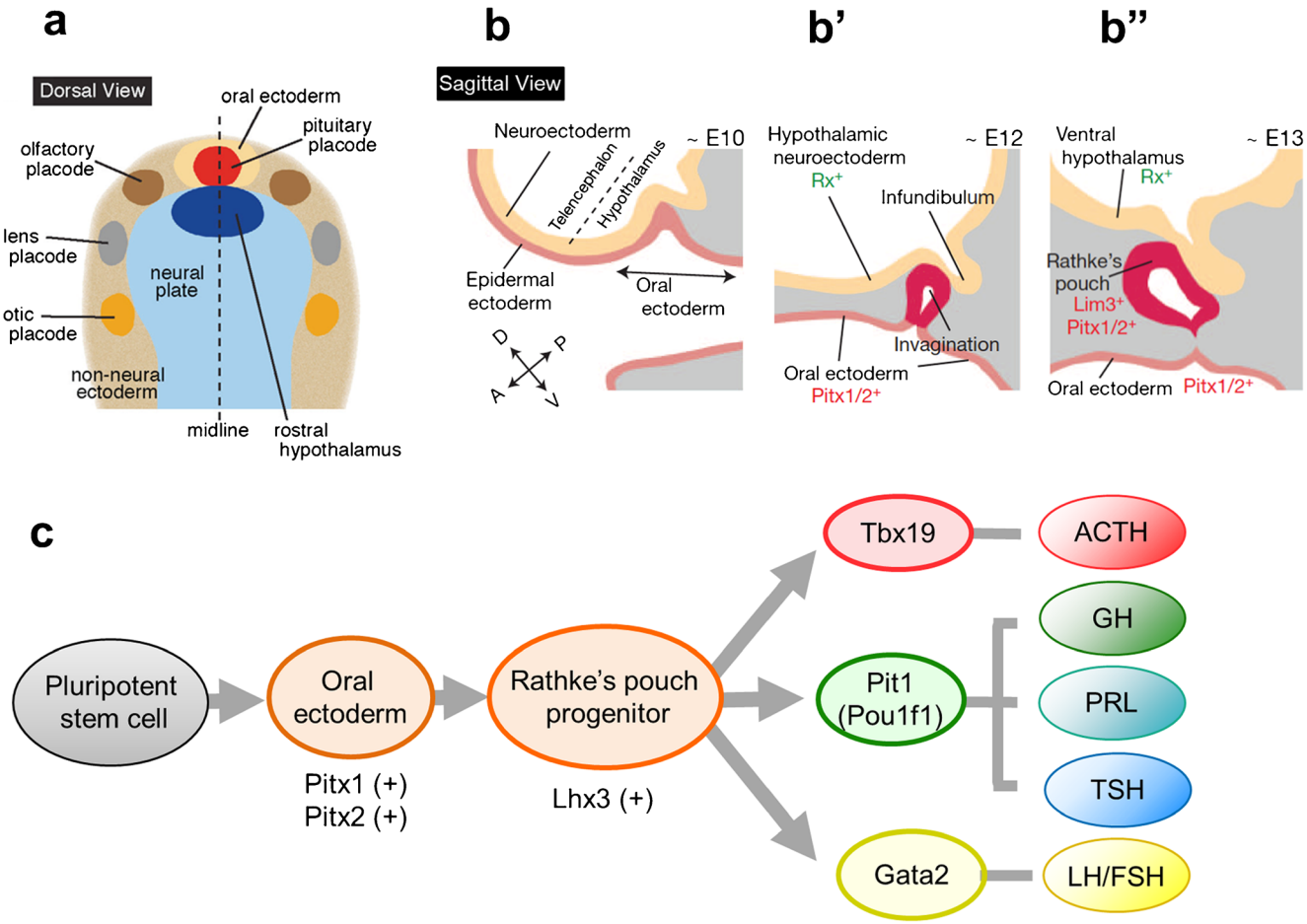

Fig. 2 Diagram of pituitary development. (a) Dorsal view of neural plate and placodes. (b–b") Sagittal view of pituitary embryogenesis. The thickened placode invaginates (b) and subsequently detaches from the oral ectoderm (b') to form a hollowed vesicle termed "Rathke's pouch" (b"). (c) Schema of pituitary differentiation

Based on the hypothalamic differentiation technique (Wataya et al. 2008), some technical modifications were added to co-induce oral ectodermal and hypothalamic differentiation. We attempted to slightly shift the positional information so that the oral ectoderm co-existed with hypothalamic tissues (Sakaguchi et al. 2015). As shown in Fig. 2 (a), the oral ectoderm is generated from the rostral and midline region adjacent to the hypothalamic region in the mouse embryo. After performing trials of many culture conditions known to affect early ectodermal patterning, we concluded that BMP4 is essential. The optimal condition was high-density cell aggregation (10,000 cells per aggregate instead of 3000 in SFEBq culture), which we refer to as large cell aggregation (LCA) (Fig. 3 a). In the LCA culture, both the oral ectoderm (Pitx1/2+) and hypothalamic tissues co-existed within one aggregate (Fig. 3 b, b') as a result of endogenous BMP4 elevation.

LCA culture allows for the formation of oral ectoderm epithelium on the surface of mouse ES cell aggregates, as well as hypothalamic neural tissue in the inner layer adjacent to the oral ectoderm (Fig. 3 b, b'). Treatment with a BMP4 antagonist, dorsomorphin, suppressed the generation of oral ectoderm (Suga et al. 2011). Quantitative polymerase chain reaction analyses revealed significantly higher internal BMP4 expression in LCA aggregates (Suga et al. 2011). Moreover, Koehler et al. succeeded in differentiating the otic placode (Fig. 2 a) (Koehler et al. 2013), which belongs to the head and oral ectoderm, following BMP treatment of mouse ES cells, which supports the reliability and robustness of this strategy. We also showed that very low concentrations of exogenous BMP4 treatment facilitated differentiation into non-neural ectoderms, which contained not only pituitary primordium but also dental germs (Ochiai et al. 2015). Taken together, an appropriate BMP4 signal appears to be important for head ectoderm induction (Wilson and Hemmati-Brivanlou 1995; Basch and Bronner-Fraser 2006; Davis and Camper 2007).

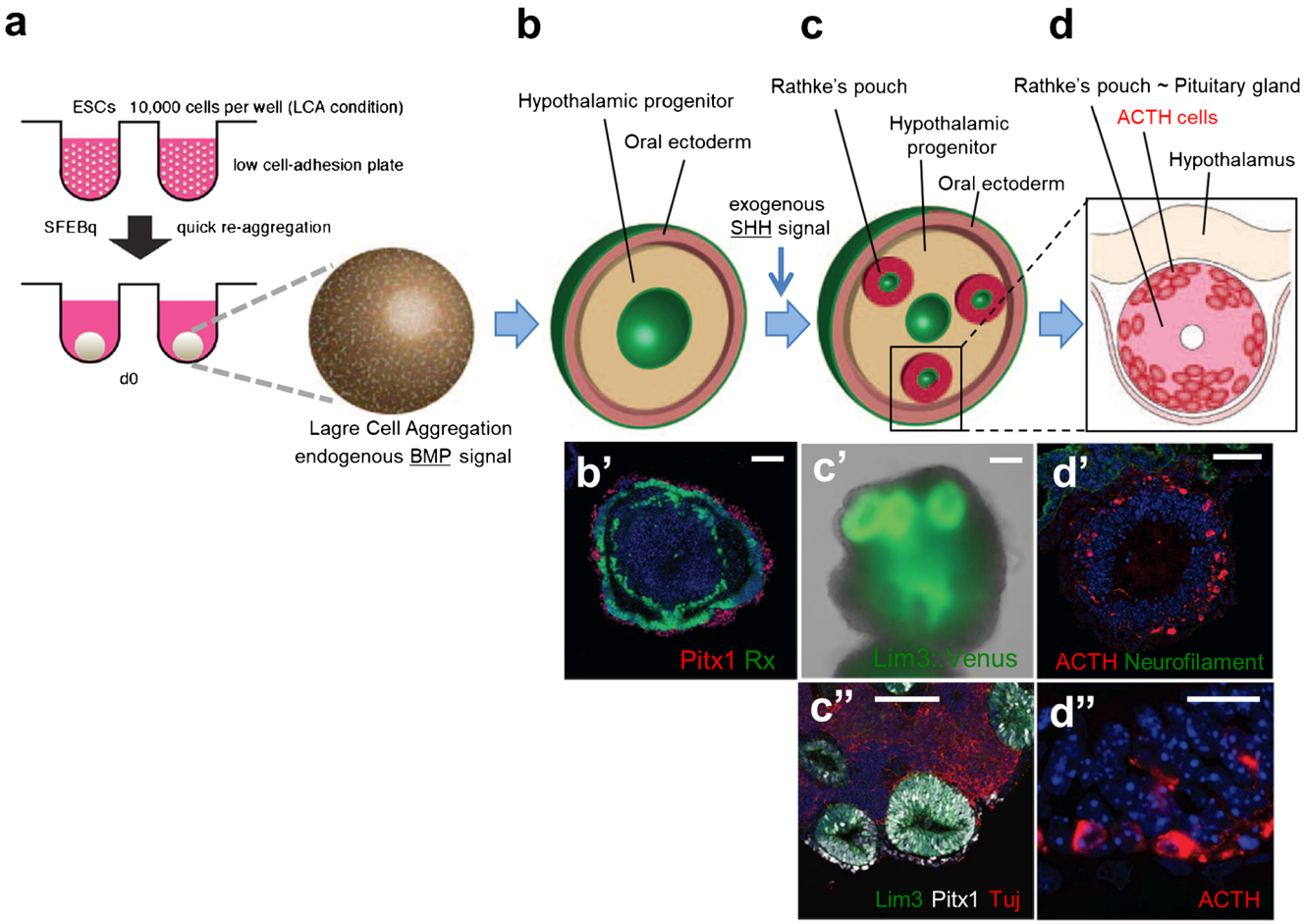

Fig. 3 In vitro differentiation into anterior pituitary from mouse ES cells. (a) Diagram of SFEBq. (b–b') Two-layer formation in LCA aggregates (b). Immunostaining of the aggregate (b', scale bar 100 μm). (c–c") Self-formation of Rathke's pouches (c). Bright field image (c', scale bar 100 μm) and immunostaining (c", scale bar 100 μm) of the aggregate. (d–d") Generation of ACTH$^+$ cells (d). Low-power field view (d', scale bar 50 μm) and high-power field view (d", scale bar 20 μm) of ACTH$^+$ area

Self-formation of Rathke's pouch in vitro

In the developing embryo, Rathke's pouch forms at the midline of the head ectoderm. SHH is expressed in the ventral diencephalon and oral ectoderm but is excluded from the invaginating Rathke's pouch (Zhu et al. 2007; Wang et al. 2010). Rathke's pouch receives SHH signals from neighboring tissues in vivo and SHH is known to provide positional information to adjust towards the midline (Zhu et al. 2007).

Also in vitro, treatment of smoothened agonist (SAG) as a strong SHH signal revealed that multiple oval structures formed in the SAG-treated LCA SFEBq aggregates (Fig. 3 c–c"). The vesicles were situated between the oral ectoderm and hypothalamic neurons (Fig. 3 c"). LHX3 (LIM homeobox 3) expression indicated that the vesicles had similar characteristics to Rathke's pouch. The LHX3$^+$ tissues appeared as a thick epithelium on the surface, which then invaginated and finally formed hollowed vesicles. The length of the major axis was about 200 μm, which is almost equal to the size of the embryonic Rathke's pouch. In contrast, the primordium of the portal vein was not formed in these in vitro aggregates, probably because they originated from the neural crest and migrated to the pituitary area in vivo.

Interactions between oral ectoderm and hypothalamic neurons appear to be critically important for in vitro induction of Rathke's pouch. Neither isolated surface ectoderm alone nor isolated hypothalamic tissues alone formed LHX3$^+$ pouches. In cases where the two divided components are re-assembled, LHX3 expression recovered to some extent (Sakaguchi et al. 2015). Our recent study showed that FGF addition to isolated surface ectoderm also recovered LHX3 expression (unpublished).

Interestingly, a single aggregate often contains several pouches, whereas there is usually only one pouch in the embryo (Sakaguchi et al. 2015). This finding suggests that several morphogenetic fields for pituitary placodes can be independently generated within the oral ectoderm epithelium on the surface of the ES cell aggregate, which is reminiscent of the *Vax1* knockout mouse (Bharti et al. 2011). A second Rathke's pouch develops in addition to the orthotopic anlage in the *Vax1* knockout mouse. Ectopic expression of FGF10, which is expressed in the infundibulum and implicated in pituitary induction, is also detected in the hypothalamic neuroepithelium overlying the second pouch. Thus, Vax1 likely limits the hypothalamic neuroepithelium area that generates pituitary-inducing signals. Indeed, *Vax1* expression in vivo is eliminated near the infundibulum, which has inducing activity for pituitary development. In the mouse ES aggregates used for pituitary differentiation in the present study, Vax1$^+$ cells did not exist in the hypothalamic area. Conversely, Wataya's aggregate for hypothalamic differentiation (Wataya et al. 2008) has been shown to contain Vax1$^+$ cells. We speculate that precise positioning in the hypothalamus slightly shifts as a result of BMP4 and SHH signals.

Differentiation into hormone-producing endocrine cells

During pituitary development in the embryo, LHX3$^+$ pituitary progenitors commit to several lineages (Davis et al. 2011; Lamolet et al. 2001), i.e., corticotroph, somatotroph, lactotroph, thyrotroph, gonadotroph and melanotroph lineages. Among them, the ACTH-producing corticotroph lineage expresses the transcription factor *Tbx19* prior to ACTH expression. Notch signaling is known to inhibit *Tbx19* expression (Zhu et al. 2006; Kita et al. 2007; DiMattia et al. 1997; Olson et al. 2006). Treatment with the Notch inhibitor DAPT revealed increased TBX19 expression in SAG-treated LCA SFEBq aggregates. A substantial number of ACTH$^+$ cells appeared in the TBX19$^+$ lesion (Fig. 3 d–d").

Previous reports have shown that canonical Wnt signaling promotes *Pit1* (formal gene name is *Pou1f1*) expression (DiMattia et al. 1997; Olson et al. 2006; Sornson et al. 1996). Consistent with this finding, treatment with the Wnt agonist BIO increased PIT1 expression, resulting in subsequent GH$^+$ and PRL$^+$ cell differentiation.

Head mesenchyme has been suggested to promote pituitary development in vivo (Gleiberman et al. 1999). Therefore, we applied conditioned medium from PA6 stromal cells to SAG-treated LCA SFEBq aggregates. As a result, LH-positive, FSH-positive and TSH-positive cells were successfully induced.

LHX3 is essential for these hormone-producing lineages. Knockdown of *Lhx3* inhibited subsequent differentiation into hormone-producing cells, which supports altered pituitary development in *Lhx3* knockout mice (Sheng et al. 1996).

These results demonstrate the competence of ES cell-derived pituitary progenitors to generate multiple endocrine lineages in vitro.

Functionality of induced ACTH$^+$ cells

Positive and negative regulations by exogenous stimuli are characteristic for endocrine cells. The achievement of responsible character is the final goal as materials for endocrine regenerative medicine.

To investigate in vitro functionality, corticotropin-releasing hormone (CRH) loading test was performed on the induced ACTH$^+$ cells generated in the SAG-treated LCA SFEBq aggregates. After CRH stimulation, substantial amounts of ACTH were secreted from SAG-treated LCA SFEBq

aggregates in vitro. Of note, the secreted ACTH concentration was at a similar level in mouse peripheral blood.

ACTH secretion from the pituitary gland is negatively regulated by the downstream glucocorticoid hormone in vivo. Consistent with this control principle, in vitro ACTH secretion as a result of CRH stimulation was suppressed by glucocorticoid pre-treatment.

Similar to in vivo endocrine systems, these data demonstrate that mouse ES cell-derived ACTH$^+$ cells respond to both positive and negative regulators. These hormonal responses to surrounding regulators are indispensable for homeostasis. For this reason, the generation of anterior pituitary tissue that retains regulatory hormonal control in vitro is an important step for the development of cell transplantation therapies for pituitary diseases.

Effect of transplantation into hypophysectomized model animals

Finally, we evaluated the transplantation effect of the induced ACTH$^+$ cells. Because of technical difficulties, we chose ectopic transplantation into the kidney subcapsule, instead of orthotopic transplantation into the sella turcica. After transplantation, blood ACTH levels were slightly but significantly increased. CRH loading induced a substantial elevation in blood ACTH levels. The downstream glucocorticoid hormone corticosterone was also significantly increased, indicating that ACTH from the graft sufficiently induced the downstream hormone.

Even without CRH loading, the basal levels of ACTH were higher. Corticosterone levels were also increased, suggesting that partial recovery of blood ACTH has a moderate but biologically significant effect (note that ED50 of the ACTH receptor MC2R for glucocorticoid production is around 9 pg/mL) (Soto-Rivera and Majzoub 2017). In accordance with this, the treated hypophysectomized mice displayed higher spontaneous locomotor activities and survived significantly longer. Although CRH, which is secreted from the hypothalamus, should be diluted in the peripheral site, mESC-derived pituitary tissues rescued survival and spontaneous activities, suggesting that basal secretion from these tissues was sufficient for those effects.

These findings showed that induced ACTH$^+$ cells derived from mouse ES cells acted as endocrine tissues and that regenerative medicine for pituitary dysfunction is feasible.

Adaptation to human ES/ iPS cell culture

Recovery from pituitary functional disorder is an important issue for medical studies because the anterior pituitary has poor potential for regeneration. Because some pituitary dysfunctions cannot be solely treated by drugs (Arima et al. 2014; Hahner et al. 2015; Sherlock et al. 2009), regenerative therapy employing stem cells should be considered as a new form of therapeutic intervention. Our SFEBq method (Suga et al. 2011) induces pituitary cells that can auto-regulate hormonal secretion and respond to changing circumstances. The application of this culture method to human ES cells is necessary for clinical purposes. However, poor survival of human ES cells in SFEB culture might limit the use of these cells for future medical applications. Our colleagues found that a selective Rho-associated kinase inhibitor, Y-27632, markedly diminished dissociation-induced apoptosis of human ES cells and enabled the cells to form aggregates in SFEB culture (Watanabe et al. 2007). Using this fundamentally important discovery, we attempted to adapt our pituitary-differentiating culture method for human ES cell culture. We recently established the differentiation method into corticotrophs and somatotrophs from human ES cells (Soto-Rivera and Majzoub 2017).

The characteristics for differentiating pluripotent stem cells into pituitary cells were as follows;

- Simultaneous induction of neighboring hypothalamic neuroectoderm and oral ectodermal tissue, similar to embryo
- Self-formation of pituitary anlage (Rathke's pouch) as a result of interaction between those two layers
- Generation of multiple endocrine lineages from LHX3(+) pituitary progenitors
- Functionality confirmation as endocrine tissue

Combining these approaches, we designed a cell culture scheme for human ES cells (Ozone et al. 2016).

Our results demonstrated that the anterior pituitary self-forms in vitro following co-induction of the hypothalamic and oral ectoderm (Fig. 4 a–a"). The juxtaposition of these tissues facilitated the formation of the pituitary placode and their features were consistent with characteristics of Rathke's pouch in vivo. These pituitary placodes subsequently differentiated into pituitary hormone-producing cells. All types of pituitary hormone-producing cells were identified (Fig. 4 b, c-c", d–d", e, e'). Among them, we confirmed that the human ES-derived corticotroph responded normally to releasing and feedback signals. Electron microscopy revealed secretory granules stored in the cytoplasm of these cells (Fig. 4 f, f').

As for hypothalamus, several reports have shown differentiation of hypothalamic neurons from human pluripotent stem cells (Wang et al. 2015; Merkle et al. 2015; Lund et al. 2016). Merkle et al. demonstrated the differentiation of AVP neurons from human pluripotent stem cells, although AVP secretion was not demonstrated (Merkle et al. 2015). Recently, we

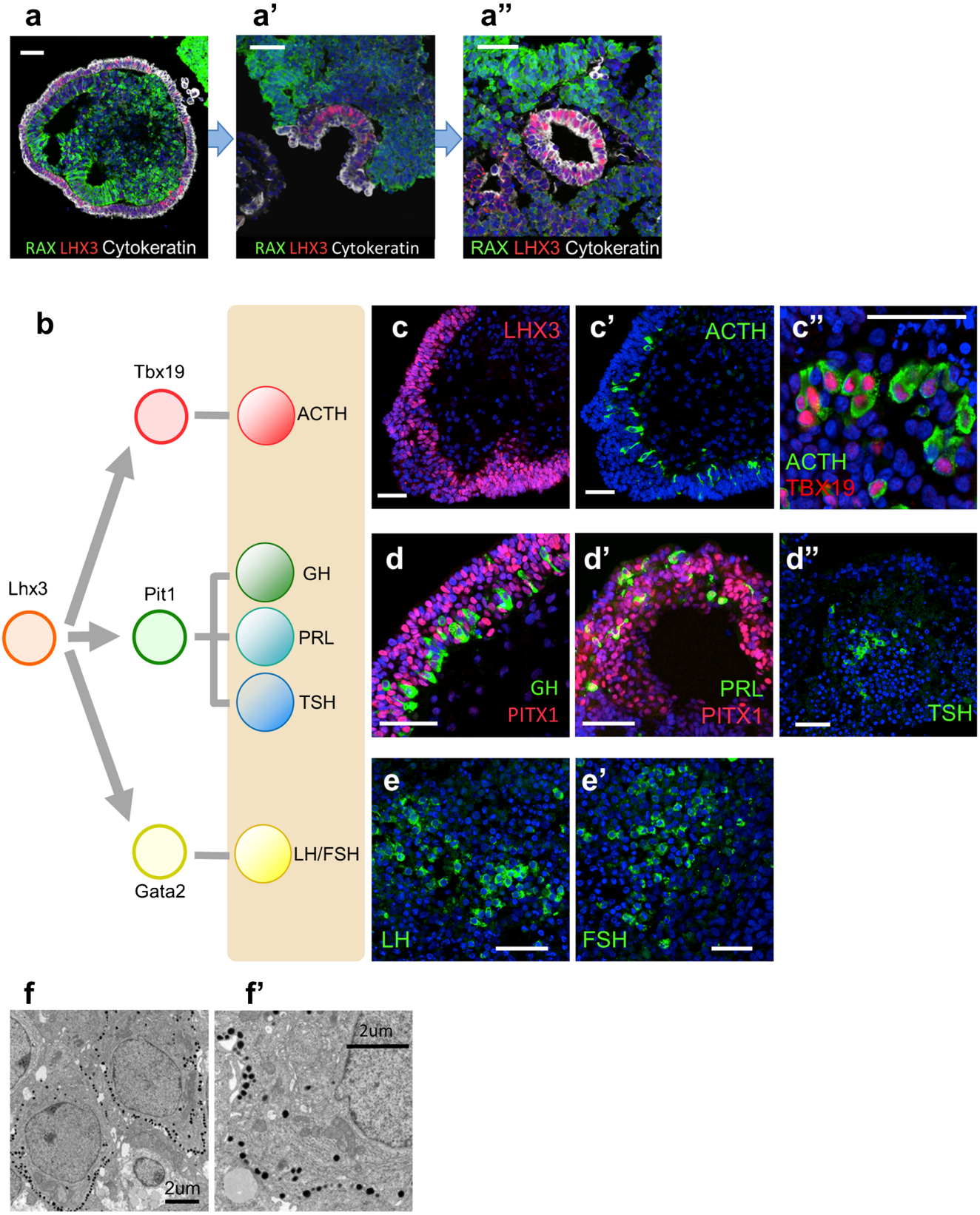

Fig. 4 Applied pituitary differentiation culture in human ES cells. (a–a") Formation of Rathke's pouch-like structure. Scale bars 50 μm. (b) Differentiation into multiple lineages. (c–c") Corticotroph. Scale bars 50 μm. (d–d") Somatotroph (d), lactotroph (d') and thyrotroph (d"). Scale bars 50 μm. (e, e') Gonadtroph. Scale bars 50 μm. (f, f') Secretory granules characteristic of endocrine cells. Scale bars 2 μm

established an induction method for AVP-secreting neurons from hES cells (Ogawa et al. 2018).

Rostral hypothalamic progenitors (Fig. 5 a) consist of two groups, a dorsal part (PAX6^{+}) (Fig. 5 b) and a ventral part (NKX2.1^{+}) (Fig. 5 c). During development of the dorsal/ventral hypothalamus in vivo, it is well known that there are gradients of BMP and SHH signals in the developing hypothalamic area. Therefore, we tuned the concentration of BMP4 and SAG in the hES cell cultures and succeeded in individual induction of dorsal or ventral hypothalamic cells. As a result, OTP and BRN2, which are precursors of AVP neurons, were strongly expressed in hES cell aggregates cultured with dorsal hypothalamic conditions (Fig. 5 b') and finally, we achieved successful differentiation of AVP neurons (Fig. 5 b"). This is the first report of induced hypothalamic AVP neurons that secrete the hormone and react to KCl stimulation. We speculate that the key points of our differentiation method are accurate recapitulation of each developmental step and scrupulous optimization of differentiation efficiency.

Taking into account reports of differentiation methods using BMP and SHH, central neuron areas around the hypothalamus are determined, as shown in Fig. 6.

For both mouse and human ES cells, SFEB culture is a favorable method that can generate functional pituitary cells. Future studies will confirm whether human iPS cells can differentiate into pituitary cells using the same culture methods.

Future perspectives

There are two primary uses for human ES/iPS cell-derived pituitary cells. One is the in vitro human model of development or

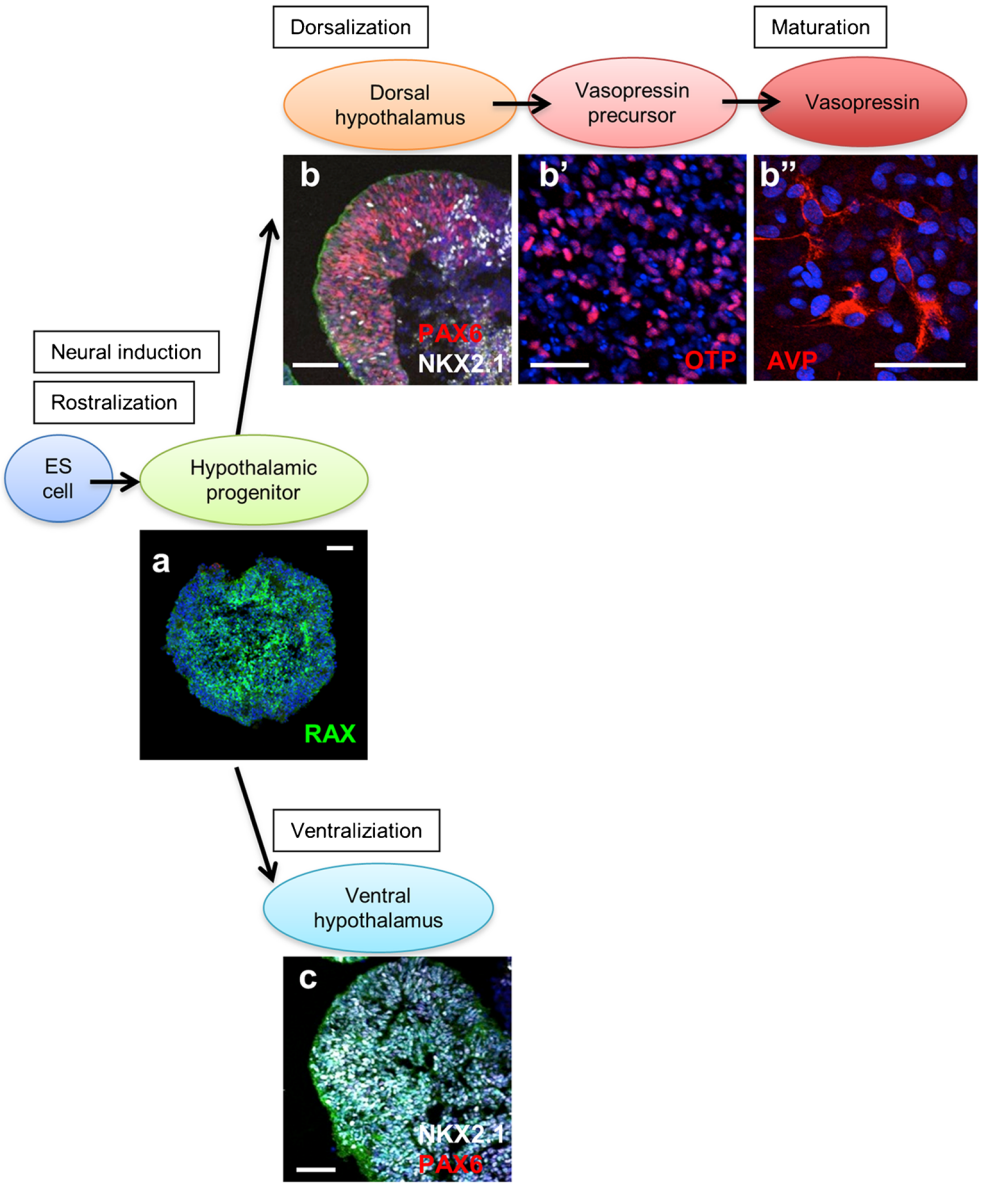

Fig. 5 Human ES cell differentiation into hypothalamic progenitors. Schematic summary of development from ES cells to vasopressin neurons. (a) Hypothalamic progenitor expressing RAX. (b–b") Dorsal hypothalamus (b), vasopressin precursor cells (b') and AVP^{+} neurons (b"). (c) Ventral hypothalamus. Scale bars 50 μm

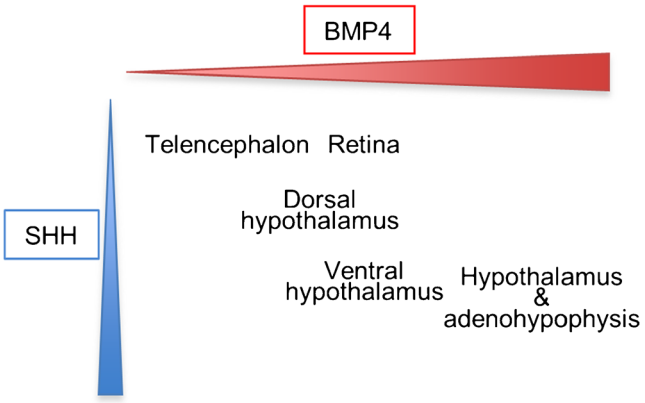

Fig. 6 Conceptual map showing the relationship between induction signals and induced areas in the human pluripotent stem cell culture

disease. Results from our study showed that the present culture methods recapitulated embryogenesis, suggesting that it could be used in the area of developmental biology. In terms of diseases due to gene mutations, tissues derived from disease-specific iPS cells can be used for therapy screenings in a human disease model.

The second major use for human ES/iPS cell-derived hypothalamic-pituitary cells is for regenerative medicine. Although stem cell-based therapeutics provide high expectations for the treatment of diabetes mellitus, the use of regenerative medicine for hypothalamus-hypophyseal dysfunctions has received little attention.

ES cell-derived ACTH-producing cells function even after ectopic transplantation. This finding raises the possibility of relatively simple grafting of artificial ES/iPS cell-derived pituitary tissues into a peripheral site. These cells can function effectively if hormone secretion can be extrinsically controlled by releasing factors or small molecule agonists. However, ectopic transplantation is not perfect, because physiological CRH released from the hypothalamus does not directly affect these grafts. Orthotopic transplantation of hormone-producing cells that are controlled by positive and negative regulators is one of the future candidates for complete therapy.

In addition to differentiation from pluripotent stem cells, direct reprogramming or transdifferentiation techniques seem to have a potential that can achieve the two major purposes. Based on accumulating reports in neuronal or endocrine fields, it is worth challenging the establishment of direct reprogramming or transdifferentiation techniques in the neuroendocrine area.

In future studies, it will be challenging to recapitulate an entire anterior pituitary gland that contains all endocrine components and to use such artificial pituitary tissues for orthotopic transplantation into the sella of a large mammal. Differentiation efficiency and secretion capacity should be optimized. To achieve this long-term goal, further studies are needed before pituitary regenerative medicine can be directly transferred to clinical use.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Appendix

Rax retina and anterior neural fold homeobox
Six3 sine oculis-related homeobox 3
Vax1 ventral anterior homeobox 1
Otp orthopedia homeobox
Brn2 Pou3f2 POU domain, class 3, transcription factor 2
Pitx paired-like homeodomain
Lhx3 LIM homeobox protein 3
Tbx19 T-box 19
DAPT *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester
Pit1 Pou1f1 POU domain, class 1, transcription factor 1
BIO (2'Z,3'E)-6-Bromoindirubin-3'-oxime

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