Laidlaw Research Report

Research Topic: Investigating the Development of a Chimeric Human Gastruloid from a Mixed Aggregate of Extraembryonic Endoderm and Epiblast Cells

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Abstract

A gastruloid is a model of human embryonic development during gastrulation used to study developmental disorders, tissue organization, and drug screening. It is developed from an aggregate of human Expanded Pluripotent Stem Cells (EPSCs) that undergo differentiation, self-organization, symmetry breaking, and body axis establishment. Mapping pattern formation is fundamental to studying genetic interventions in gastruloid models. Despite recent progress, major challenges remain because of high variation within and across batches, which leads to low reproducibility. Introducing extraembryonic endoderm cells (XEN) could preserve anterior pole structure and enhance neural development in a mouse model.[4] This approach offers a promising strategy to generate robust human gastruloids for downstream study on neurodevelopmental disorders. This research investigates the development of chimeric human gastruloids by introducing different levels of XEN to pre-differentiated human EPSCs in the epiblast lineage. Qualitative analysis methods were performed to map out gastruloid structures. Hereby, the immunofluorescence staining of day 8 gastruloids showed the development of multiple yolk sac structures extending from the amniotic cavity and multiple notochords developing within the yolk sac region. The nucleofection of TCF and CSL plasmids also demonstrated the presence of Wnt/β-catenin signaling, but no Notch signaling was observed.

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1. Introduction

1.1. Gastruloid model

1.1.1. Human embryonic development

In humans, after fertilization, cells divide without increasing in size during the blastula stage. This gives rise to a blastocyst, a hollow sphere in which the outer layer develops into the trophectoderm and the inner cell cluster develops into the inner cell mass (ICM). The trophectoderm facilitates implantation and contributes to the formation of the placenta and chorion, which supply nutrients to the embryo, whereas the ICM segregates into two specialized lineages: the hypoblast and the epiblast (EPI). The hypoblast differentiates into visceral endoderm, which remains intact with the epiblast and forms cuboidal epithelium, and parietal endoderm, which forms an inner lining of the trophoblast. Both of them contribute to the formation of the primary yolk sac. (Ross & Boroviak, 2020) The epiblast subsequently undergoes cavitation to form the amniotic cavity, with the embryonic portion organizing into a bilaminar disc and the extra-embryonic portion forming the amniotic ectoderm. (Lin et al., 2026)

During gastrulation, the embryo produces three germ layers: endoderm, mesoderm, and ectoderm. The process begins at the primitive streak of the single-layered epiblast and is driven by the Nodal and Wnt signaling pathways located at the posterior end. Anterior-posterior (A-P) axis establishment occurs with the contribution of antagonistic signals from anterior visceral endoderm (AVE). AVE is a subpopulation of visceral endoderm, which is specified prior to the primitive streak formation. It secretes NODAL inhibitors, CER1 and LEFTY1; and WNT3 inhibitors, DKK1, SFRP1 and SFRP5. (Ross & Boroviak, 2020) This restricts gastrulation to the posterior pole and preserves anterior structures in the mammalian embryo. As epiblast cells migrate inward through the primitive streak via epithelial-to-mesenchymal transition (EMT), they produce the endodermal and mesodermal layers. The cells remaining on the surface layer form the ectoderm, which subsequently undergoes neural induction to form the neural tube and develop into the central nervous system.

1.1.2. Gastruloid as a platform to study embryonic development *in vitro*

In the past, studying early lineage decisions, genetic defects, and phenotypic development in human embryos was severely restricted by technical and ethical challenges. The development of stem cell-derived *in vitro* models, such as gastruloid, has provided researchers with scalable, controllable platforms to study early developmental events in isolation. Gastruloids partly mimic the gastrulation stage of human embryonic development. A conventional gastruloid is

a three-dimensional aggregate of pluripotent stem cells (PSCs) that self-organizes, proliferates, and undergoes symmetry breaking and specification into the three germ layers under appropriate culture conditions. (Arias et al., 2022) Because these standard gastruloids are typically derived from embryonic stem cells in the absence of extraembryonic counterparts, they robustly recapitulate posterior gastrulation and early organogenesis but lack the extraembryonic lineages to form the yolk sac and fail to develop anterior head and brain structures. A defined number of initial PSCs self-organizes into a spherical structure in AggreWell, and engages in cell-cell communication and signaling crosstalk to produce distinct cell lineages. The absence of maternal cues is compensated by precise biochemical supplementation. Ultimately, this allows gastruloids to develop mature structures and establish multi-axial patterning.

1.1.3. Challenges in gastruloid formation

Gastruloid formation is highly variable and difficult to control, even under the same experimental protocol and the same EPSC cell lines. Gastruloid structures were found to be inconsistent within and between batches; for example, some did not develop mature morphology, such as a yolk sac, or establish body axes. This necessitates an approach to optimize gastruloid formation and better understand the mechanisms that drive embryonic development. Note that gastruloid models also vary widely depending on which aspects of embryos are being modeled; thus, the suitable model also depends on the questions being explored. (Arias et al., 2022)

One challenge is the development of anterior and neural structures in gastruloids. Standard gastruloid culture medium contains Chiron that acts as a Wnt agonist and induces symmetry breaking. In cell culture, a spherical cell aggregate is equally exposed to this external cue, but cell fate specification varies with the distance molecules diffuse into the center of the cell aggregate. As a result, hEPSCs can self-organize into an embryoid body (EB) and specify its anterior-posterior axis without extraembryonic cells. However, prolonged exposure to Chiron can hinder anterior development. (Turner et al., 2017)

1.1.4. Mixed aggregate of extraembryonic endoderm and epiblast cells

Extraembryonic endoderm (XEN) cells can function as a signaling domain that antagonizes the effects of Wnt and Nodal signaling from the posterior end. This provides developmental potential for anterior structures and controls gastruloid A-P axis organization. Bérenger-Currias et al. (2022) showed that XEN-enhanced gastruloids (XEGs) of mouse embryonic stem cells (mESCs) and XEN cells exhibit the formation of neural epithelia with an optimal ratio of 3:1 mESC: XEN. They also showed that most XEN cells adopt a visceral endoderm-like state

due to co-differentiation with mESCs, which can inhibit local WNT signaling. This suggests that cells of different types can form a mixed aggregate and interact to develop a more robust gastruloid model. It is hypothesized that applying this approach to human XEN and EPI cells will allow gastruloids to develop more mature neural characteristics and anterior structure. A modified FTW medium, which contains components that activate the FGF, TGF-β, and WNT pathways, is utilized to generate a population of XEN from hEPSCs. (Wei et al., 2023)

1.2. Signaling pathway in the early stage of embryonic development

Most current knowledge about mammalian gastruloids is based on mouse models. However, key differences exist between murine and human gastrulation in both structure and gene activity due to their evolutionary divergence. (Lin et al., 2026) Further investigation of signaling patterns in the human gastruloid model is necessary to support its use as a platform for studying developmental diseases.

1.2.1. Notch signaling pathway and 12xCSL-d1EGFP reporter plasmid

Notch signaling plays an important role in cell-fate determination during embryonic development. It can be activated in two ways: ligand-dependent and ligand-independent. For ligand-dependent activation, Notch ligands (JAG1/2 and DLL1/3/4) bind to Notch receptors (Notch1/2/3/4) on the cell membrane. This interaction generates a pulling force that extends the negative regulatory region (NRR), allowing ADAM to cleave the receptor at the S2 and S3 sites and release the Notch intracellular domain (ICD). Notch ICD either remains in the cytoplasm or is translocated into the nucleus. In the cytoplasm, it interacts with other pathways, such as NFkB, mTORC2, AKT, Hippo, and Wnt. In the nucleus, it binds the DNA-binding protein CSL, which was initially bound to corepressors to inhibit the transcription of target genes. Their interaction releases corepressors, recruits mastermind-like proteins (MAMLs) and coactivators, and promotes the transcription of NOTCH target genes. (Shi et al., 2024) For ligand-independent activation, Notch signaling can be triggered by the chelating agent ethylenediaminetetraacetic acid (EDTA). EDTA diffuses into cells and removes Ca^{2+} from the Lin-12/Notch Repeat (LNR) domain of the NRR, exposing the structure to ADAM17, which cleaves the S2 site. Subsequent cleavage at the S3 site releases Notch ICD. (Rand et al., 2000)

In the embryo, Notch signaling is active in many processes during embryogenesis, including neurogenesis, cardiogenesis, and hematopoiesis. (Zhou et al., 2022) For example, during definitive hematopoiesis, it helps generate hematopoietic stem cells. (Ditadi et al., 2015) Hemogenic endothelium (HE) requires Notch signaling to drive endothelial-to-hematopoietic transition (EHT) and

produce myeloid, erythroid, and lymphoid progeny *in vitro*. $CD34^+$ hemogenic endothelial cells are present in gastruloids cultured under cardiovascular-inducing conditions. (Rossi et al., 2022) They are found within a vascular-like network marked by $SOX17^+$ and give rise to CD41 hematopoietic precursors. It is hypothesized that Notch signaling can be exploited to study gastruloid development by using the 12xCSL-d1EGFP reporter plasmid.

2. Methodology

2.1. Plasmid validation

2.1.1. Bacterial Transformation and Maxi-prep

Plasmids were introduced to *E. coli* by heat-shock transformation. *E coli.* mixture was added to an ampicillin-coated agar plate and cultured overnight at 37°C. The next day, a single colony was collected using a P200 pipette tip, and the tip was ejected into an Erlenmeyer flask containing 200 mL LB medium with 1% ampicillin, and cultured overnight in a shaking incubator at 220 rpm. Finally, amplified plasmids were extracted from bacterial solution using the Maxi Prep kit and stored at -20°C.

2.1.2. Validation of 12XCSL-d1EGFP plasmid

HEK293T cells were transfected using PEI under four different conditions. Because 293T cells are known to express low endogenous Notch signaling, the first condition was co-transfection of DLL4 and CSL plasmid. To induce DLL4 expression, cells were treated with doxycycline (dox) 8 hours after PEI transfection. The second culture was co-culture of CSL-expressing and DLL4-expressing cells. 293T cells were transfected separately and mixed together at 24 hours post-transfection. This condition is designed to prevent cis-inhibition, which can occur when DLL4-expressing cells bind to their own Notch receptor. The third condition induced Notch signaling by chemical activation: add 0.5 mM EDTA in PBS, then incubate for 15 minutes at 37°C.

2.2. EPSC culture and gastruloid formation

2.2.1. EPSC cell culture and *in vitro* differentiation

EM3 hEPSCs were cultured on feeder-coated plates in EPSC medium and kept in a humidified incubator at 37°C, 5% carbon dioxide (CO_2). The medium was changed daily, and colony morphologies were observed under a light microscope. Cells were passaged when the confluency reached 70%.

To perform *in vitro* differentiation, EPSCs were cultured until they reached 30-50% confluency and switched to *in vitro* differentiation media under two conditions. The first condition used DFK medium, a 1:1 mixture of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) and Keratinocyte Serum-Free Medium (KSFM), to generate epiblast-primed cells (EPI). The second condition used FTW medium to generate extra-embryonic-primed cells (XEN). They were cultured for another 2 days on feeder plates, during which colony morphologies gradually changed.

2.2.2. Embryoid (EB) formation

Pre-differentiated EPSCs were collected from the plate by trypsinization, gentle pipetting, and centrifugation at 300g for 3 minutes to collect a cell pellet. Cells were resuspended in tHDM medium supplemented with 0.1% Y-27632 and counted for cell density by hemocytometer. The initial seeding number was 10,000 cells per round-bottom Aggrewell. Cells were diluted to the desired amount as described in Table 1 in 200 µl tHDM and mixed by gentle pipetting to obtain a homogeneous single-cell suspension. They were cultured in a humidified incubator at 37°C in 5% carbon dioxide (CO_2) without changing medium.

Table 1. Conditions for forming a mixed aggregate of EPI and XEN cells.

Condition (EPI: XEN)	Approximate number of cells per aggregate	
	EPI	XEN
9:1	9,000	1,000
3:1	7,500	2,500

2.2.3. Gastruloid Formation

On day 3 after seeding cell aggregates, EBs were collected from Aggrewells and transferred to a tissue culture plate in gastruloid medium A. The plate was kept in a shaking incubator at 37 °C with 5% carbon dioxide (CO_2). After 3 days, the medium was changed to gastruloid medium B. Gastruloids were observed daily under a microscope. On day 8, gastruloids with good morphology were selected for nucleofection and immunofluorescence staining.

2.2.4. Nucleofection

Gastruloids were collected and transferred to a microtube containing PBS. Nucleofector solution was prepared according to the P3 Primary Cell 4D-Nucleofector® X Kit S protocol. 1 µg of reporter plasmid was added per 10 µL nucleofector solution. In a microtube containing 4-5 gastruloids, PBS was removed and substituted with 40 µL nucleofector solution before transferring all gastruloids to a Nucleocuvette® Strip. The nucleofection program for h9 cells was selected. After completion, gastruloids were transferred back to a 6-well plate and observed for reporter signals at 24 hours.

3. Results

3.1. Validation of 12XCSL-d1EGFP plasmid

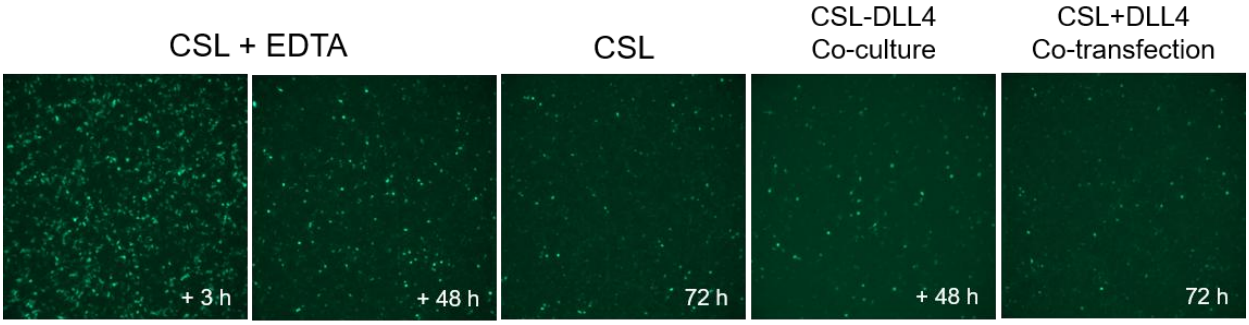

Figure 1. GFP signals in HEK293T cells under four conditions.
Note. + sign indicates the time after EDTA treatment in the CSL+EDTA condition and after mixing CSL and DLL4-expressing cells in the CSL-DLL4 co-culture condition.

The strongest GFP signal was observed at 3 h post-EDTA treatment and weakened by 48 h. CSL-expressing and CSL+DLL4 co-culture conditions showed similar GFP expression levels, and CSL-DLL4 co-transfection showed the weakest GFP signal. This suggests that naïve Notch signaling in 293T cells is sufficient to induce plasmid expression without additional stimulation from DLL4-expressing cells or EDTA.

3.2. Gastruloid formation

Multiple lobes were observed in chimeric gastruloids, in line with the early observation of multiple protrusions or dark spots at EB stage.

3.2.1. EPSC colony

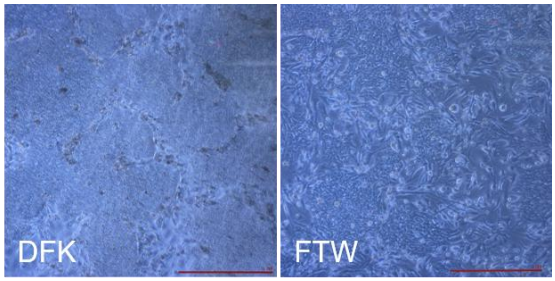

Figure 2. Pre-differentiated EPSCs in DFK and FTW media.

After *in vitro* differentiation, EPSCs showed differentiated morphology with distinct features. In DFK medium, which primes cells to the epiblast lineage, EPSCs grew into dense, flat colonies with small nuclei, remaining in a pluripotency state. In FTW medium, which primes cells to the extraembryonic endoderm lineage, EPSCs grew into split colonies with clear individual cells and nuclei.

3.2.2. Embryoid formation and development

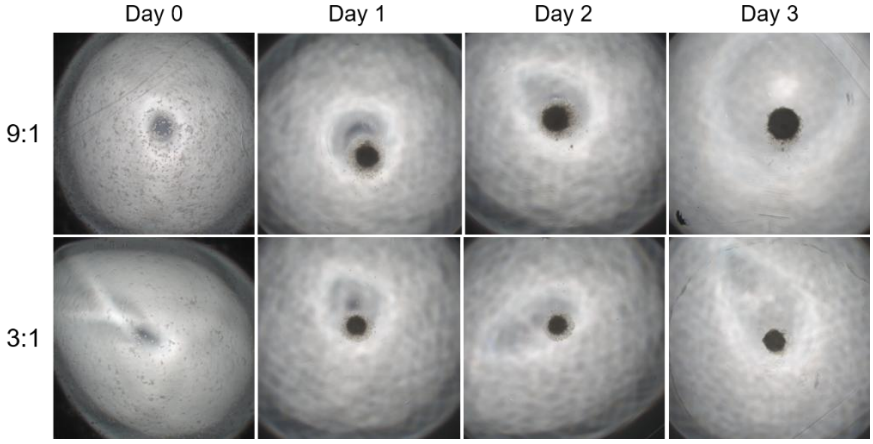

Figure 3. Day 0-3 embryoids from 9:1 and 3:1 conditions.

A mix of epiblast (EPI) and extraembryonic endoderm (XEN) cells successfully aggregated into a spherical embryoid (EB) with a dense core. Each mixed aggregate was surrounded by a dispersed cell layer, potentially remnants of feeder cells that were not fully dissociated. EB structures were consistent across conditions. Initial seeding density was expected to be approximately 10,000 cells per aggregate. However, aggregates in the 3:1 condition had lower cell density

and were smaller than those in the 9:1 condition, which may have resulted from an error in cell counting or aggregate seeding.

3.2.3. Gastruloid development

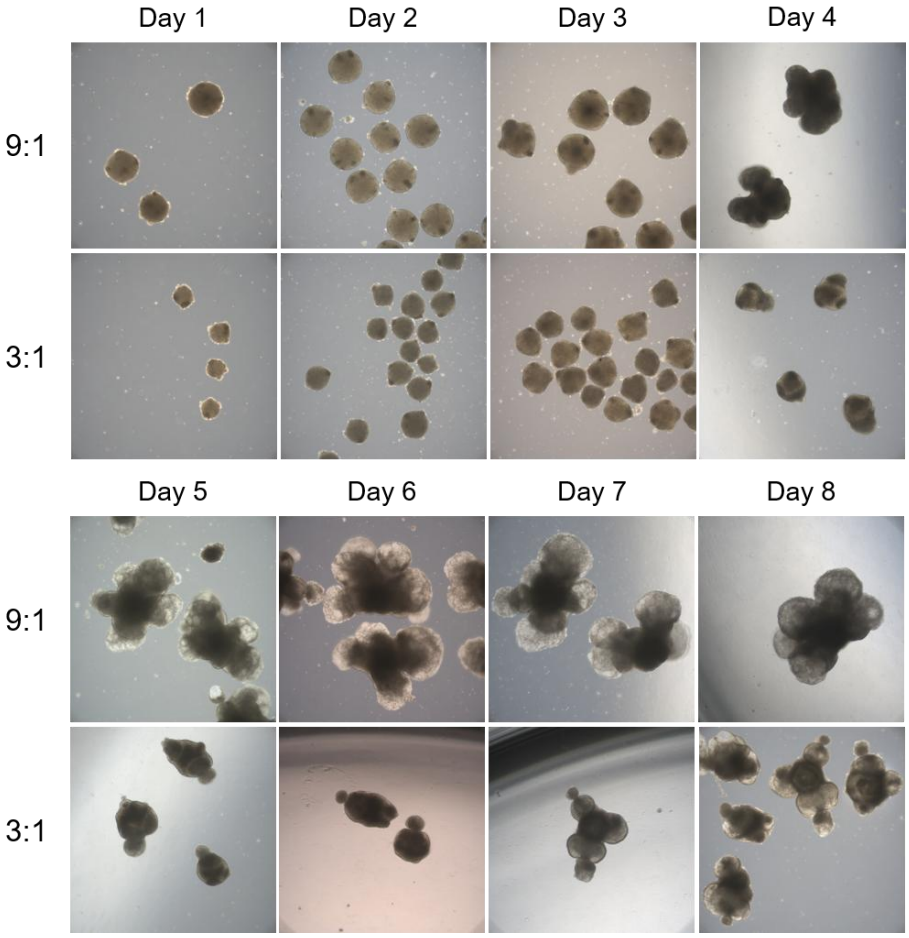

Figure 4. Day 1-8 gastruloids from 9:1 and 3:1 conditions. Gastruloids were supplemented with medium A on days 1-3 and with medium B from day 4 onwards.

On days 1-3, several dark spots appeared on the spherical aggregates and protruded, as clearly observed on day 3. After the medium change, gastruloids grew larger and developed into a structure with multiple lobes (9:1 gastruloids on day 4 and 3:1 gastruloids on days 4-6). Lobes extended in different directions from the core structure. A yolk sac structure became morphologically visible on day 5 in 9:1 gastruloids and later on day 7 in 3:1 gastruloids. The number of developing yolk sacs varied among gastruloids, ranging from 2 to more than four. Although no absolute conclusion could yet be drawn about the differences between the two conditions, 9:1 gastruloids showed a more defined yolk sac, with each one separated from the others. In contrast, most 3:1 gastruloids had fused yolk sacs and varied significantly in size.

3.2.4. Immunofluorescence staining

3.2.4.1. PODXL, SOX2, and SOX17 for amniotic and yolk sac structure

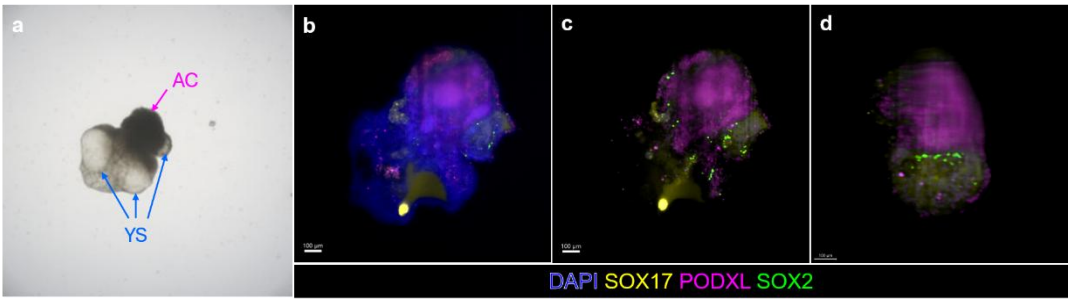

Figure 5. Yolk sac and amniotic cavity of day 8 3:1 gastruloid. **a** Bright-field image with blue arrows pointing to the yolk sac (YS) and a pink arrow pointing to the amniotic cavity (AC). Immunofluorescence staining of SOX2 (green), SOX17 (yellow), and PODXL (magenta): **b** 3D image and **c-d** cross-sectional images, showing three yolk sac compartments (c) and epiblast cells (SOX2) between AC (PODXL) and YS (SOX17) (d).

The expression of PODXL and SOX17 in distinct regions supported that gastruloids developed an amniotic cavity and multiple yolk sacs. $SOX2^+$ expression was found in the region between the amniotic cavity and the yolk sac, indicating epiblast-like cells (Fig. 5d).

3.2.4.2. T, FOXA2, and SOX2 for notochord and neuroectoderm structure

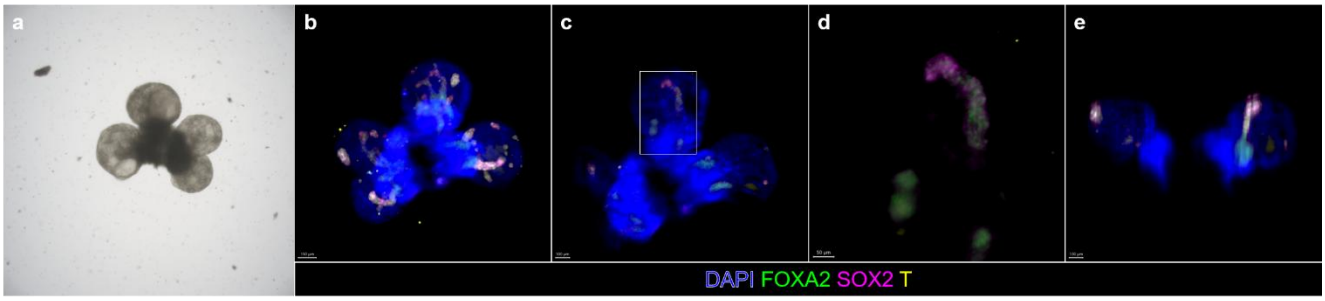

Figure 6. Notochord structure of day 8 9:1 gastruloid. **a** Bright-field image shows four yolk sacs. Immunofluorescence staining of FOXA2 (green), SOX2 (pink), and T (yellow): **b** 3D image and **c-e** cross-sectional images: multiple developing notochords in yolk sacs marked by co-localization of T/FOXA2 (c); zoomed in on the white box area (d); and a developing notochord inside the yolk sac structure (e).

The result showed multiple notochords (T^+/$FOXA2^+$) developed within the yolk sacs (Fig. 6b,e). This observation requires further investigation to explain why notochords develop inside the yolk sac and how larger aggregate sizes result in multiple notochords. Additionally, the localization of $SOX2^+$ with notochords is not ideal (Fig. 6b-e) based on the fact that $SOX2^+$ is a marker of neuroectoderm and thus should be present in a different region.

3.2.4.3. PAX7, Olig2, NKX6.1 for dorsoventral neural tube patterning

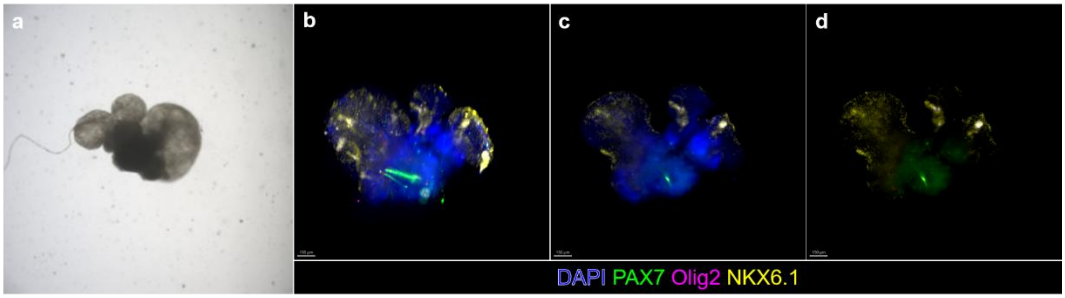

Figure 7. Staining for dorsoventral neural tube patterning in day 8 9:1 gastruloid. **a** Bright-field image shows three yolk sacs. Immunofluorescence staining of PAX7 (green), Olig2 (pink), and NKX6.1 (yellow): **b** 3D image and **c-e** cross-sectional images show co-localization of Olig2/NKX6.1 around yolk sac structure and PAX7 at the core structure.

The dorsoventral neural tube patterning staining (Fig. 7) is not sufficient to explain chimeric gastruloid development. Ventral neural progenitors ($Olig2^+/NKX6.1^+$) and dorsal neural progenitors ($PAX7^+$) are expected to form as cell layers, but based on the observations, they were found in different regions of the amniotic cavity and yolk sac structures.

3.2.5. Signals from nucleofection of CSL and TCF plasmids

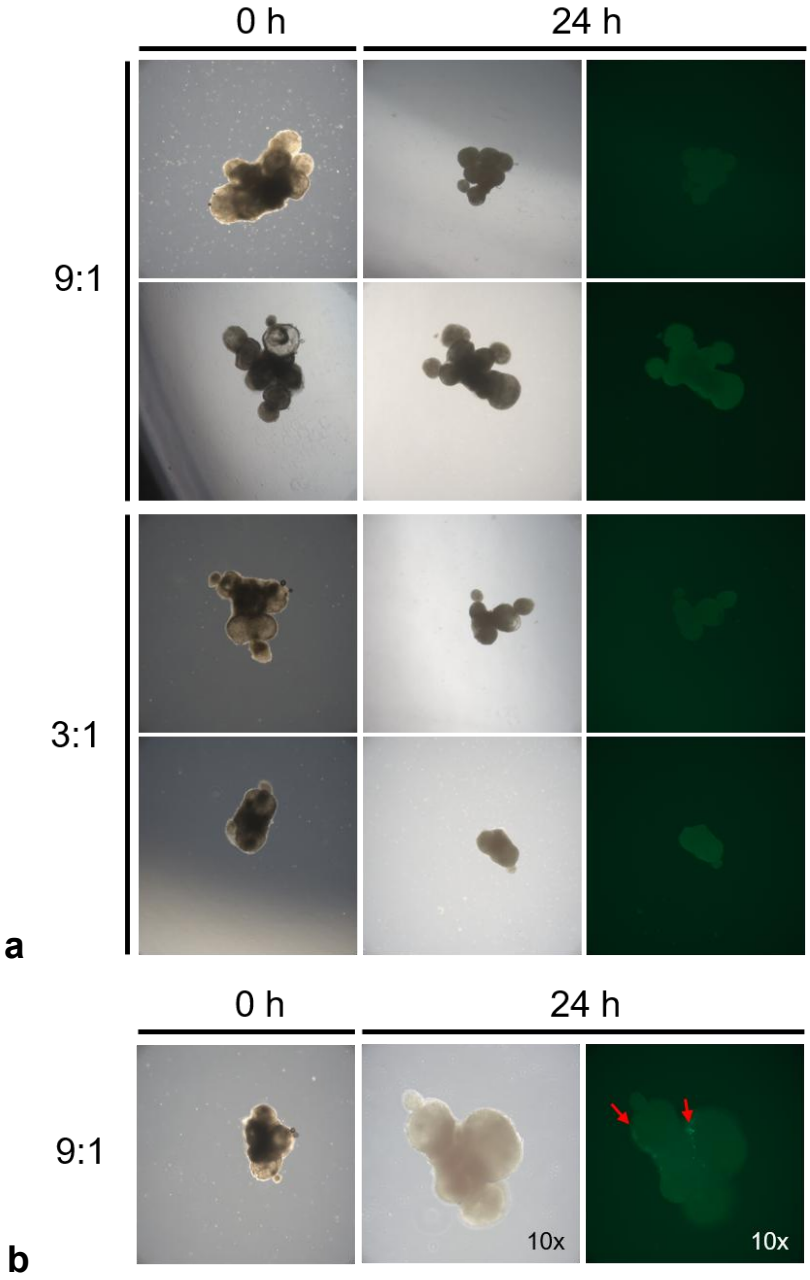

Figure 8. Gastruloids after nucleofection of **a** CSL and **b** TCF reporter plasmids, showing bright-field images at 0 and 24 h and GFP signals at 24 h post-transfection.

No GFP signal from the CSL plasmid was observed in any gastruloids. The plasmid may not be diffused deeply enough to reach regions that express Notch signaling, such as hemogenic endothelium. However, a weak GFP signal from the TCF plasmid was observed, indicating the presence of Wnt/β-catenin signaling near the boundary between the amniotic cavity and the yolk sac.

4. Discussion

In validating 12xCSL-d1EGFP plasmid functionality, the plasmid can be transfected directly into HEK293T cells without additional steps to address cis-inhibition or enrich endogenous Notch signaling. Chemical activation with EDTA can enhance Notch signaling in a ligand-independent manner. Co-culture can minimize cis-inhibition, which usually occurs under co-transfection when cells expressing DLL4 inhibit their own Notch signaling pathway.

In a generation of mixed aggregates, we observed an increasing number of lobes with aggregate size. This supports a Turing patterning mechanism, which describes how the number of periodic patterns scales proportionally with tissue length. (Ishihara et al., 2018) After switching to media A and B, gastruloids seemed to develop clear yolk sacs and core structure under a bright-field microscope; nevertheless, the immunofluorescence staining of notochord and dorsoventral patterning doesn't align with normal developmental patterning, especially with multiple notochords in yolk sac structures and an unusual distribution of dorsal and ventral neural progenitors. Therefore, further experiments are needed to determine whether these results were due to a technical error or the real influence of XEN cells, since XEN cells can serve as a signaling domain, releasing molecules that alter developmental patterning from wild-type gastruloids.

Nucleofection of reporter plasmids aims to track signaling pathways present in gastruloids. The GFP signal from the TCF plasmid was observed, indicating the presence of Wnt/β-catenin signaling (Fig. 8b), but no signal was observed from the CSL plasmid, indicating the lack of Notch signaling in transfected cells. The nucleofection method can introduce plasmids into gastruloid structures but is limited to the outer region. Thus, this method is not suitable for studying all signaling pathways but is sufficient to provide evidence for pathways expressed at the margin of the gastruloid structure.

The effects of introducing XEN at different levels on gastruloid development remain inconclusive due to the differences in initial seeding cell numbers between the 9:1 and 3:1 conditions. The aggregate size significantly affects signaling dynamics and gastruloid axis formation. (Fiuza et al., 2025) As observed in Fig. 3-4, larger gastruloids in the 9:1 condition started to develop yolk sacs earlier on day 5, whereas smaller gastruloids in the 3:1 condition showed an elongated pattern on day 5-6 before developing a yolk sac from day 7. Because a high seeding density (10,000 cells/aggregate) was chosen for the experiment, this can result in multiple axes and multiple notochords.

5. Limitations and suggestions for future research

Overall, forming a chimeric human gastruloid from EPI and XEN cells offers valuable insights into tissue patterning and warrants further investigation; it showed significant deviations from wild-type human gastruloids with multiple notochords inside yolk sacs. There were several limitations that should be addressed. First, we unfortunately had a media shortage during the experiment, so a control group of gastruloids formed from EPI-lineage only was excluded. It is strongly recommended that a control group be included in future experiments to enable more accurate comparisons between conditions and clarify the role of XEN. Second, the technical error with the initial seeding density precludes the comparison between 9:1 and 3:1 conditions. The seeding density should be made consistent across conditions, and different aggregate sizes can also be tested to study how they affect pattern formation.

Building on this preliminary study, a plasmid reporter can be integrated into XEN cells to help distinguish XEN from EPI cells. Thus, their influence on gastruloid patterning can be better understood based on their localization. Furthermore, quantitative analysis should be carried out to elucidate the findings, for example, using single-cell RNA analysis to identify specific cell populations.

6. Acknowledgment

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