

Article

Generation of Functional GnRH-Responsive Pituitary-like Organoids from Ovine Embryonic Stem Cells

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Abstract

The pituitary gland is a master regulator of endocrine and reproductive physiology; however, the lack of physiologically relevant in vitro models in livestock species has limited studies of pituitary development and endocrine function. Although pituitary organoids have been generated from human and mouse pluripotent stem cells, comparable models remain unavailable in large animal species. Here, we established functional pituitary-like organoids from ovine embryonic stem cells (oESCs) using a three-dimensional differentiation strategy. The resulting organoids self-organized into Rathke's pouch-like structures and acquired pituitary progenitor characteristics, as demonstrated by the robust expression of LHX3, PITX1, ISLET1 and PROP1. Compared with conventional two-dimensional differentiation, the organoids displayed enhanced structural organization and improved lineage specification. Notably, the organoids exhibited functional gonadotroph maturation, with GnRH treatment significantly upregulating FSHB and LHB expression, as confirmed by immunohistochemistry, qPCR and Western blot. SMART-Seq transcriptome analysis revealed progressive loss of pluripotency-associated signatures and activation of pituitary developmental programs, including sustained expression of lineage-associated regulators such as PAX6. Collectively, this study establishes the first ovine pituitary-like organoid model and demonstrates its capacity to generate functional GnRH-responsive gonadotroph cells. This platform provides a valuable resource for investigating pituitary development, reproductive endocrinology, livestock fertility, and endocrine disease mechanisms in a large-animal context.

Keywords: ovine embryonic stem cells; pituitary-like organoids; gonadotroph differentiation; reproductive endocrinology

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1. Introduction

The pituitary gland, a central regulator of endocrine functions, is anatomically divided into the adenohypophysis and neurohypophysis. Pituitary hormones play essential

roles in regulating a wide range of physiological processes, including growth, metabolism, homeostasis, and reproduction in both males and females [1]. In the adenohypophysis, somatotrophs are the predominant cell type, playing a crucial role in muscle and bone growth through the secretion of growth hormone (GH) [2]. Lactotrophs secrete prolactin (PRL), which stimulates mammary gland development and lactation [3]. Gonadotrophs regulate reproductive function through the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [4,5]. Despite the pivotal role of the pituitary gland in both physiological regulation and disease development, studies investigating the mechanisms underlying pituitary cell differentiation and endocrine function remain challenging. Traditional two-dimensional (2D) culture systems fail to fully recapitulate the complex cellular interactions, spatial organization, and microenvironment of native pituitary tissue. Moreover, the limited proliferation and transient hormone secretion of isolated primary pituitary cells *in vitro* constrain research potential. The rapid progress in stem cell research has established a cellular basis for pituitary organoid development, paving new avenues for understanding and treating associated disorders. However, most studies to date have focused on human and mouse systems, while comparable models in large animal species remain largely unexplored. As an important agricultural animal, the growth and reproduction regulation of ovine are closely related to economic benefits. Therefore, establishing an ovine stem cell-derived pituitary model would not only address a significant gap in the field but also provide a novel platform for studying pituitary development and function in large animals.

Embryonic stem cells (ESCs) have the remarkable ability to differentiate into various cell types, including those of the brain. The generation of brain organoids from ESCs and induced pluripotent stem cells (iPSCs) has greatly advanced our understanding of human neurodevelopment and neurological disorders [6–8]. The pituitary gland, which is central to the hormonal system, is regulated by the release of hormones from the hypothalamus. To date, substantial progress has been made in generating pituitary organoids from ESCs and iPSCs [9–11]. Using the SFEBq method (serum-free, floating culture of embryoid-body-like aggregates), both mouse and human ESCs have been successfully differentiated into pituitary organoids, which, upon renal capsule implantation, rescued hypopituitary phenotypes in mice [12,13]. Human iPSC-derived pituitary organoids have similarly shown potential for treating hypopituitarism, with the combined addition of BMP4 and SAG significantly enhancing the expression of the pituitary progenitor marker LHX3. Moreover, 3D-cultured organoids harbor more diverse cell types and exhibit richer cell–cell communications than 2D cultures [14], underscoring their value as *in vitro* models for studying the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in fertility and endocrine research [15]. In the livestock field, organoid platforms have been established for multiple organs including the intestine, liver, lung, kidney, testis, mammary gland, and reproductive tract, offering valuable tools for veterinary disease modeling [16]. However, livestock pluripotent stem cell-derived organoids remain less explored; notable exceptions include the generation of photoreceptor-dominant retinal organoids from porcine iPSCs [17], lung progenitor-like organoids from porcine PSC [18], and multinucleated myotubes from bovine iPSCs [19], as well as blastocyst-like structures from both porcine and bovine ESCs [20,21]. Despite these advances in other livestock species, the generation of pituitary organoids from large domestic animals, such as sheep, has not been systematically explored. Therefore, in this study, we aimed to establish a protocol for directing ovine ESCs toward pituitary organoid differentiation and to characterize their cellular identity and developmental dynamics via transcriptomic profiling.

In the present study, we drew upon previous studies to optimize differentiation protocols for generating pituitary-like cells *in vitro* [22–24]. We cultured oESCs in three-dimensional (3D) suspension or 2D adherent conditions, using growth factors, agonists, or

antagonists related to the FGF, BMP, Wnt, and SHH signaling pathways to replicate the developmental process of the pituitary gland in vitro. This study provides the first ovine stem cell-derived pituitary-like organoid model and offers a physiologically relevant platform for investigating pituitary development, reproductive endocrinology, and endocrine regulation in large-animal species.

2. Materials and Methods

2.1. oESCs Culture and Maintenance

Ovine ESCs were isolated by our laboratory. Briefly, ovine blastocysts were seeded onto mitomycin C-treated mouse embryonic fibroblasts (MEFs) for ESCs derivation. The cell culture medium was adapted from bovine EPSCs (expanded potential stem cells) protocols [25] and consisted of mTeSR1 basal medium (Stemcell, 85850, Vancouver, BC, Canada) supplemented with 5 μ M IWR1 (Selleck, S7086, Houston, TX, USA), 1 μ M CHIR99021 (Selleck, S2924, Houston, TX, USA), 50 μ g/mL Vitamin C (Sigma, 49752, St. Louis, MI, USA), 10 ng/mL LIF (Peprotech, 96-300-05, Waltham, MA, USA), and 20.0 ng/mL Activin A (Peprotech, 96-120-14, Waltham, MA, USA). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂. After cells were derived from the blastocysts, they were initially passaged at a 1:1 ratio. Following two passages, ESCs colonies emerged, and the passaging ratio was gradually increased for expansion. Once the cells were stabilized, they were routinely passaged every four days at a ratio of 1:10 to 1:15. For passaging, cells were dissociated with TrypLE Express (Invitrogen, 12604013, Waltham, MA, USA) for 3 min, centrifuged, and reseeded onto fresh MEFs. Additionally, when cells were passaged as single cells, 10 μ M Rock inhibitor (Selleck, S1049, Houston, TX, USA) was added to the medium to improve cell survival.

2.2. 2D oESCs Differentiation of Pituitary Lineage

The pituitary differentiation of oESCs in 2D was performed following the protocol described in previous studies [15]. The oESCs were cultured in a 12-well plate and dissociated into a single-cell suspension using TrypLE Express upon reaching 80% confluence. Subsequently, the cells were seeded onto a 0.1% gelatin-coated (Beyotime, C0316, Shanghai, China) 12-well plate at a 1:3 dilution with gfCDM medium contained 10% KSR (Gibco, 10828010, Waltham, MA, USA) and 10 μ M Rock inhibitor for pituitary lineage induction at 37 °C in a humidified atmosphere with 5% CO₂. The gfCDM formulation included IMDM (Gibco, 31980030, Waltham, MA, USA) and Ham's F12 (Gibco, 11765054, Waltham, MA, USA) in a 1:1 ratio, 1% chemically defined lipid concentrate (Gibco, 11905031, Waltham, MA, USA), 450 μ M 1-thioglycerol (Sigma, M6145), 1% BSA (Sigma, B2064, St. Louis, MI, USA), and 1% GlutaMAX (Gibco, 35050-061, Waltham, MA, USA). From day 7 of culture, 200 ng/mL SAG (Smoothed Agonist, Stemcell, 73412, Vancouver, Canada), a Sonic Hedgehog (SHH) signaling pathway agonist, and 5 ng/mL BMP4 (Bone Morphogenetic Protein 4, Peprotech, 120-05-5 μ g, Waltham, MA, USA) were added to the medium to promote pituitary lineage specification. On day 15, the KSR concentration was increased to 15%, and 20 ng/mL FGF2 (Recombinant human FGF-basic, Peprotech, 96-100-18B, Waltham, MA, USA) was supplemented to support further differentiation and proliferation until day 21 [15]. Throughout the entire differentiation period, 1 μ M SB431542 (MCE, HY-10256, Brunswick Township, NJ, USA), a TGF- β signaling pathway inhibitor, and 2 μ M IWP2 (MCE, HY-13912, Brunswick Township, NJ, USA), a Wnt signaling pathway inhibitor, were maintained in the culture medium to suppress unwanted signaling and facilitate proper anterior pituitary lineage specification.

2.3. 3D oESCs Differentiation of Pituitary-Like Organoid

The SFEBq [22,26] method was used for 3D differentiation culture. The culture start date was defined as day 0. The oESCs were dissociated into single cells using TrypLE

Express and subsequently seeded in a 96-well round-bottom ultra-low attachment microplate (Corning, 7007, Corning, NY, USA). The differentiation medium consisted of gfCDM supplemented with a fixed concentration of KSR.

The initial number of seeded cells is important for the stable differentiation of the organoids. We designed four gradients to select the optimal number on day 0 as follows: 2500, 5000, 10,000, and 20,000 cells per well. We observed that the 10,000-cell aggregates initiated differentiation into ectoderm-like structures by day 3 and, upon continued culture, developed smooth edges and a dense spherical shape by day 6 (Figure S1). On day 0, 10,000 oESCs were seeded in 100 μ L of differentiation medium (gfCDM + 10% KSR + 10 μ M Y-27632) in each well. Half of the medium was replaced daily during the first week. In order to mimic the *in vivo* hypothalamic–pituitary interaction by co-inducing both neural and oral ectodermal tissues, starting from day 7, gfCDM was supplemented with 2 μ M SAG and 5 nM BMP4, with half of the medium replaced every 48 h. From days 15 to 21, 20 ng/mL of FGF2 was added to the culture medium to induce the expression of LHX3, a key marker for pituitary progenitor commitment [22]. To overcome the limited oxygen diffusion in 3D culture and ensure sufficient oxygenation for the core regions of the aggregates during the subsequent high-energy differentiation and maturation stages [9], we began replacing the BMP4-free medium daily from day 18, gradually diluted the BMP4 concentration, and cultured the aggregates under high-oxygen conditions (40% O₂). Additionally, 1 μ M SB431542 and 2 μ M IWP2 were added to the culture medium throughout the experiment to suppress endogenous TGF β and Wnt signals, thereby facilitating proper endocrine lineage commitment and preserving forebrain identity.

2.4. Ovine Pituitary Tissue Collection

All experimental animals were obtained from Jiangsu Qianbao Sheep Industry Limited Company in Yancheng, China. The developmental period of the embryonic lamb can be estimated based on the mating records of the ewe. At embryonic day 90 (E90) and day 120 (E120) of pregnancy, pregnant ewes ($n = 3$ per time point) underwent surgical abortion. The fetal heads were dissected to expose the cranial cavity, and the pituitary glands were carefully collected. The collected tissues were immediately snap-frozen in liquid nitrogen and subsequently stored at -80°C for long-term preservation. For RNA extraction, frozen pituitary tissues were homogenized in TRIzol reagent (Invitrogen, 15596026, Waltham, MA, USA) following the manufacturer's instructions. Total RNA was quantified using a spectrophotometer, and cDNA was synthesized using a reverse transcription kit (Vazyme, R323-01, Nanjing, China) for subsequent quantitative real-time PCR analysis.

2.5. Immunohistochemistry

We selected 6 to 10 3D aggregates, which were transferred from a 96-well plate to 1.5 mL conical tubes and allowed to settle for 5 min. The culture supernatant was then discarded, and the aggregates were washed with 1 $\times$ phosphate-buffered saline (PBS) prior to fixation in a 4% formaldehyde solution at 4°C overnight. Immunohistochemistry on 3D organoids was conducted using frozen section slides, following these steps: antigen retrieval with a heated pH 6.0 citric acid repair buffer for 20 min. After washing, sections were permeabilized with 0.3% Triton X-100 for 10–20 min at room temperature, blocked with 3% bovine serum albumin (BSA) for 40 min at room temperature, and incubated with primary antibodies overnight at 4°C . After washing, sections were incubated with the appropriate fluorescently labeled secondary antibodies for 40 min at room temperature in the dark. Nuclei were counterstained with DAPI (Invitrogen, D1306, Waltham, MA, USA) for 5 min. Finally, the sections were mounted and examined under a fluorescence microscope. Detailed information on all antibodies is provided in Table 1.

Table 1. Details of antibodies.

Antibodies	Cat No.	Source	Dilution of WB	Dilution of IF
E-Cadherin	60335-1-Ig	Proteintech, Wuhan, China	1:500	1:200
PITX1	DF3224	AFFinity, Cincinnati, USA	-	1:100
Pan-keratin	26411-1-AP	Proteintech, Wuhan, China	-	1:200
LHX3	sc-293411	Santa cruz, Dallas, USA	-	1:50
ISLET1	15661-1-AP	Proteintech, Wuhan, China	-	1:50
PROP1	DF2609	AFFinity, Cincinnati, USA	-	1:100
SOX2	AF5140	AFFinity, Cincinnati, USA	1:500	-
GAPDH	1E6D9	Proteintech, Wuhan, China	1:10,000	-
HRP-conjugated Goat Anti-Mouse IgG (H + L)	SA00001-1	Proteintech, Wuhan, China	1:5000	-
HRP-conjugated Goat Anti-Rabbit IgG (H + L)	SA00001-2	Proteintech, Wuhan, China	1:5000	-
CoraLite594-conjugated Goat Anti-Rabbit IgG (H + L)	SA000134	Proteintech, Wuhan, China	-	1:200
CoraLite488-conjugated Goat Anti-Mouse IgG (H + L)	RGAM002	Proteintech, Wuhan, China	-	1:200

2.6. Western Blot Analysis

Preparation of protein lysate was conducted using a ratio of RIRA lysis buffer (Beyotime, P0013B, Shanghai, China) supplemented with protease and phosphatase inhibitor cocktail (Beyotime, P1046, Shanghai, China) at a ratio of 100:2 (RIPA to inhibitor cocktail). After adding the prepared RIPA buffer, samples were lysed at 4 °C for 30 min. The lysates were then centrifuged at 4 °C, 12,000 rpm for 10 min, and the supernatants (total protein extracts) were collected and stored at −80 °C for further use. Protein concentrations were determined using a BCA protein assay kit (Beyotime, P0010), and all samples were adjusted to the same concentration to ensure consistency in subsequent experiments. For Western blot analysis, 100 µg of protein sample was aliquoted, to which 15 µL of 4× LDS Sample Buffer (Invitrogen, NP0007, Waltham, MA, USA) and 10 µL of 10× Sample Reducing Agent (Invitrogen, NP0009, Waltham, MA, USA) were added. Nuclease-free water was then added to bring the final volume to 100 µL. For example, if the protein concentration of a sample is 2 µg/µL, then 50 µL of the protein sample is required to obtain 100 µg of protein; in this case, 50 µL of protein sample would be mixed with 15 µL of 4× LDS Sample Buffer, 10 µL of 10× Sample Reducing Agent, and 25 µL of nuclease-free water to reach a total volume of 100 µL. The mixture was then denatured at 70 °C for 10 min using a PCR instrument. Denatured proteins were subsequently separated by SDS-PAGE (ACE Biotechnology, ET12008Gel, Nanjing, China) according to molecular weight and transferred onto PVDF membranes (Millipore, IPVH00010, Billerica, USA). The membranes were blocked with 5% non-fat milk in TBST (Tris-Buffered Saline with Tween 20) for 1 h at room temperature, followed by overnight incubation with primary antibodies at 4 °C. After three washes with TBST, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using an ECL chemiluminescence substrate (Maido Biotechnology, BL520B, Nanjing, China) and imaged with a chemiluminescence imaging system.

2.7. Synthesis and Amplification of cDNA Derived from Minimal Cellular RNA and Quantitative Real-Time PCR

For the analysis of 3D aggregates, 3–4 aggregates per sample were utilized to extract trace DNA using the CellAmp™ Whole Transcriptome Amplification Kit (Real Time) Ver.2 (Takara, No. 3734, Kusatsu, Japan). The procedures were conducted according to the kit's

instructions. Briefly, the aggregates were first lysed in the provided Lysis Buffer. Subsequently, a reverse transcription reaction was carried out using the RT dT Primer 2 to synthesize first-strand cDNA from the released mRNA. Following cDNA synthesis, Terminal Deoxynucleotidyl Transferase (TdT) was used to add a poly(dA) tail to the 3' end of the synthesized cDNA. The dA-tailed cDNA then served as the template for PCR amplification using the provided PCR Primer Mix 2. To minimize nonspecific amplification derived from primers and enhance the yield for low-expression genes, an additional Exonuclease I treatment step was included. Finally, the amplified cDNA products were collected and stored for further analysis. The qPCR reaction was used AceQ qPCR SYBR Green Master Mix (Vazyme, Q711-02, Nanjing, China) and was performed using the QuantStudio 5 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA). The qPCR cycling parameters included an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The final step consisted of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s, utilizing specific primers as detailed in Table 2, using *GAPDH* as an internal reference gene.

Table 2. Primer sequences used for this study.

Genebank No.	Items	Primer Sequence (5'–3')	Product Size (bp)
XM_015095690.4	<i>PITX1</i>	F: ATCGCCGTGTGGACCAACCTC R: CCGCCCTTGCACAGGTCCAG	113
XM_027966195.3	<i>LHX3</i>	F: TCAAGGCTCTGGACCGCCACT R: CACGCAGCGCACTTGGTCCC	148
XM_042245193.2	<i>POMC</i>	F: GGACCTCACCACGGAAGTA R: GGAAATGGCCCATGACGTAC	143
XM_042236731.2	<i>TFAP2A</i>	F: GTGTCTCAACGCTTCGCTGCT R: CGGTTTCGCACACGTACCCAA	206
XM_027957858.3	<i>GATA2</i>	F: AAGGAAGGCATCCAGACCCGGAA R: ACCGGGACAGCTCCTCGAAGCA	91
XM_042236046.1	<i>HESX1</i>	F: ACGAAGATTACTTTGCACCCTC R: CACGCCGATTTTGAACCAG	222
XM_060418092.1	<i>OTX2</i>	F: TCCCATGACCTATACTCAGGCTT R: AAGTTGAGCCAGCATATCCTTG	56
XM_004009615.5	<i>PITX2</i>	F: ACCAACCTAACGGAAGCACGAGT R: CTTTCTCCATTGGCCCGGCGAT	63
XM_060396669.1	<i>LHX4</i>	F: TCCGCAAGGCACAGGACTTCGTCT R: AGCTCCAGCCTCTGAGTCATCGTT	167
XM_004017002.6	<i>ISL1</i>	F: ACTCAGTGACTTCGCCTTGACAG R: TCAATTGGACTGGCTACCATGCTGT	165
NM_001009767.1	<i>PROP1</i>	F: CGTCACCGCACCACTTCAGTCCA R: TGACCGCTCTTGCTTCCTCTGCT	180
XM_027959742.2	<i>SF-1</i>	F: CTCATCACGGAATGGTTGCCCTC R: TGTTCCCTCTTGCCCCAATCAGC	154
NM_001009350.1	<i>POU1F1</i>	F: AGAACAATATCAGTATTGCTGCT R: GGTTTAGTTCTTCAGCCATCCTCA	106
XM_060415955.1	<i>TBX19</i>	F: AAGGCACCCATCTCCTTCAGC R: ACATTATCTGCCACCTCCGTTG	67
NM_001318074.1	<i>SOX2</i>	F: GGCGGAAAACCAAGACGCTCAT R: CTGGAGTGGGAAGAAGAGGTAA	448
XM_004018968.6	<i>OCT4</i>	F: CCGTCGCCAGAAAGGGCAAAC R: GTGACAGACACCGAGGGAAA	213
XM_004006901.5	<i>NANOG</i>	F: TACCCGGGCTTCTATTCTTAC R: AGGAGTGGTTGCTCCAAGAC	139

XM_015097249.4	<i>LIN28</i>	F: GCAGCACCGGAAGAACAAAG R: CTTCCCAATCGGGGAGAGAAA	100
XM_012142500.2	<i>GAPDH</i>	F: CGACTTCAACAGCGACACTCAC R: CCCTGTTGCTGTAGCCGAATTC	136
XM_004015363.3	<i>ACTB</i>	F: TCAGCAAGCAGGAGTACGAC R: AGCCATGCCAATCTCATCTCG	123
XM_004004910.5	<i>Fibronectin</i>	F: AGATGATGAGCTGCACGTGTC R: GCACCAAGATATTCCTTCTGCC	130
XM_012183418.5	<i>NESTIN</i>	F: AAGGCCTCCAGAGCCAAATC R: GGAGTAGGGCTCAGGACAGA	250
XM_012132137.4	<i>Neurod</i>	F: TCTTTCAAACACGAACCGTCCG R: CGTGAAAGATGGCATTGAGCTG	217
XM_042234806.1	<i>NFH</i>	F: GAGTGAGGCTGGACCGACTG R: AGCTCAGAGCGCTGCCTCTC	166
XM_012179355.4	<i>KDR</i>	F: GGGTCCTGAAATCACGTTGCA R: AGTCTTCCTGTCCTGAGCAAAGC	301
XM_004010972.5	<i>ACTC1</i>	F: AAGCAAGAGTATGATGAGGCAGGCC R: TTTGTAGGTTGCAAGTCCTGATCTGG	240
XM_015094556.3	<i>MYF5</i>	F: GAATGCCATCCGCTACATTGAGAG R: AATCCAGGTTGCTCTGAGTTGGT	291
XM_027971404.2	<i>AFP</i>	F: TGTCAAGACAAATCGCAGAGTGCTG R: GAAGAAAGTTGGTT- GAAATCTCTCTCTCCT	151
XM_012159321.4	<i>DCN</i>	F: AACAATATCTCTGCAATCGGCTC R: AGTTTCCAAGCTGAACAGCAGC	169
XM_027964616.2	<i>GATA4</i>	F: GGAAGAAAGACGACGAGCGATGAG R: CGGGTCACGGGTAGAAGAGTAGAG	122

2.8. GnRH Treatment

Pituitary-like organoids were treated with 1 µg/mL GnRH (NSHF, Ningbo, China) for 3 h on the final day of culture. Total RNA was extracted and converted to cDNA, followed by analysis of changes in the expression of the FSHB and LHB genes.

2.9. SMART-Seq Method of 3D Pituitary-like Organoid Differentiation

Smart-seq was performed in accordance with a previously published research study [27]. Briefly, 3D aggregates cultured for 7, 14, and 21 days (one aggregate per sample) and logarithmically growing oESC clones (approximately 10 clones per sample, designated as day 0 controls) were collected, with 4 biological replicates per group. The samples were directly lysed for cDNA synthesis following the protocol for the Single Cell Full-Length mRNA Amplification Kit (Vazyme, N712-01, Nanjing, China). Then, 1 ng of cDNA was used to prepare sequencing libraries using the TruePrep DNA Library Prep Kit V2 for Illumina (Vazyme, TD503, Nanjing, China). The libraries were paired-end sequencing on an Illumina NovaSeq 6000 platform with 150 bp read length, generating approximately six million reads per sample. Reads were aligned to the sheep reference genome Oar_rambouillet_v1.0. Differential gene expression and functional enrichment analyses were performed using the day 0 oESC samples as the control group.

2.10. Statistics Analysis

All data are expressed as the mean ± SEM. All experiments were conducted at least three times. For statistical significance, data were analyzed using SPSS software (version 24.0) with a two-tailed Student's t-test or one-way ANOVA (followed by the Tukey post hoc test). A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. In Vitro Differentiation Capacity of oESCs

To assess the in vitro differentiation potential of oESCs, embryoid bodies (EBs) were generated under suspension culture conditions. EBs are three-dimensional cellular aggregates that recapitulate early embryonic development. Under these conditions, oESCs efficiently formed spherical three-dimensional structures that progressively increased in size and density over the culture period (Figure 1a).

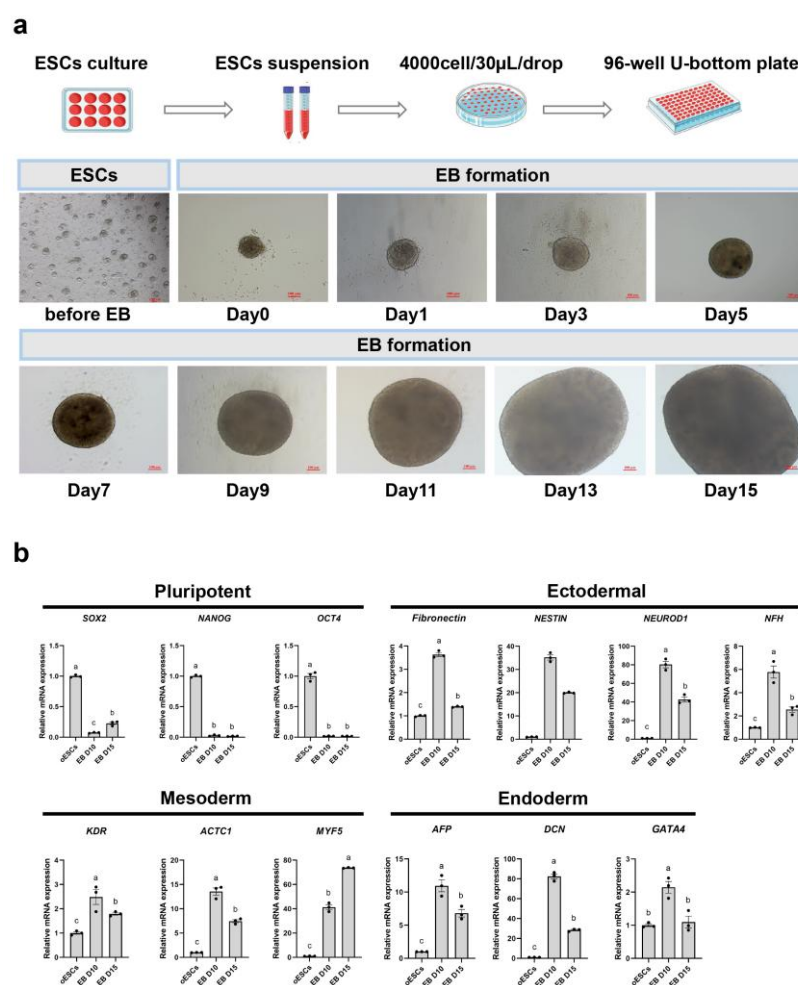


Figure 1. In vitro Differentiation Capacity of Ovine ESCs. **(a):** EB (embryoid body) differentiation of oESCs. EB differentiation time course. **(b):** RT-qPCR of germ layer gene expression in oESCs and EBs (Day 10 and Day 15): Pluripotency (SOX2, NANOG, OCT4), Ectoderm (NESTIN, NEUROD1, NFH), Mesoderm (KDR, ACTC1, MYF5), Endoderm (AFP, DCN, GATA4). Mean $\pm$ SEM, $n = 3$. Different letters indicate significant differences between groups, $p < 0.05$.

Lineage-specific gene expression analysis revealed a marked increase in ectodermal markers (*Fibronectin*, *NESTIN*, *NEUROD1*, and *NFH*), mesodermal markers (*KDR*, *ACTC1*, and *MYF5*), and endodermal markers (*AFP*, *DCN*, and *GATA4*) at Day 10 and Day 15 compared with undifferentiated oESCs. In contrast, pluripotency-associated genes were significantly downregulated or showed minimal expression during differentiation (Figure 1b), indicating successful loss of stemness and acquisition of multilineage differentiation potential.

3.2. Pituitary Lineage Differentiation from oESCs in 2D Cultures

ESCs possess the ability to differentiate into any cell lineage. The experimental strategy for 2D pituitary differentiation is outlined in Figure 2a. We observed morphological changes in oESCs during the differentiation process. After dissociation into single cells and replating under feeder-free conditions, the cells initially grew as clusters within one day. By day 6, they formed sheet-like aggregates, which gradually became more compact by day 11. From day 15 onward, placode-like structures emerged, and upon further culture, these structures proliferated and gave rise to ectoderm-like tissues, with cells progressively increasing in number and density (Figure 2b).

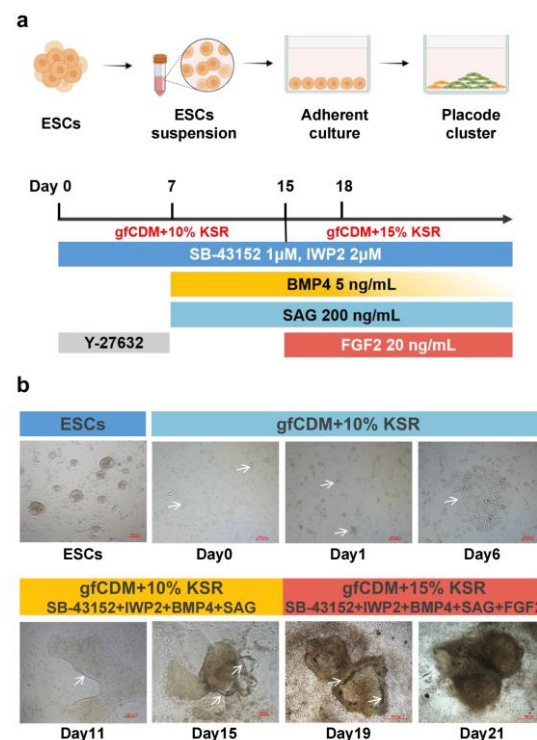


Figure 2. Pituitary Lineage Differentiation from oESCs in 2D Cultures. (a): Culture protocol for the 2D differentiation of pituitary lineage induction. (b): Representative bright-field images of undifferentiated oESCs and 2D differentiation from day 0 to day 21 (scale bar: 100 μ m and 500 μ m). White arrows indicate morphological changes of cells during the induction process.

The oESCs gradually differentiated into the pituitary cell lineage through a sequential process: (1) a significant downregulation of pluripotency markers, including *Oct4*, *Nanog*, and *LIN28*; (2) the induction of non-neural ectoderm (NNE) markers was noted, with the upregulation of *ISL1*, *GATA2*, *TFAP2A* and *OTX2*; (3) the emergence of pituitary placode markers like *SOX2*, *PROP1*, *LHX3*, *LHX4*, *HESX1*, *PITX1*, and *PITX2*; (4) the expression of key transcription factors or precursors involved in hormone-producing cell differentiation, namely *TBX19* (a transcription factor for adrenocorticotrophic hormone cell differentiation), *SF-1* (a transcription factor for gonadotropin cell differentiation), and *POMC* (a precursor gene for ACTH). Quantitative PCR analysis revealed a rapid decline in pluripotency marker expression by Day 7, indicating efficient exit from the undifferentiated state. In parallel, NNE markers and pituitary progenitor markers were progressively upregulated throughout differentiation compared with Day 0 controls (Figure 3a), suggesting the successful acquisition of pituitary lineage identity. The differentiation trajectory was further validated at the protein level. Western blot analysis (Figures 3b and S2) demonstrated that E-cadherin, a marker associated with non-neural ectoderm specification, was undetectable in undifferentiated oESCs but became markedly expressed from

Day 7 onwards and remained elevated through Day 21. SOX2 exhibited a dynamic expression pattern during differentiation. High SOX2 expression was detected in undifferentiated oESCs at Day 0, followed by a substantial reduction at Day 7. Subsequently, SOX2 expression gradually increased from Day 14 to Day 21, consistent with its established role in pituitary progenitor maintenance and early pituitary development.

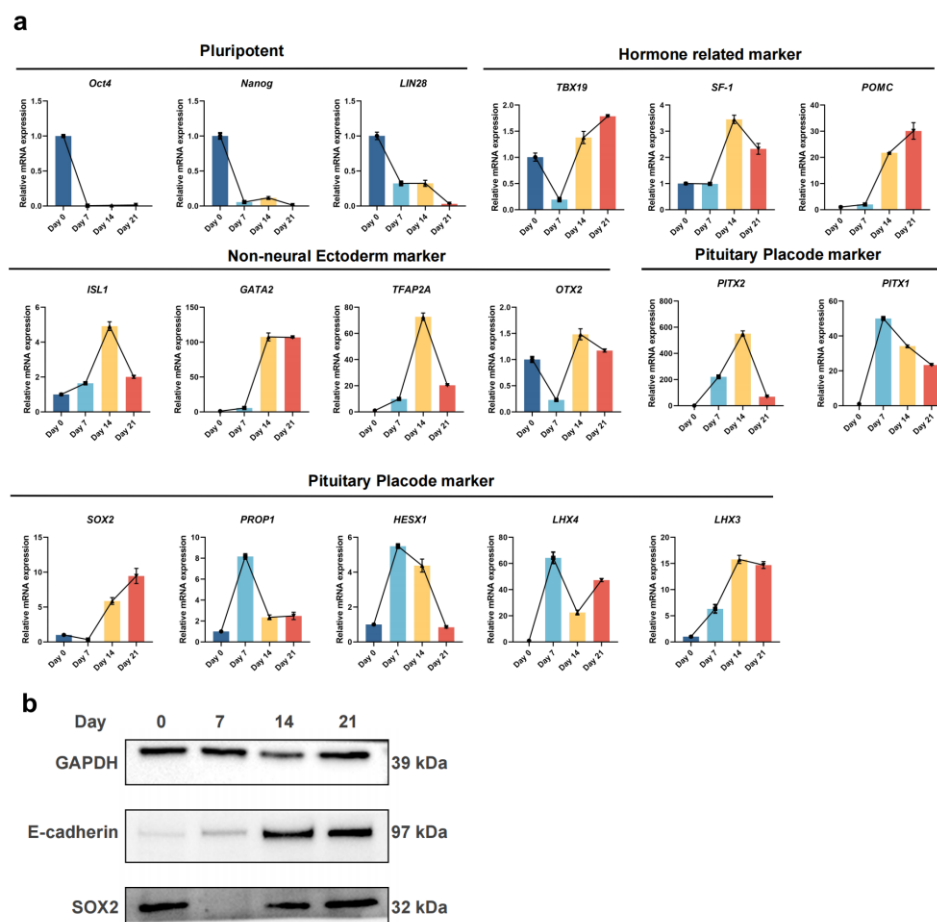


Figure 3. Molecular Characterization of Differentiated oESCs. **(a):** RT-qPCR analysis of RNA expression for pluripotency (*SOX2*, *NANOG*, *OCT4*), non-neural ectoderm (*TFAP2A*, *GATA2*, *ISL1*, *OTX2*), pituitary placode (*PROP1*, *HESX1*, *PITX1*, *PITX2*, *SOX2*, *LHX3*, *LHX4*), and other markers (*TBX19*, *SF-1*, *POMC*) during differentiation process. Mean $\pm$ SEM, $n = 3$. **(b):** Western blot analysis of pituitary placode markers: E-cadherin, SOX2, See Figure S2 for original Western blot images.

3.3. Generation of a Pituitary-like Structure in 3D Culture of oESCs Aggregates

Pituitary-like organoids were generated from oESC-derived EBs using a three-dimensional differentiation protocol (Figure 4a). During differentiation, a distinct outer epithelial-like layer gradually formed around the aggregates and became progressively thicker with prolonged culture, while the central region became increasingly compact (Figure 4b).

Gene expression analysis revealed a rapid decline in pluripotency markers (*Oct4*, *Nanog*, and *LIN28*) after Day 7 of differentiation. In contrast, the expression of non-neural ectoderm and pituitary placode markers increased progressively from Day 7 to Day 21, indicating successful pituitary lineage specification (Figure 5). Although *POMC* expression was detected at later stages, its level remained substantially lower than that observed in E90 and E120 ovine pituitary tissues (Figure 5).

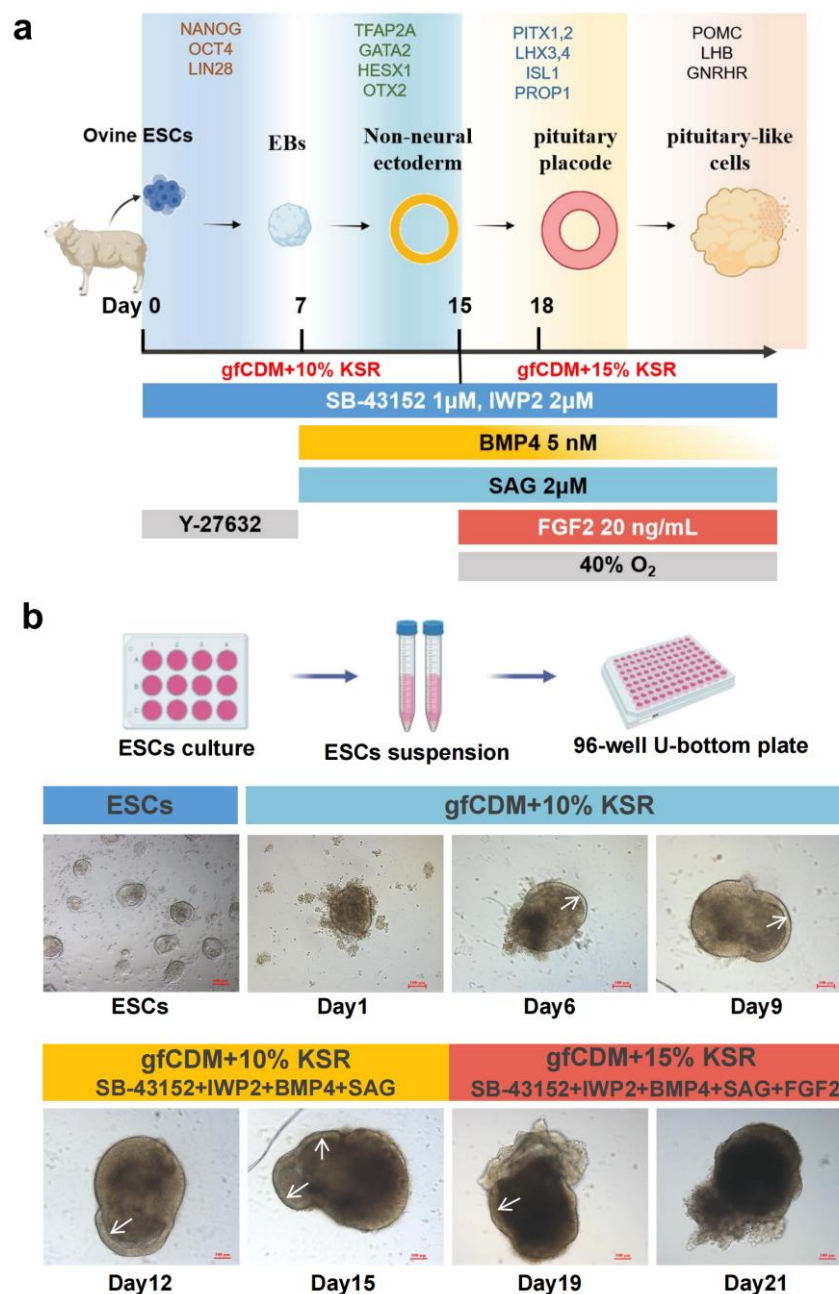


Figure 4. Generation of a pituitary-like structure in 3D culture of oESCs aggregates. (a): Culture protocol for the 3D differentiation of pituitary-like organoid induction. (b): Representative bright-field images of undifferentiated oESCs and 3D differentiation from day 1 to day 21 (scale bar: 100 μm). White arrows indicate morphological changes of cells during the induction process.

Morphological features resembling early pituitary development emerged during organoid maturation. By Day 15, invaginated structures resembling Rathke's pouch and multiple vesicle-like cavities were observed within the aggregates (Figure 6a–d). Immunofluorescence analysis demonstrated the localization of the non-neural ectoderm marker Pan-CK and the pituitary progenitor marker LHX3 within both the outer epithelial layer and Rathke's pouch-like vesicles, suggesting successful recapitulation of key events during pituitary organogenesis (Figure 6a–d).

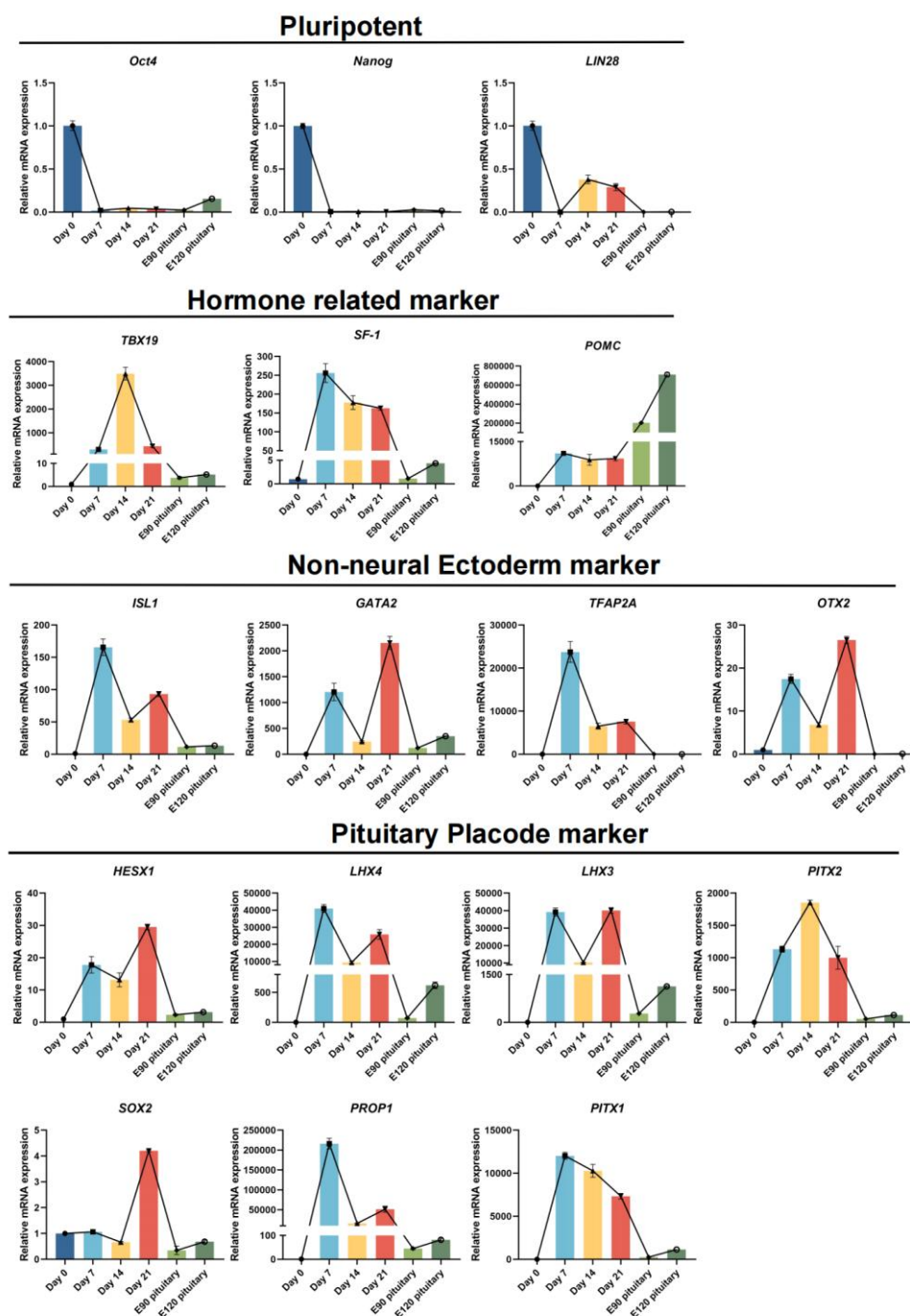


Figure 5. RT-qPCR analysis of marker gene expression in 3D-differentiated organoids. RT-qPCR analysis of RNA expression for pluripotency (*SOX2*, *NANOG*, *OCT4*), non-neural ectoderm (*TFAP2A*, *GATA2*, *ISL1*, *OTX2*), pituitary placode (*PROP1*, *HESX1*, *PITX1*, *PITX2*, *SOX2*, *LHX3*, *LHX4*), and other markers (*TBX19*, *SF-1*, *POMC*) compared with Pituitary tissue of female fetal ovine at 90 days and 120 days of embryonic development (E90, E120 Pituitary). Mean $\pm$ SEM, $n = 3$.

As differentiation progressed, pituitary progenitor markers *LHX3* and *PITX1* were detected in Rathke's pouch-like structures and surrounding regions by Day 19 (Figure 6e–h). By Day 21, the early pituitary markers *ISLET1*, *PROP1*, and *PITX1* were further identified within the organoids, supporting continued pituitary lineage development (Figure 6i–l).

To evaluate functional maturation, organoids were stimulated with gonadotropin-releasing hormone (GnRH). GnRH treatment significantly increased the expression of

FSHB and LHB, demonstrating the acquisition of gonadotroph-like characteristics and responsiveness to physiological endocrine stimulation (Figure 6m,n).

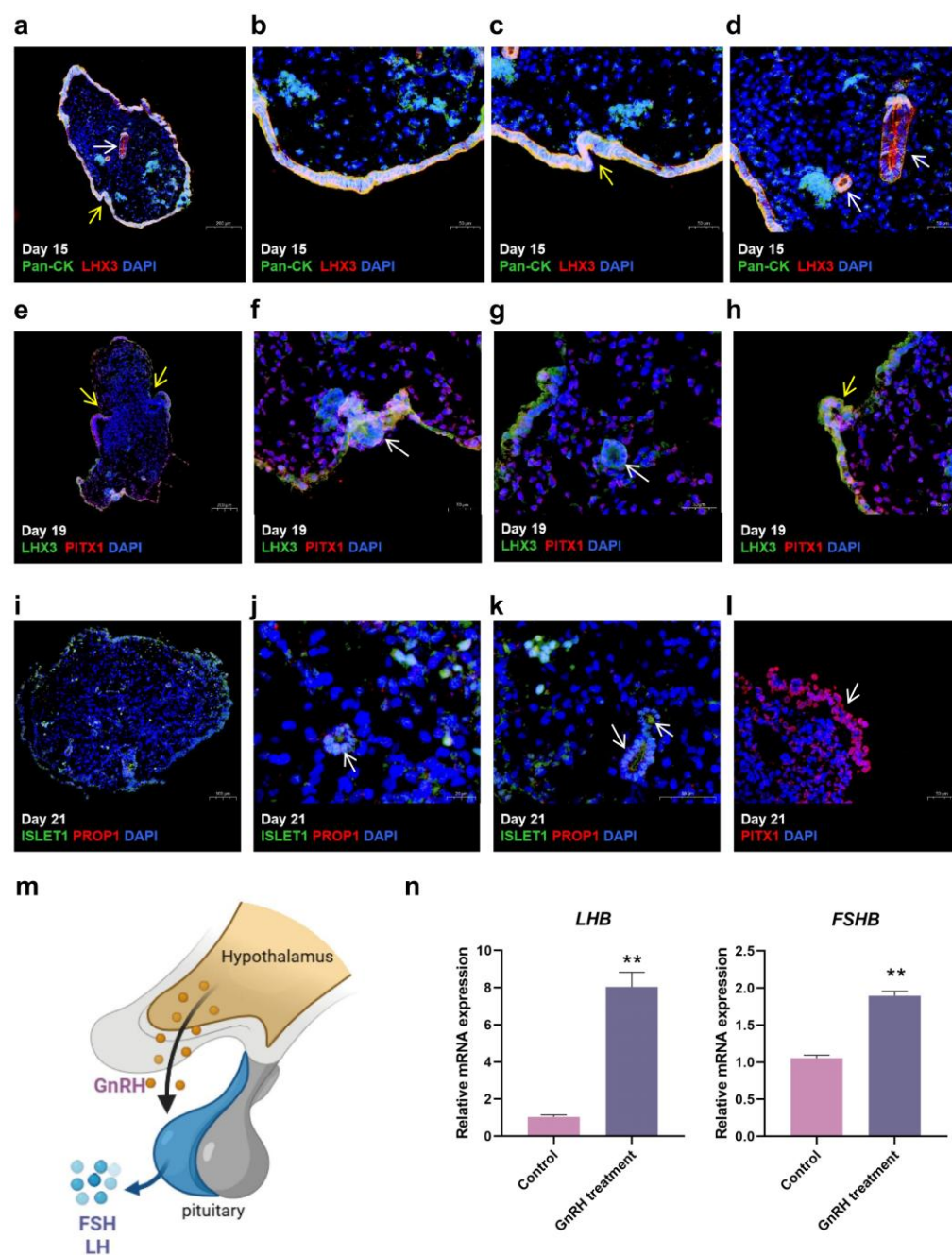


Figure 6. Generation of a Rathke's pouch in 3D culture of oESC aggregates. (a–d): Aggregates were stained with Pan-CK (green), LHX3 (red), and DAPI (blue) at the beginning of differentiation Day 15. (e–h): Aggregates were stained with LHX3 (green), PITX1 (red), and DAPI (blue) at the beginning of differentiation Day 19. (i–l): Aggregates were stained with ISLET1 (green), PROP1 (red), and DAPI (blue) at the beginning of differentiation Day 21. The white arrows mark Rathke's pouch, while the yellow arrows denote vesicle-like cavities. (m,n): Treat the aggregates on Day 21 with 1 μ g/mL GnRH for 3 h in vitro. Perform RT-qPCR analysis to assess the RNA expression of the gonadotropin marker genes FSHB and LHB. Mean $\pm$ SEM, $n = 3$ and ** indicates a significant difference ($p < 0.01$).

3.4. SMART-Seq Analysis of Pituitary-like Organoid Differentiation

To characterize the transcriptional dynamics of pituitary-like organoid differentiation, SMART-Seq analysis was performed on oESCs (Day 0) and organoids collected at

Days 7, 14, and 21. Principal component analysis (PCA) revealed a clear separation between undifferentiated oESCs and differentiated organoids, indicating substantial transcriptional remodeling during differentiation (Figure 7a). Differential gene expression analysis showed that the number of DEGs gradually increased during differentiation, peaking at day 14 with a twofold increase compared to day 7, relative to day 0 ESCs (Figure 7b).

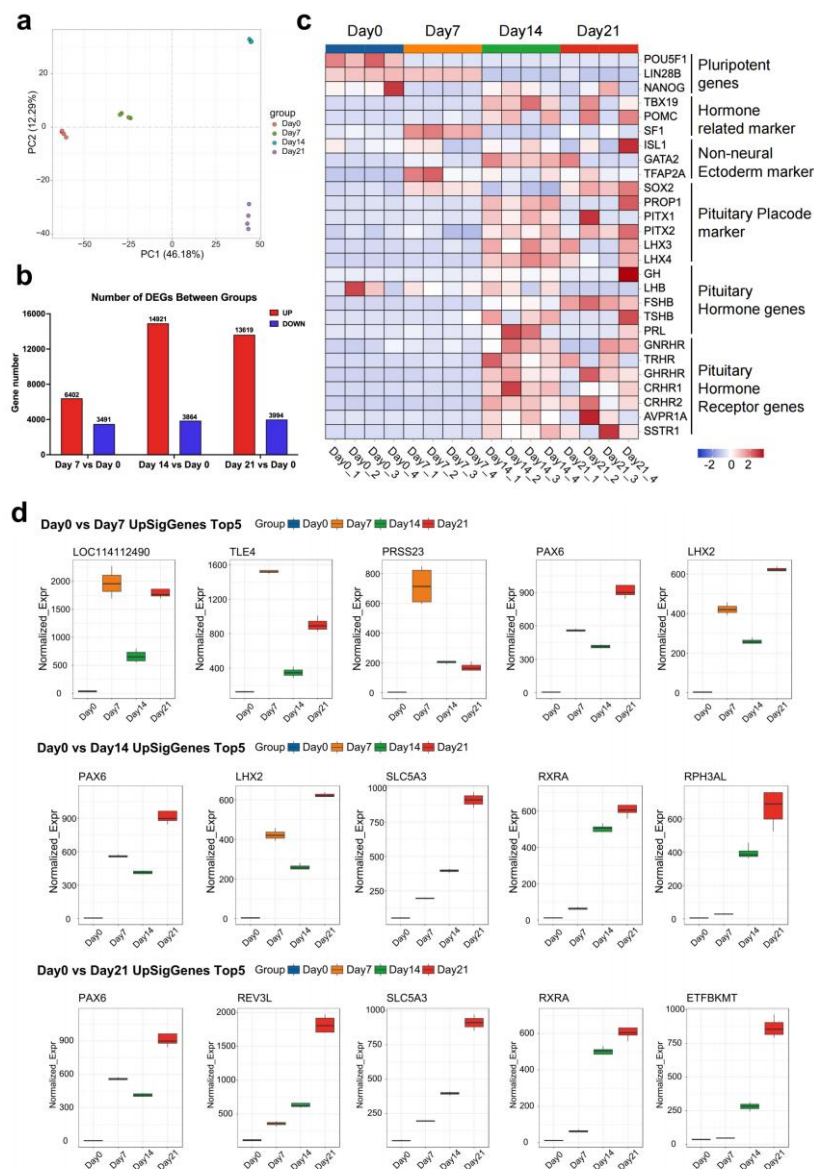


Figure 7. SMART-Seq Method for Single-Cell Transcriptomic Analysis of pituitary-like organoid Differentiation. (a): PCA analysis of differentiation aggregates on Days 0, 7, 14, and 21. (b): Bar chart of the number of differentially expressed genes (DEGs) between groups. (c): Heatmap showing the expression of genes that changed between Days 0, 7, 14, and 21. Each vertical column represents an individual organoid sample ($n = 4$ per time point). Red indicates the maximum value within a row, while blue represents the minimum value. (d): Boxplot showing TOP5 significantly up-regulated genes at different time points (Days 7, 14, and 21) relative to Day 0.

Transcriptomic analysis revealed a progressive decline in pluripotency-associated genes, including POU5F1 and LIN28, from Day 0 to Day 21. In contrast, the expression of pituitary-related genes, including non-neural ectoderm markers (ISL1 and GATA2) and pituitary placode markers (SOX2, PITX1, PITX2, LHX3, and LHX4), increased progres-

sively during differentiation. Furthermore, the expression of pituitary hormone genes, including GH, FSHB, TSHB, and PRL, was significantly upregulated after differentiation, although LHB expression did not show a marked increase. Additionally, hormone receptor genes expressed in pituitary tissues, such as GNRHR, TRHR, GHRHR, and CRHR1/2, were also upregulated following differentiation. Collectively, these results indicate that we have successfully induced oESCs toward a pituitary tissue identity (Figure 7c),.

Analysis of the most significantly upregulated genes at each time point revealed dynamic transcriptional changes throughout differentiation (Figure 7d). Among these genes, PAX6 was consistently upregulated from Day 7 to Day 21, while LHX2 was significantly induced at Days 7 and 14 but became undetectable by Day 21. These findings are consistent with the progressive activation of developmental programs associated with pituitary organogenesis and lineage specification.

4. Discussion

Our research successfully generated 3D ovine pituitary-like organoids, representing the first report of such a system in a large animal model and addressing a significant gap in the field. Previous studies have successfully induced and differentiated pituitary like organoids in vitro, utilizing sources that can be categorized into three groups: ESCs [22,28], iPSCs [24,29] and pituitary stem cells [28]. However, these studies have predominantly focused on humans and mice, with limited attention on large livestock. The importance of this study is underscored by the fact that ovine are major agricultural animals, and our oESC-derived pituitary organoids, which exhibit GnRH-responsive gonadotroph activity, provide a valuable in vitro platform for studying reproductive endocrine regulation and other pituitary-mediated endocrine functions in ovine and other large animals.

During early embryonic development, Rathke's pouch arises from the ectoderm and serves as the precursor to the anterior pituitary gland. Additionally, the neurohypophysis develops from the neuroectoderm [30,31]. Consistent with the previous studies [22,32], we have successfully recapitulated the formation of Rathke's pouch in vitro with the use of BMP4 and SAG (SHH agnotion) to induce the formation of non-neuroectoderm (oral ectodermal) and neuroectoderm, respectively. The Sonic Hedgehog (SHH) signaling pathway is crucial for initiating the adenohypophyseal placode and promoting the alternative adenohypophyseal fate. SHH remains active in the oral ectoderm before Rathke's pouch forms [33], further contributing to our understanding of ovine pituitary development. Inspired by the establishment of pituitary organoid models derived from human pluripotent stem cells, we optimized our protocol by incorporating the TGF β signaling pathway inhibitor SB431542 and the Wnt signaling pathway inhibitor IWP2 throughout the culture process. These inhibitors were used to suppress mesoderm development and promote non-neural ectodermal differentiation [24]. We also conducted a comparative analysis between 3D spheroids and the pituitary glands of ovines at days 90 and 120 of the embryonic phase. Notably, POMC expression was upregulated at the later time point but remained significantly lower than the expression levels in actual ovine pituitary glands. This observation is consistent with the results of pituitary lineage differentiation induced by human iPSCs [15]. Pituitary organoids derived from ESCs differentiate more simply than those from pituitary stem cells [28], eliminating the need for fluorescent-activated cell sorting and reducing dependence on live animals, thereby reducing experimental costs. Notably, on Day 21 of differentiation, PAX6 was the most significantly upregulated gene compared to undifferentiated oESCs. As a transcription factor crucial to pituitary development [34], and an early marker of both the anterior pituitary [35] and Rathke's pouch [36], the continuous significant upregulation of the PAX6 gene indicates that our differentiated organoids are progressively adopting an adenohypophysis fate rather than posterior pituitary, which is consistent with the results of embryonic development induced by human ESCs

[37]. And we also note that our protocol yields a similar efficiency in terms of placode marker expression (e.g., PITX1 and LHX3) and the formation of Rathke's pouch-like structures, which is consistent with published studies [23]. Furthermore, our transcriptomic data also demonstrated that the expression of pituitary hormone genes was significantly upregulated after differentiation, with the exception of LHB, which showed no marked change. This may be attributed to the limited maturity of the organoids within the 21-day differentiation window, as well as the potential requirement for additional inductive signals or extended culture duration to achieve full maturation of gonadotroph cells. Indeed, brief stimulation with GnRH was sufficient to upregulate LHB expression in the organoids, suggesting that the cells have acquired the capacity to respond to hormonal cues, yet their full functional maturation may require a longer or more refined differentiation environment. Our transcriptome results also indicate the presence of heterogeneity during the differentiation process of organoids. Since three-dimensional culture itself has the ability to induce heterogeneous cell populations, it is challenging to maintain a constant differentiation efficiency [38]. Additionally, single-cell RNA sequencing further reveals the diverse cell clusters, complex cell–cell interactions, and spatial information within organoids [14]. GH was only detected with significantly high expression in one of the four organoid replicates on day 21, which may reflect the differences in the maturity levels among individual organoids. Moreover, previous studies have shown that the expression of GH, PIT1, and ACTH only appears in certain cells after 100 days of culture [29]. Extending the culture time of our organoids beyond 21 days may lead to more robust and consistent hormone expression. Previous research has demonstrated that pituitary organoids can be maintained in extended culture. The differentiation of corticotropes becomes obvious at 70 days of culture [39]. Importantly, pituitary cell aggregates can be maintained in culture for several months without losing the representativeness and functionality of tissues and cell types, and the cells within pituitary-like spheroids remain viable during the 90-day differentiation process [15]. These findings suggest that our organoids are likely to remain viable beyond the current 21-day time point, which requires further investigation in our future work. Previous studies utilizing primary pituitary cells isolated in vitro have been constrained by the cells' limited proliferative capacity and short-lived hormone secretion. Consequently, these have been largely replaced by immortalized pituitary cell lines, such as the mouse gonadotrope cell lines α T3-1 and L β T2 [40,41], and rat pituitary adenoma-derived cells like GH, MMQ, RC-4B/C, as well as human pituitary adenoma-derived HP75, PDFS, HPA, and GX cells [42]. However, a stable pituitary cell line for livestock species, including pigs, cattle, and ovine, remains elusive, thereby limiting research in these areas. In this study, we induced differentiation of oESCs in a 2D culture system, leading to the generation of cells expressing pituitary progenitor markers. However, these cells were not viable for extended culture periods due to a tendency to detach prematurely. In contrast, 3D culture conditions were more conducive to long-term differentiation and the maturation into pituitary organoids.

Traditional endocrine research typically relies on animal models or primary pituitary cells; however, organoid models offer a viable alternative. These models not only mitigate ethical controversies but also enhance the relevance of research findings. Examination of the functions of pituitary organoids can enhance existing reproductive technologies, such as artificial insemination (AI) and in vitro fertilization (IVF), thereby improving reproductive efficiency. The structure and function of the ovine pituitary gland closely resemble those of the human pituitary gland. Therefore, constructing pituitary organoids enables the development of more accurate disease models, facilitating the study of disorders related to pituitary dysfunction, including pituitary tumors and hormonal secretion disorders.

While we describe a method for the directed differentiation of oESCs into pituitary cell lineages using specific extrinsic signaling molecules, and successfully direct oESCs

commitment toward pituitary progenitor identities, the organoids generated within the 21-day differentiation window likely represent an early developmental stage, and whether they can give rise to fully mature hormone-secreting cell types—such as somatotropes, lactotropes, or thyrotropes—under extended culture remains to be determined. Furthermore, a more comprehensive molecular and functional comparison with native ovine pituitary tissue will be essential to assess the fidelity of our system in recapitulating *in vivo* development. Future efforts directed at optimizing long-term culture conditions, incorporating single-cell resolution analyses, and establishing functional readouts for multiple hormonal axes will be necessary to fully realize the potential of this platform for studying pituitary biology and endocrine regulation in large animals.

5. Conclusions

In this study we successfully generated ovine pituitary-like organoids *in vitro* from oESCs. The resulting organoids formed Rathke's pouch-like structures and expressed key pituitary progenitor markers, including LHX3, PITX1, ISLET1, PROP1, and E-cadherin. Compared with conventional two-dimensional cultures, the three-dimensional culture system provided enhanced structural stability and sustained differentiation. Importantly, the oESC-derived organoids exhibited functional responsiveness to GnRH stimulation, indicating the acquisition of gonadotroph-like endocrine activity. Collectively, our findings established a valuable large-animal platform for studying pituitary development and reproductive endocrine regulation.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cells15151360/s1>, Figure S1: Preliminary experiments for screening the initial number of seeded cells used to initiate organoid formation in 3D culture; Figure S2: Original Western blot Images for Figure 3b: Expression of GAPDH, SOX2, and E-cadherin.

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Institutional Review Board Statement: All animal experiments were approved by the Animal Care and Use Committee of Nanjing Agricultural University, China (Approval date: 10 June 2022; Approval ID: SYXK2022-0031; the ethics committee is the Department of Science and Technology of Jiangsu Province), and the experimental methods in our study were conducted in accordance with the approval of the Animal Experiments of Nanjing Agricultural University, China.

Data Availability Statement: The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

oESCs	ovine embryonic stem cells
GH	growth hormone
PRL	Prolactin
FSH	follicle-stimulating hormone
LH	luteinizing hormone
2D	two-dimensional
iPSCs	induced pluripotent stem cells
MEFs	mouse embryonic fibroblasts
EPSC	expanded potential stem cells
3D	three-dimensional
EBs	embryoid bodies
GnRH	gonadotropin-releasing hormone
GSEA	Gene set enrichment analysis

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