



# Generation of Hypothalamus and Adenohypophysis from Human Pluripotent Stem Cells

# 3

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## Abstract

The hypothalamus orchestrates various essential physiological and behavioral processes via neuropeptide secretion. The adenohypophysis (anterior pituitary) is a major center for peripheral endocrine organs, which secretes systemic hormones responding to hypothalamic neuropeptides as releasing factors. This functional connection between the hypothalamus and adenohypophysis is indispensable for the endocrine system and homeostasis. Pluripotent stem cells are promising tools for studying the process of human organ development, disease modeling, and regenerative medicine. Differentiation methods derived from pluripotent stem cells have been studied over the last quarter of a century. Recent studies have succeeded in the differentiation into hypothalamus and adenohypophysis from mouse and human pluripotent stem cells by a three-dimensional floating culture method of embryonic bodies. The induced hypothalamic-like progenitors generate hypothalamic neurons such as vasopressin neurons. In the induction of adenohypophysis, Rathke's pouch-like structures are self-organized as seen in embryogenesis *in vivo*, and functional anterior pituitary hormone-producing cells are subsequently differentiated.

## Keywords

Hypothalamus · Adenohypophysis · Pituitary · Embryonic stem cell · induced pluripotent stem cell · Differentiation

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## Abbreviations

ACTH	adrenocorticotrophic hormone
AVP	arginine vasopressin
BIO	6-bromo-3-[(3E)-1,3-dihydro-3-(hydroxyimino)-2H-indol-2-ylidene]-1,3-dihydro-(3Z)-2H-indol-2-one
BMP	bone morphogenetic protein
CPH	congenital pituitary hypoplasia
CRH	corticotropin-releasing hormone
DAPT	(2S)-N-[N-(3,5-Difluorophenacetyl)-l-alanyl]-2-phenylglycine tert-butyl ester
ES	embryonic stem
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GATA2	GATA-binding factor 2
gfCGM	growth factor-free chemically defined medium
GH	growth hormone
iPS	induced pluripotent stem
IRX3	Iroquois homeobox gene 3
KSR	knockout serum replacement
LCA	large-cell aggregate
LH	luteinizing hormone
LHX3	LIM-homeobox 3
NKX2.1	NK2 homeobox 1
OTP	orthopedia homeobox
OTX2	orthodenticle homeobox 2
PAX6	paired box 6
PI3K	phosphoinositide 3-kinase
PIT1	pituitary transcription factor 1
PRL	prolactin
Rock	Rho-associated kinase
SAG	smoothened agonist
SF1	steroidogenic factor 1
SFEBq	serum-free culture of embryoid body-like aggregates with quick re-aggregation
SHH	sonic hedgehog
SIX3	SIX homeobox 3
SOX1	SRY-box transcription factor 1
TBX19	T-box transcription factor
TSH	thyroid-stimulating hormone
VAX1	ventral anterior homeobox 1

### 3.1 Introduction

The hypothalamus and pituitary gland are essential for fundamental physiological processes such as stress responses, growth, sexual development, reproduction, regulation of food intake and energy expenditure and circadian rhythms. In the central part of the brain, hypothalamic neurons integrate afferent information from the periphery and respond by releasing neuropeptides and neurotransmitters. The pituitary gland consists of the adenohypophysis (anterior pituitary gland) and neurohypophysis (posterior pituitary gland). Some of the hypothalamic neuropeptides reach the adenohypophysis through the pituitary portal vein and regulate anterior pituitary hormones, including adrenocorticotrophic hormone (ACTH), growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Through the regulation of these anterior pituitary hormones, the adenohypophysis, in turn, stimulates or inhibits hormone secretion from peripheral endocrine glands such as the adrenal cortex, thyroid, and gonads. The neurohypophysis contains axon terminals of hypothalamic neurons producing arginine vasopressin (AVP) and oxytocin, the somas of which are located in the supraoptic and paraventricular nuclei in the hypothalamus.

Dysfunction of the hypothalamus and pituitary gland causes various systemic symptoms (Schneider et al. 2007). Currently, the only available treatment for hypothalamic and pituitary disorders is hormone replacement therapy, which is not able to fully and precisely meet the demands of hormone requirements, which may change from moment to moment under various physiological conditions. Indeed, adrenal crisis (a life-threatening condition due to glucocorticoid deficiency) occurs in a substantial proportion of patients with hypopituitarism (deficiency of pituitary hormones), and also adrenal crisis-associated death could occur even in educated patients treated with glucocorticoid replacement therapy (Hahner et al. 2015). Moreover, patients with ACTH-dependent adrenal insufficiency (glucocorticoid deficiency) have higher risks of diabetes mellitus, hypertension, hyperlipidemia, depression, and anxiety, presumably due to glucocorticoid overreplacement (Stewart et al. 2016). Furthermore, adipsic patients with diabetes insipidus (polyuria due to antidiuretic hormone AVP) exhibit severe fluctuations in serum sodium levels, resulting in poor prognosis (Arima et al. 2014). These still unsatisfactory results of conventional hormone replacement therapies are attributed to the lack of a hormonal feedback mechanism, which is an essential feature of the endocrine system.

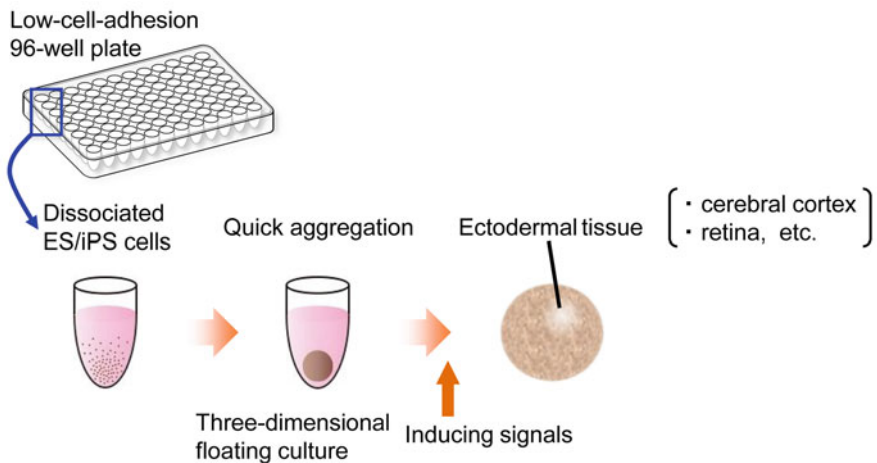
To overcome the difficulties in the current treatment for hypothalamic and pituitary disorders, high expectations have been growing for regenerative medicine using hypothalamic and pituitary hormone-producing cells differentiated from human embryonic stem (ES) and induced pluripotent stem (iPS) cells. Theoretically, ES/iPS cells are able to differentiate into all types of cells in our body. In addition, these pluripotent stem cells provide an unlimited cell source because of their self-renewal properties. Since recent studies have succeeded in the differentiation into hypothalamic neurons and adenohypophysis cells from mouse and human ES/iPS cells (Wataya et al. 2008; Suga et al. 2011; Dincer et al. 2013; Wang et al. 2015; Merkle et al. 2015; Ozone et al. 2016; Zimmer et al. 2016; Lund et al. 2016;

Yamada-Goto et al. 2017; Ogawa et al. 2018; Kano et al. 2019; Mitsumoto et al. 2019; Kasai et al. 2020), regenerative medicine is coming closer to reality in the neuroendocrine field. Furthermore, these differentiated hypothalamic neurons and adenohypophysis cells can also be used for developmental basic research and disease modeling. In this chapter, we introduce achievements to date of differentiation into hypothalamic neurons and adenohypophysis cells from mouse and human ES/iPS cells.

## 3.2 Three-Dimensional Culture Method for Embryoid Body

Organ formation during embryogenesis consists of complicated and sophisticated processes with various local interactions among distinct cells and tissues. To imitate these developmental processes during embryogenesis, a three-dimensional culture is an ideal approach. An efficient three-dimensional culture method has been established for selective neural differentiation from ES cells, which is called “serum-free culture of embryoid body-like aggregates with quick re-aggregation (SFEBq)” (Watanabe et al. 2005; Eiraku et al. 2008). Dissociated ES/iPS cells are autonomously and quickly aggregated in low-cell adhesion well plates in the differentiation medium (Fig. 3.1).

The SFEBq method is appropriate for differentiation into various ectodermal derivatives from ES/iPS cells. In the SFEBq method, the ES/iPS cell aggregates exhibit self-organization (Sasai et al. 2012) and spontaneous formation of a highly ordered structure and patterning. This floating culture has revealed intrinsic programs driving locally autonomous modes of organogenesis and homeostasis.



**Fig. 3.1** Schema of SFEBq method. Dissociated ES/iPS cells are seeded into the low-cell-adhesion 96-well plate and quickly aggregated. Using inducing signals in the culture medium, the aggregates differentiate into aimed ectodermal tissues. Reproduced from Suga (2019), with permission

Based on the SFEBq method, cortex neurons (Eiraku et al. 2008; Danjo et al. 2011; Kadoshima et al. 2013), the optic cup (Ikeda et al. 2005; Osakada et al. 2008; Eiraku et al. 2011; Nakano et al. 2012), cerebellar neurons (Muguruma et al. 2010) and hippocampal neurons (Sakaguchi et al. 2015) have been generated from ES cells.

### 3.3 Mouse ES Cells as a Pioneer of a Human Model

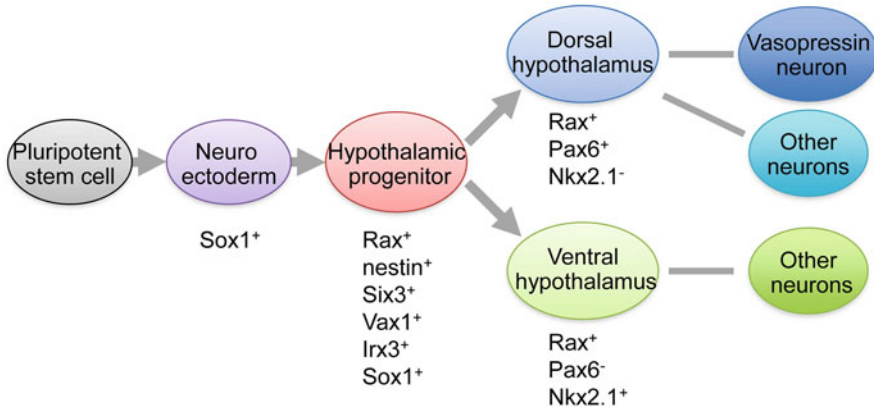
Fundamental processes in differentiation studied in mouse ES cells can apply to human ES cells. For example, the retinal differentiation process in human ES cells (Nakano et al. 2012) has been established based on a previous report using mouse ES cells (Eiraku et al. 2011). Furthermore, the duration of mouse fetal development is approximately 20 days, which is considerably shorter than the 300 days of human fetal development. Since numerous trials and errors are required to establish a novel differentiation method, we should take advantage of mouse ES cells as a first step.

#### 3.3.1 Hypothalamic Neuron Differentiation from Mouse ES Cells

SFEBq-cultured ES cells spontaneously differentiate into neural progenitors of the rostral forebrain and efficiently generate telencephalic progenitors (Watanabe et al. 2005). In the SFEBq method, ES cell aggregates are cultured in serum-free medium containing knockout serum replacement (KSR) without major exogenous inductive factors such as fibroblast growth factor (FGF), bone morphogenetic protein (BMP) or Wnt. However, the serum-free medium used in the original SFEBq culture still includes some exogenous signals that might affect the differentiation pathway. In particular, KSR, widely used for the maintenance and differentiation of ES cells, contains bioactive growth factors, including a high concentration of insulin and lipid-rich albumin purified from bovine serum.

Wataya et al. have established the differentiation method for hypothalamic neurons from mouse ES cells using a growth factor-free, chemically defined medium (gfCDM). Strict removal of exogenous patterning factors during early differentiation steps induces efficient generation of rostral hypothalamic progenitors ( $Rax^+/Six3^+/Vax1^+$ ) in mouse ES cell aggregates. The addition of insulin to the SFEBq/gfCDM culture strongly inhibits differentiation into  $Rax^+$  hypothalamic progenitors from mouse ES cells via the PI3K/Akt pathway. The ES cell-derived  $Rax^+$  progenitors generate rostral-dorsal hypothalamic precursors ( $Pax6^+/Nks2.1^-$ ) and subsequently AVP neurons that efficiently release the hormone upon stimulation. Besides, rostral-ventral hypothalamic precursors ( $Pax6^-/Nks2.1^+$ ) and neurons are differentiated from ES cell-derived  $Rax^+$  progenitors by treatment with the Sonic hedgehog (SHH) (Wataya et al. 2008).

ES cell differentiation into hypothalamic progenitors in SFEBq/gfCDM culture is summarized in Fig. 3.2. When cultured in the chemically defined medium without additional growth factors, including insulin, FGF, BMP, Wnt, and retinoic acid, ES cells frequently differentiate into  $Sox1^+$  naïve neuroectodermal cells and subsequent

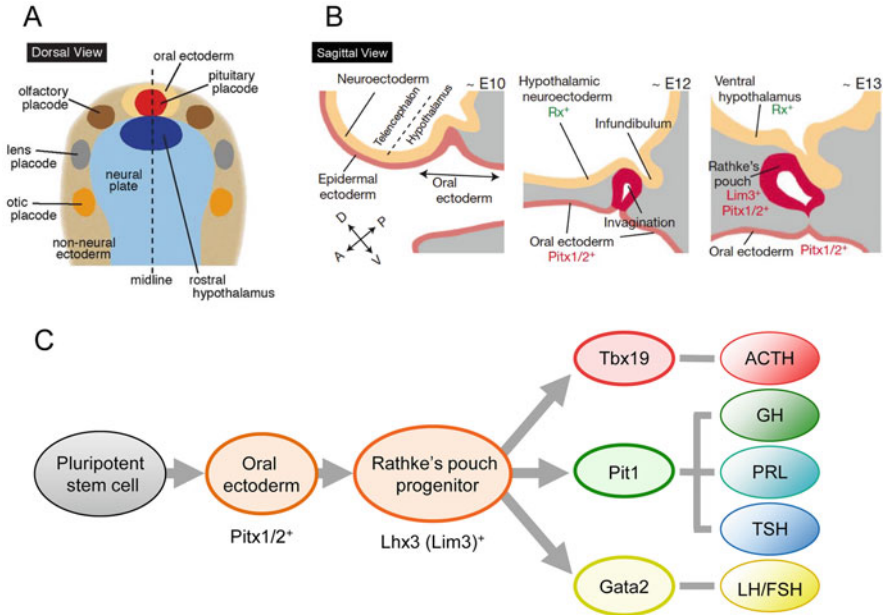


**Fig. 3.2** Schema of hypothalamic differentiation. When cultured in the chemically defined medium without additional growth factors, pluripotent stem cells frequently differentiate into Sox1<sup>+</sup> naïve neuroectodermal cells and subsequent rostral hypothalamic progenitors (Rax<sup>+</sup>/nestin<sup>+</sup>/Six3<sup>+</sup>/Vax1<sup>+</sup>/Irx3<sup>+</sup>). Without SHH treatment, these rostral hypothalamic progenitors have the characteristic of dorsal hypothalamic progenitors (Pax6<sup>+</sup>/Nks2.1<sup>-</sup>), while SHH treatment promotes differentiation into ventral hypothalamic progenitors (Pax6<sup>-</sup>/Nks2.1<sup>+</sup>). Dorsal hypothalamic progenitors generate AVP neurons. SHH-treated ventral hypothalamic progenitors give rise to neurons characteristic of the ventral hypothalamus. Modified from Suga (2019), with permission

rostral hypothalamic progenitors (Rax<sup>+</sup>/nestin<sup>+</sup>/Six3<sup>+</sup>/Vax1<sup>+</sup>/Irx3<sup>+</sup>). Without SHH treatment, these rostral hypothalamic progenitors have the characteristics of dorsal hypothalamic progenitors (Pax6<sup>+</sup>/Nks2.1<sup>-</sup>), while SHH treatment promotes differentiation into ventral hypothalamic progenitors (Pax6<sup>-</sup>/Nks2.1<sup>+</sup>). ES cell-derived dorsal hypothalamic progenitors generate AVP neurons, presumably via Otp<sup>+</sup>/Brn2<sup>+</sup> intermediate precursors. SHH-treated ES cell-derived ventral hypothalamic progenitors give rise to neurons characteristic of the ventral hypothalamus (e.g., SF1<sup>+</sup> glutamatergic neurons in the ventromedial hypothalamic nucleus, A12 dopaminergic neurons, and neuropeptide Y/agouti-related peptide neurons in the arcuate nucleus).

### 3.3.2 Adenohypophysis Differentiation from Mouse ES Cells

The principal aspect of the SFEBq method is replicating the embryonic differentiation environment and imitating complicated and sophisticated processes during embryogenesis. Therefore, a detailed understanding of developmental biology is essential for the establishment of differentiation methods from ES cells.



**Fig. 3.3** Diagram of adenohypophysis differentiation. **(a)** Dorsal view of neural plate and placodes. The adenohypophysis anlage (pituitary placode) is located in the non-neural ectoderm adjacent to the hypothalamus anlage (rostral hypothalamus). **(b)** Sagittal view of adenohypophysis embryogenesis. The thickened placode invaginates and subsequently detaches from the oral ectoderm, leading to the formation of a hollowed vesicle termed Rathke's pouch. The epithelial cells of Rathke's pouch express *Lim3* (also called *Lhx3*). **(c)** Schema of adenohypophysis differentiation and subsequent generation of anterior pituitary hormone-producing cell lineages. *Lhx3*<sup>+</sup> Rathke's pouch progenitors (adenohypophysis progenitors) are derived from the oral ectoderm (*Pitx1/2*<sup>+</sup>) and subsequently generate several lineages of anterior pituitary hormone-producing cells. Among them, the ACTH-producing cell lineage requires a transcription factor *Tbx19*. GH-, PRL-, and TSH-producing cell lineages are differentiated from *Pit1*<sup>+</sup> intermediate precursors. The third lineage differentiates into LH- and FSH-producing cells. Modified from Suga (2019), with permission

### Box 3.1 Adenohypophysis Development In Vivo

The adenohypophysis anlage originates as a placode in the non-neural ectoderm adjacent to the hypothalamic anlage situated in the top of the anterior neural plate (Fig. 3.3a). The adenohypophysis placode and hypothalamic anlage interact with each other during early development. In particular, the thickened placode invaginates and subsequently detaches from the oral ectoderm, leading to the formation of a hollowed vesicle termed Rathke's pouch (Fig. 3.3b) (Zhu et al. 2007). The molecular nature of local inductive interaction underlying the initial phase of adenohypophysis formation has been

(continued)

**Box 3.1** (continued)

intensively investigated, revealing that FGF, BMP, and SHH signals are involved as important factors (Takuma et al. 1998; Brinkmeier et al. 2007). Also, epithelial cells of Rathke's pouch express Lhx3 (also called Lim3). Lhx3<sup>+</sup> adenohypophysis progenitors generate several lineages of anterior pituitary hormone-producing cells (Fig. 3.3c). Among them, the ACTH-producing corticotroph lineage requires a transcription factor, Tbx19. GH-, PRL-, and TSH-producing cell lineages are differentiated from Pit1<sup>+</sup> intermediate precursors. The third lineage differentiates into LH- and FSH-producing cells. Lhx3 knockout mice reveal that Lhx3 is essential for these anterior pituitary hormone-producing cell lineages (Sheng et al. 1996).

**3.3.2.1 Two-Layer Formation In Vitro as the First Step of Adenohypophysis Differentiation**

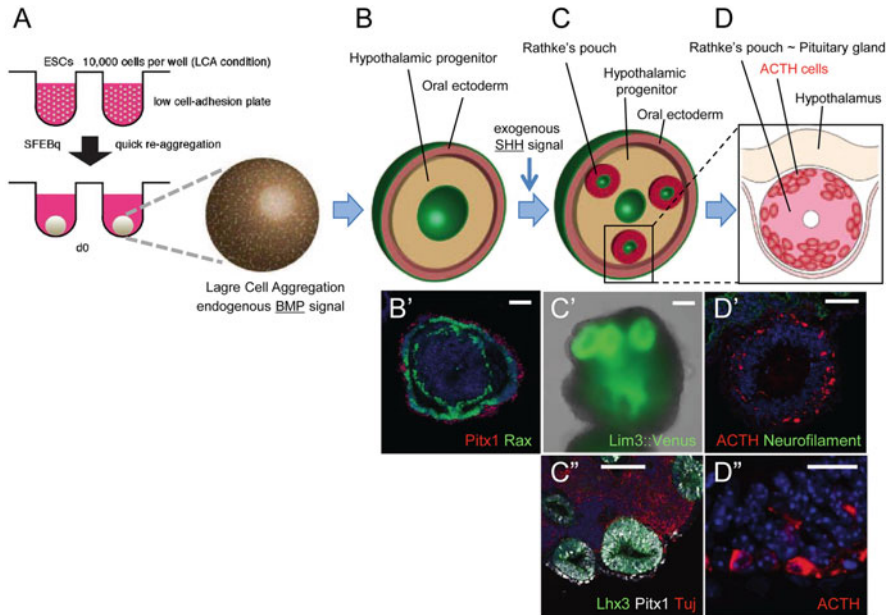
As discussed above, the formation of Rathke's pouch is attributed to interactions between the hypothalamus and neighboring oral ectoderm. By inducing the hypothalamus and oral ectoderm simultaneously within the same ES cell aggregate *in vitro* to recapitulate these developmental processes in embryos, Suga et al. have established the differentiation method for adenohypophysis from mouse ES cells (Suga et al. 2011).

In the ES cells cultured by the SFEBq method for hypothalamic differentiation (Wataya et al. 2008), the expression of an oral ectoderm marker Pitx2 is low. Since oral ectoderm and hypothalamic progenitors are adjacent, a slight shift in the positional information is expected to promote the simultaneous generation of both tissues within the same aggregate in SFEBq culture. A key positioning factor for the oral ectoderm is BMP4. BMP4 treatment increased Pitx2 expression in SFEBq-cultured ES cells, whereas the BMP4 antagonist dorsomorphin suppresses the generation of the oral ectoderm. However, treatment with exogenous BMP4 in contrast inhibits hypothalamic differentiation even at a low dose. An optional condition is a large-cell aggregation (LCA) culture (Fig. 3.4a; starting differentiation culture at 10,000 cells per aggregate, instead of 3000 in the original SFEBq culture). In the LCA-SFEBq culture, both the oral ectoderm (Pitx1/2<sup>+</sup>) and hypothalamic progenitors (Rax<sup>+</sup>) are differentiated simultaneously within the same aggregate (Fig. 3.4b, b') as a result of the moderate elevation of endogenous BMP4 (Suga et al. 2011).

**3.3.2.2 Self-Formation of Rathke's Pouch In Vitro**

SHH signals are known to provide positional information to adjust towards the midline *in vivo* (Zhu et al. 2007). During embryogenesis, Rathke's pouch receives SHH signals from neighboring tissues and develops in the middle of the rostral head ectoderm. Also *in vitro*, treatment of smoothed agonist (SAG), a hedgehog agonist induces multiple oval epithelial clusters in the LCA-SFEBq-cultured mouse EB cell aggregates (Fig. 3.4c, c'). These oval structures are located between the oral





**Fig. 3.4** *In vivo* differentiation into adenohypophysis from mouse ES cells. (a) Schema of LCA-SFEBq method. (b, b') Two-layer formation of the oral ectoderm and hypothalamic progenitors in the LCA-SFEBq-cultured aggregates (b). Immunostaining of the aggregates (b', scale bar 100  $\mu$ m). (c-c') Self-formation of Rathke's pouches (c). Bright field images (c', scale bar 100  $\mu$ m) and immunostaining of the aggregates with Rathke's pouches (c'', scale bar 100  $\mu$ m). (d-d'') Generation of ACTH<sup>+</sup> cells in the differentiated adenohypophysis (d). Low-power field view (d', scale bar 50  $\mu$ m) and high-power field view (d'', scale bar 20  $\mu$ m) of ACTH<sup>+</sup> area. Modified from Suga (2019), with permission

ectoderm and hypothalamic neurons (Fig. 3.4c''). Rathke's pouch marker Lim3 (also called Lhx3) is expressed in epithelial cells comprising these oval structures. The long axis of the Lim3<sup>+</sup> (Lhx3<sup>+</sup>) pouches reaches a diameter of 150–200  $\mu$ m, comparable to that of early Rathke's pouch *in vivo*. Interactions between the oral ectoderm and hypothalamic neurons are critical for *in vitro* induction of Rathke's pouch. Neither isolated surface ectoderm nor hypothalamus alone generates Lim3<sup>+</sup> (Lhx3<sup>+</sup>) pouches (Suga et al. 2011).

### 3.3.2.3 Generation of Multiple Endocrine Lineages

During early adenohypophysis development, Lhx3<sup>+</sup> adenohypophysis progenitors commit to several hormone-type-specific lineages (Davis et al. 2011). Among them, the ACTH-producing corticotroph lineage requires the transcription factor Tbx19 (Lamolet et al. 2001), the expression of which is inhibited by Notch signaling (Zhu et al. 2006; Kita et al. 2007). Treatment with the Notch inhibitor DAPT increases Tbx19 expression in SAG-treated LCA-SFEBq-cultured ES cell aggregates.

Substantial numbers of ACTH<sup>+</sup> cells accumulate in the Tbx19<sup>+</sup> domains of DAPT-treated pouch tissues (Fig. 3.4d–d'') (Suga et al. 2011).

Previous reports have shown that canonical Wnt signaling promotes Pit1 expression (DiMattia et al. 1997; Olson et al. 2006). Consistent with these findings, treatment with the Wnt agonist BIO increases Pit1 expression, leading to subsequent GH<sup>+</sup> and PRL<sup>+</sup> cell differentiation in the SAG-treated LCA-SFEBq-cultured ES cell aggregates. Head mesenchyme has been suggested to promote adenohipophysis development *in vivo* (Gleiberman et al. 1999). In the SAG-treated LCA-SFEBq-cultured ES cell aggregates with conditioned medium of PA6 stromal cells, LH<sup>+</sup>, FSH<sup>+</sup>, and TSH<sup>+</sup> cells are successfully differentiated (Suga et al. 2011).

Positive and negative feedback systems are characteristic of endocrine cells. The complete recapitulation of these properties is the ultimate goal for endocrine regenerative medicine. To investigate *in vitro* functionality, a corticotropin-releasing hormone (CRH) loading test has been performed on induced ACTH<sup>+</sup> cells generated in SAG+DAPT-treated LCA-SFEBq-cultured aggregates of mouse ES cells. From the SAG+DAPT-treated LCA-SFEBq-cultured aggregates, substantial amounts of ACTH are secreted, comparable to peripheral ACTH levels in mice. ACTH secretion from the adenohipophysis is negatively regulated by the downstream glucocorticoid hormone. Consistent with this hormonal regulation *in vivo*, ACTH release *in vitro* after CRH stimulation is suppressed by glucocorticoid pre-treatment. These findings demonstrate that the ES-cell-derived endocrine cells in the adenohipophysis actively secrete ACTH and respond to both positive and negative regulators that work for endocrine homeostasis *in vivo* (Suga et al. 2011).

### 3.3.2.4 Functional Rescue in Hypophysectomized Model Animals

In hypophysectomized mice with the SAG+DAPT-treated LCA-SFEBq-cultured aggregates transplanted into the kidney capsule, CRH loading induces a substantial elevation of blood ACTH levels and downstream glucocorticoid hormone corticosterone, indicating that ACTH from the graft sufficiently induced the downstream hormone. Even without CRH loading, the basal levels of ACTH and corticosterone are also increased after transplantation, suggesting that partial recovery of blood ACTH has a moderate but biologically significant effect. With hormonal recovery, the transplanted hypophysectomized mice exhibit higher spontaneous locomotor activities and survive longer. Although CRH, secreted from the hypothalamus, should be diluted at the peripheral site, mouse ES cell-derived adenohipophysis tissues improve survival and spontaneous activities even when transplanted into the kidney capsule (Suga et al. 2011).

### 3.3.3 Application to Human ES/iPS Cell Culture

Although human ES cells are vulnerable to apoptosis upon cellular detachment and dissociation, it has been demonstrated by Watanabe et al. that Y-27632, a selective inhibitor of Rho-associated kinase (Rock), markedly diminishes dissociation-induced apoptosis of human ES cells, which enables human ES cells to aggregate

in the SFEBq method (Watanabe et al. 2007). Based on differentiation methods into hypothalamic neurons (Wataya et al. 2008) and adenohypophysis (Suga et al. 2011) from mouse ES cells using the SFEBq culture, differentiation methods have been established for human hypothalamic neurons (Merkle et al. 2015; Ogawa et al. 2018) and adenohypophysis cells (Ozone et al. 2016) from human ES cells. Very recently, a functional hypothalamic-adenohypophysis unit has been generated using human iPS cells (Kasai et al. 2020).

### 3.3.3.1 Hypothalamic Neuron Differentiation from Human ES Cells

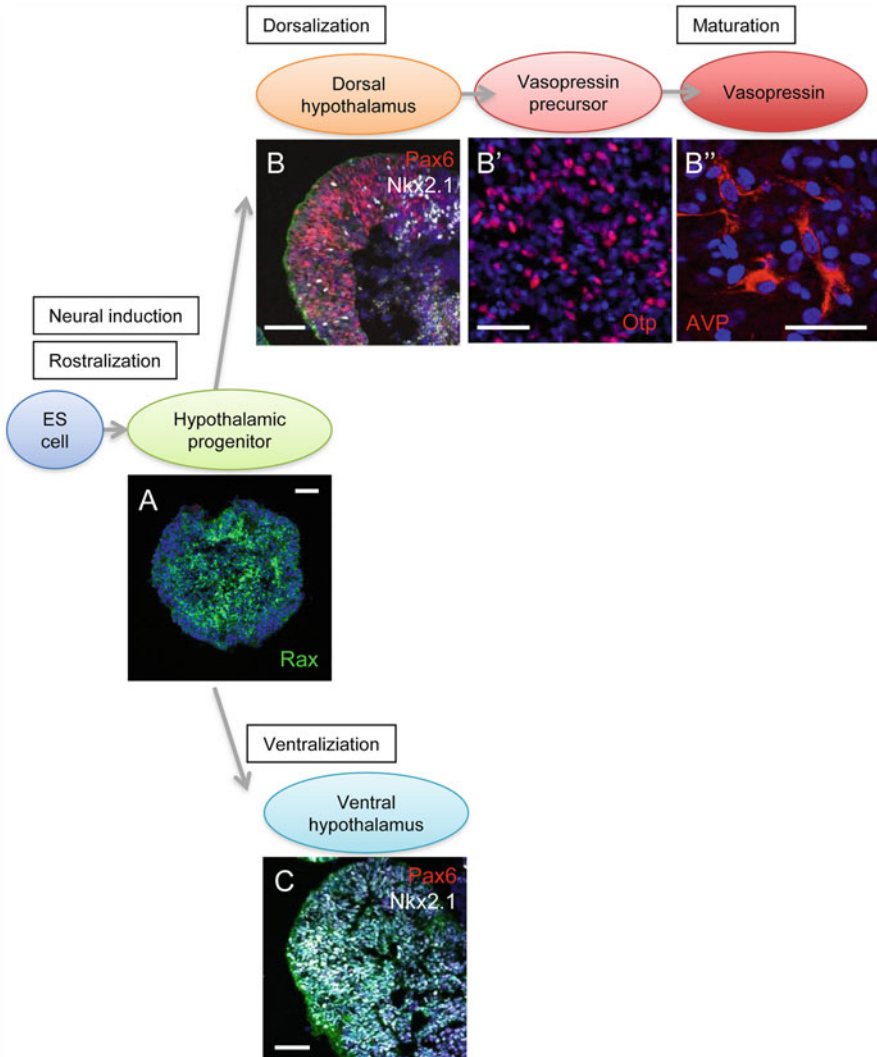
Merkle et al. have reported hypothalamic neuronal differentiation from human pluripotent stem cells using the SFEBq method, although hormonal secretion from differentiated hypothalamic neurons has not been demonstrated (Merkle et al. 2015). Recently, Ogawa et al. established a differentiation method for functional hypothalamic neurons from human ES cells (Ogawa et al. 2018).

In the development of hypothalamic neurons from mouse ES cells, strict removal of exogenous patterning factors is essential in the SFEBq/gfCDM culture (Wataya et al. 2008); however, human ES cells fail to aggregate and die within several days in this method. A small amount of KSR as well as Rock inhibitor Y-27632 enables human ES cells to aggregate in the gfCDM, although the cells differentiate towards the telencephalon under these conditions. Therefore, by the slight modification of positional information with BMP4, SAG, and Akt inhibitor, SFEBq/gfCDM-cultured human ES cell aggregates differentiate into rostral hypothalamic progenitors (Rax<sup>+</sup>). Furthermore, the tuning of SHH signals induces dorsal (Pax6<sup>+</sup>/Nks2.1<sup>-</sup>) or ventral (Pax6<sup>-</sup>/Nks2.1<sup>+</sup>) hypothalamic progenitors. Via Otp<sup>+</sup>/Brn2<sup>+</sup> intermediate precursors, dorsal hypothalamic progenitors generate AVP neurons that efficiently release the hormone upon stimulation (Ogawa et al. 2018). A schematic summary of human ES cell differentiation into hypothalamic neurons is shown in Fig. 3.5.

### 3.3.3.2 Adenohypophysis Differentiation from Human ES/iPS Cells

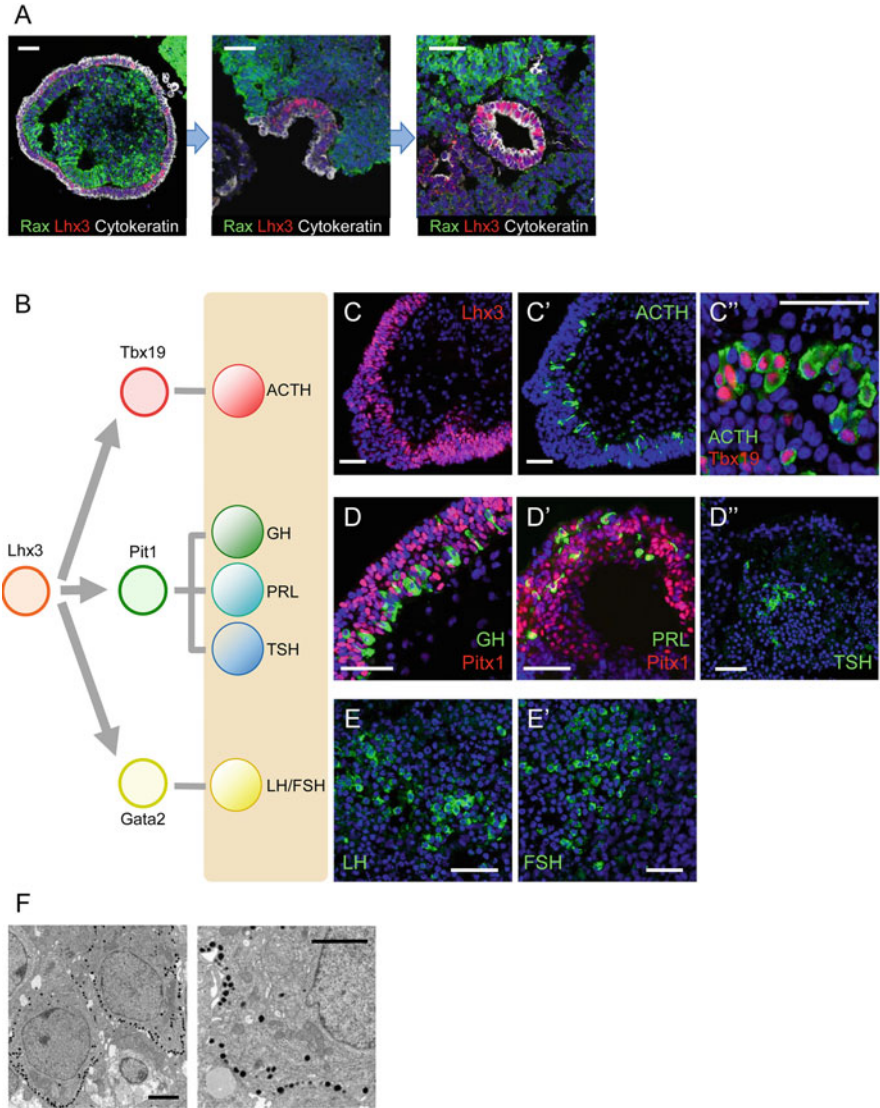
As demonstrated in the adenohypophysis differentiation method from mouse ES cells (Suga et al. 2011), the stages of differentiating pluripotent stem cells into adenohypophysis are as follows; (1) simultaneous induction of neighboring hypothalamic neuroectoderm and oral ectoderm, (2) self-formation of adenohypophysis anlage (Rathke's pouch) as a result of interaction between two layers of the hypothalamic neuroectoderm and oral ectoderm, (3) generation of multiple endocrine lineages from Lhx3<sup>+</sup> adenohypophysis progenitors, and (4) differentiation into functional anterior pituitary hormone-producing cells. By following the above steps and mimicking the differentiation method in mice (Suga et al. 2011), Ozone et al. have established the adenohypophysis differentiation method from human ES cells (Ozone et al. 2016).

In SFEBq-cultured human ES cell aggregates with gfCDM/KSR/Y-27632 medium, the addition of SAG, BMP4, and FGF2 to the differentiation medium induces hypothalamic neuroectoderm and oral ectoderm, following self-formation of Pitx1<sup>+</sup>/Lhx3<sup>+</sup> Rathke's pouch-like oval structures (Fig. 3.6a). These structures



**Fig. 3.5** Human ES cell differentiation into hypothalamus. Schematic summary of development from human ES cells to AVP neurons. Hypothalamic progenitors expressing Rax (a). Turning of SHH signals induces dorsal hypothalamus ( $Pax6^+/Nkx2.1^-$ , b), the subsequent generation of AVP precursor cells (Otp, b') and AVP neurons (b''), or ventral hypothalamus ( $Pax6^-/Nkx2.1^+$ , c). Scale bars 50  $\mu$ m. Modified from Suga (2019), with permission

subsequently differentiate into all lineages of anterior pituitary hormone-producing cells (Fig. 3.6b, c–c'', d–d'', e–e'). Among them, human ES cell-derived ACTH-producing corticotrophs and GH-producing somatotrophs have been demonstrated feedback systems. Moreover, electron microscopy reveals secretory granules characteristic of endocrine cells stored in the cytoplasm of these cells (Fig. 3.6f).



**Fig. 3.6** Applied adenohypophysis differentiation in human ES cells. **(a)** Self-formation of Rathke's pouch-like structures. Scale bars 50  $\mu\text{m}$ . **(b)** Schema of differentiation into multiple lineages of anterior pituitary hormone-producing cells. **(c–c'')** Corticotroph. Scale bars 50  $\mu\text{m}$ . **(d–d'')** Somatotroph **(d)**, lactotroph **(d')**, and thyrotroph **(d'')**. Scale bars 50  $\mu\text{m}$ . **(e, e')** Gonadotrophs. Scale bars 50  $\mu\text{m}$ . **(f)** Secretory granules characteristic of endocrine cells in electron microscopy. Scale bars 2  $\mu\text{m}$ . Modified from Suga (2019), with permission

Furthermore, transplantation with human ES cell-derived adenohypophysis tissues into the kidney capsule improved survival and spontaneous activities in hypophysectomized mice (Ozone et al. 2016).

By following the above differentiation method with slight modifications, Kasai et al. have succeeded in generating a functional hypothalamic-adenohypophysis unit from human iPS cells (Kasai et al. 2020). This hybrid organoid exhibits simultaneous differentiation and maturation of the hypothalamic neurons and anterior pituitary hormone-producing cells within the same aggregates. Therefore, ACTH secretion capacity is comparable to that *in vivo* since CRH from the hypothalamic area regulates ACTH-producing cells in analogy with the hypothalamic-pituitary axis (Kasai et al. 2020).

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### **3.4 Perspectives: Applications of Human ES/iPS Cell-Derived Hypothalamic Neurons and Adenohypophysis**

#### **3.4.1 In Vitro Human Model of Development and Disease**

Human iPS cells are promising tools for studying the process of human organ development and its disorders. Recently, Matsumoto et al. have established a disease model of congenital pituitary hypoplasia (CPH) using iPS cells derived from patients with a heterozygous mutation in the orthodenticle homeobox 2 (OTX2) gene. The patient-derived iPS cells retain the potential to differentiate into the oral ectoderm but exhibit a severely impaired adenohypophysis differentiation. OTX2 in the hypothalamus is essential for progenitor cell maintenance by regulating Lhx3 expression in the ectoderm via FGF10 expression in the hypothalamus (Matsumoto et al. 2020).

iPS cell lines from patients with various hereditary diseases have been generated so far. Regarding hypothalamic and pituitary diseases, besides CPH described above, iPS cell lines from patients afflicted with familial neurohypophysial diabetes insipidus (Yoshida et al. 2020b) and multiple endocrine neoplasia type 1 (Yoshida et al. 2020a) have recently been generated.

#### **3.4.2 Transplantation of Human ES/iPS Cell-Derived Hypothalamic Neurons and Adenohypophysis**

ES cell-derived ACTH-producing cells function with hormonal regulation and improve survival and spontaneous activities in hypophysectomized mice even ectopically transplanted in the kidney capsule (Suga et al. 2011; Ozone et al. 2016). These findings raise the possibility of simple grafting in a peripheral site; however, physiological CRH release from the hypothalamus does not directly affect these peripheral grafts. Therefore, orthotopic transplantation into the sella or hypothalamus is one of the future candidates.

There are several challenges for regenerative medicine using human ES/iPS cell-derived hypothalamic neurons and adenohypophysis. First, the differentiation method still needs to be optimized: the maturity of ACTH- and GH-producing cells and the differentiation of other lineages are not enough. Also, xeno-free culture



systems are required for clinical application. Second, ensuring safety is essential for clinical use. Contamination of immature cells increases the risk of tumorigenesis. Therefore, purification methods for target cells need to be developed. Third, ethical issues should be considered appropriately, even though human iPS cells have fewer issues than ES cells in general.

### Key References

- Watanabe et al. (2005) The first paper to introduce a method of serum-free suspension culture (SFEBq; serum-free floating culture of embryoid body-like aggregates with quick re-aggregation).
- Wataya et al. (2008) This paper demonstrates that strict removal of exogenous patterning factors during early differentiation step induces hypothalamic progenitors in mouse ES cells.
- Suga et al. (2011) Innovative work on self-formation of Rathke's pouch-like adenohypophysis progenitors and subsequent differentiation into functional adenohypophysis from mouse ES cells.
- Ozone et al. (2016) This paper illustrates the differentiation method into adenohypophysis from human ES cells.
- Kasai et al. (2020) Seminal work generating hypothalamic-adenohypophysis functional units from human iPS cells.

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