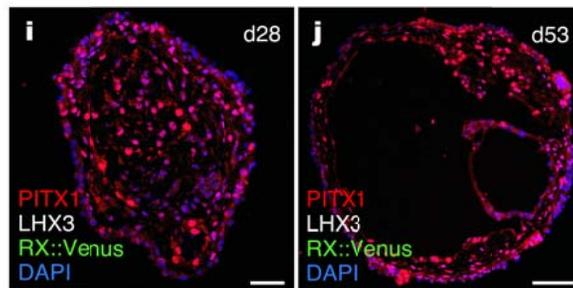
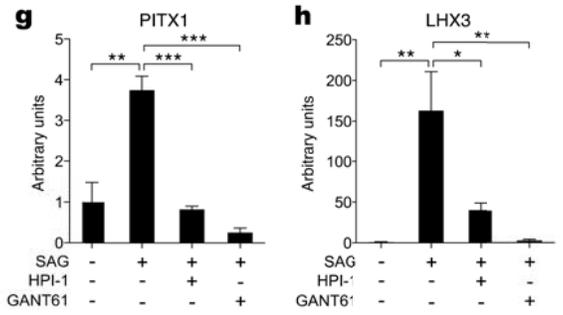
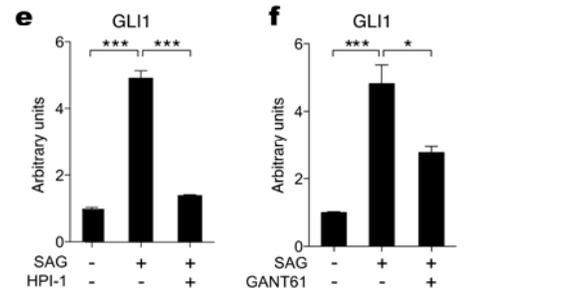
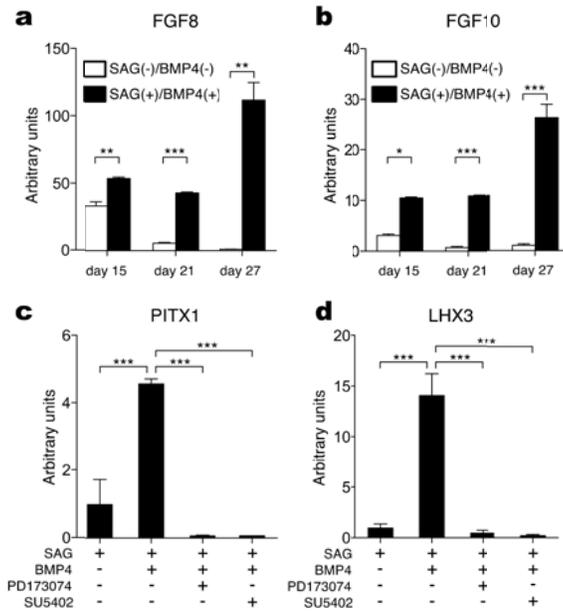


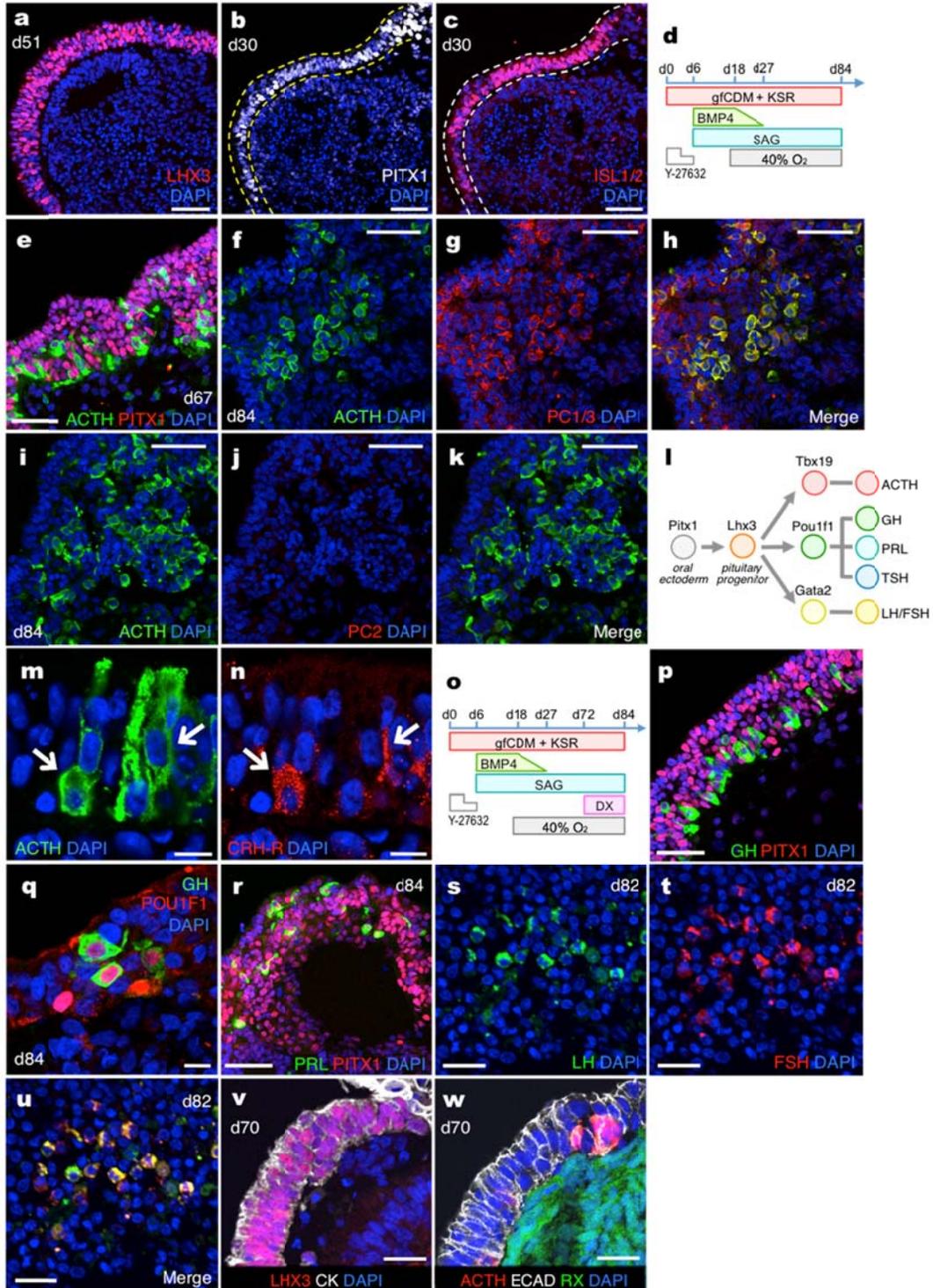
Supplementary Figure 1 | Simultaneous generation of non-neural oral ectoderm and hypothalamic tissue in three-dimensional human embryonic stem cell (hESC) culture.

(a) Schematic diagram of mouse pituitary development, sagittal view. (b) Schematic diagram of serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq) culture. (c-e) Immunostaining of a day-24 aggregate without smoothed agonist (SAG) treatment for FOXG1 (c), TUJ (d) and N-cadherin (NCAD; e). The aggregate without SAG co-expressed FOXG1, TUJ and NCAD, indicating that the telencephalic neuroectoderm (NE) was induced. c and d; the same section, c and e; serial sections. (f,g) Non-neural oral ectoderm (pan-Cytokeratin⁺; f) also expressed E-cadherin (ECAD; white; g). (h-k) Induced *RX::Venus*⁺ tissue expressed the neural markers NCAD (i) and TUJ (j), but not the retinal marker CHX10 (k). (l) Atypical kinase C (aPKC) immunostaining of a sagittal section of the E13.5 mouse pituitary primordium demarcated by a white dotted line and oral ectoderm (arrowheads). This is a magnification of the white outlined area. (m) The number of Rathke's pouch-like structures (LHX3⁺) of human versus mouse ESC aggregates (per 8 aggregates; hESC; *n* 4, mESC; *n* = 3 experiments). n.s., not significant. Error bars represent s.e.m. Student's *t*-test. Scale bars; 200 μm (c-e); 50 μm (f-k).



Supplementary Figure 2 | Roles of fibroblast growth factor (FGF) and hedgehog signals in the hESC culture for the differentiation of PITX1⁺ and LHX3⁺ epithelium.

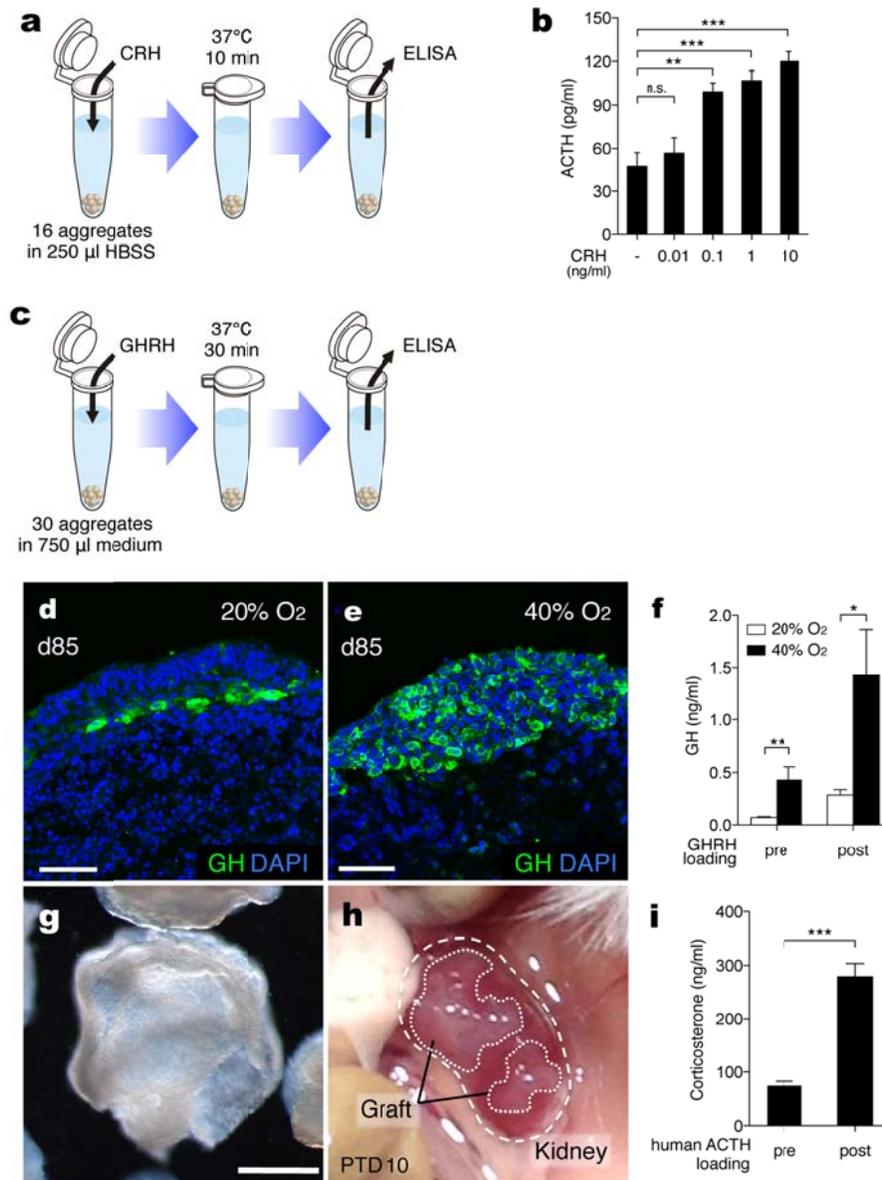
(a,b) The endogenous expression of *FGF8* (a) and *FGF10* (b) during days 15-27 in the aggregates treated or untreated with SAG (2 μ M) and bone morphogenetic protein 4 (BMP4, 5 nM; qPCR analysis; $n = 3$ experiments). (c,d) The aggregates treated with SAG (2 μ M) and BMP4 (5 nM) expressed *PITX1* and *LHX3*, but FGF inhibitor treatment, PD173074 (10 μ M) and SU5402 (0.5 μ M) on days 15-27, decreased their expression on day 27 (qPCR; $n = 3$ experiments). (e,f) qPCR analysis for expression of *GLII*. SFEBq culture on day 8. SAG treatment increased the *GLII* expression, but treatment with GLI inhibitors (HPI-1 at 50 μ M for 48 hours; GANT61 30 μ M for 36 hours) decreased *GLII* expression of the SAG-treated aggregates ($n = 3$ experiments). (g,h) Inhibition of *PITX1* (g) and *LHX3* (h) induction (on day 27) by treating hESC aggregates with the GLI inhibitors during days 6-27 (qPCR; $n = 3$). (i,j) PITX1⁺ oral ectoderm (red) alone did not express LHX3 (white). (i) on day 28. (j) on day 53. (These aggregates, which consisted of only oral ectoderm, were quite rare in our culture.) Error bars represent s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using one-way ANOVA with Bonferroni's test (a-h). Scale bars, 50 μ m (i); 100 μ m (j).



Supplementary Figure 3 | Generation of multiple endocrine lineages in 3D hESC culture.

(a) LHX3 immunostaining of day-51 aggregates. (b, c) PITX1⁺ surface ectoderm

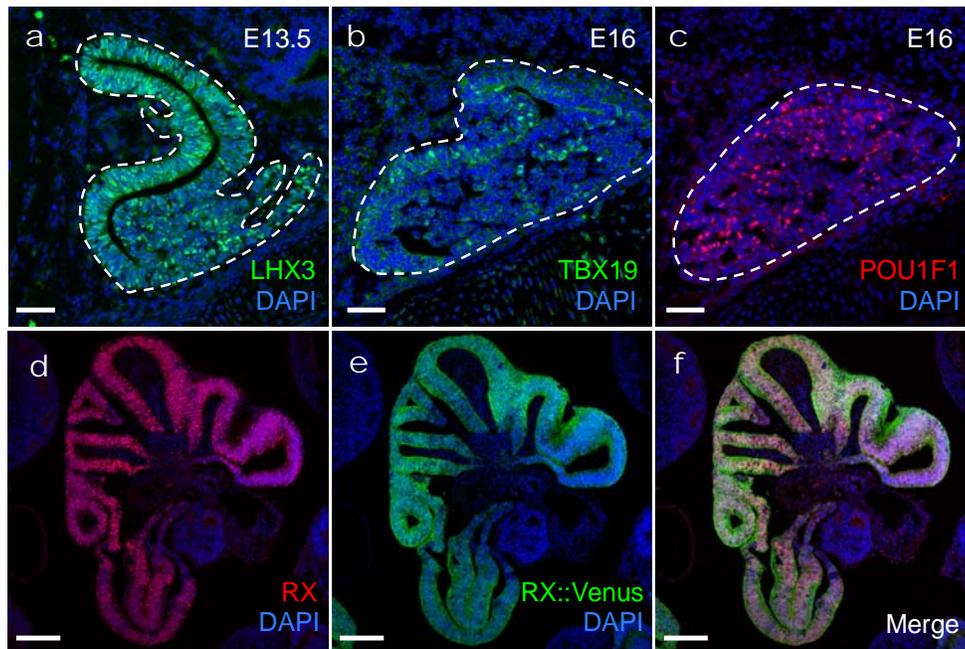
(white; **b**) expressed pituitary progenitor marker ISL1/2 (red; **c**), on day 30. (**d**) Culture protocol for adrenocorticotrophic hormone (ACTH)-producing cells. (**e**) Double-staining of a day-67 aggregate for ACTH (green) and PITX1 (red). (**f-k**) Induced ACTH⁺ cells expressed the corticotroph marker PC1/3 (**f-h**) but not the melanotroph marker PC2 (**i-k**). (**l**) Schematic of pituitary lineage development *in vivo*. (**m,n**) ACTH⁺ cells (green; **m**) expressed corticotropin releasing hormone receptors (CRH-R; **n**). (**o**) Culture protocol for growth hormone (GH)-secreting cells. DX, dexamethasone. (**p**) A human ESC aggregate immunostained for GH (green) and PITX1 (red). (**q**) Immunostaining of day-84 dexamethasone-treated aggregates for GH (green) and the lineage marker POU1F1 (red). (**r**) Prolactin (PRL; green) co-expressed with PITX1 (red) in hESC aggregates. (**s-u**) The majority of hESC-derived gonadotropin cells co-expressed luteinizing hormone (LH; green) and follicle stimulating hormone (FSH; red). (**v,w**) Immunostaining of aggregates derived from a hESC line, KhES-3. (**v**) LHX3 (red) and pan-Cytokeratin (white). (**w**) ACTH (red), ECAD (white) and RX (green). In this report, all the hESC-aggregates' data except for Supplementary Fig. 3s,t was derived from the KhES-1 cell line. Scale bars, 50 μm (**a-c,e-k,p,r**); 10 μm (**m,n,q**); 20 μm (**s-w**).



Supplementary Figure 4 | *In vitro* hormone secretion test and transplantation of hESC-derived corticotrophs.

(a) Schematic of *in vitro* CRH-loading test for ACTH secretion. (b) ACTH secretion from day-89 aggregates at different CRH doses ($n = 3$ experiments). (c) Schematic of growth hormone releasing hormone (GHRH)-loading test for GH secretion. (d, e) GH immunostaining of day-94 aggregates cultured in atmospheric (20% O₂; d) versus high-oxygen (40% O₂ from day 18; e) environment. (f) GHRH loading test showed that

the day-94 aggregates cultured in hyperoxic (40% O₂) condition secreted more GH versus control ($n = 7$). (g) Pituitary epithelial lesion excised from hESC aggregates, i.e. NE and debris were removed to reduce the graft volume. (h) The grafts 10 days after transplantation under the mouse renal capsule. (i) Increased blood corticosterone levels of wild-type SCID mice 60 min after intraperitoneal administration of human ACTH (5 ng/g; $n = 6$ experiments). Scale bars, 50 μm (d,e); 200 μm (g). Error bars represent s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using one-way ANOVA with Bonferroni's test (b) and Student's t -test (f,i).



Supplementary Figure 5 | The specificity of antibodies.

(a-c) The anti-human LHX3 antibody was raised against a synthetic peptide whose amino acid sequence was nearly the same in human and mouse. The anti-human TBX19, and POU1F1 antibodies were raised against synthetic peptides whose amino acid sequence was the same in human and mouse. These antibodies (LHX3; a, TBX19; b, POU1F1; c) selectively recognized mouse embryonic adenohypophysis. (d-f) The specificity of anti-RX antibody was confirmed by immunostaining of RX::Venus hESC reporter line-derived neural retina. Scale bars, 50 μm (a-c); 200 μm (d-f).