Investigating the Development of Chimeric Human Gastruloids from a Mixed Aggregate of Extraembryonic Endoderm and Epiblast Cells

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Introduction

Gastruloids recapitulate human embryonic development during gastrulation and serve as a valuable platform for studying developmental disorders, tissue organization, and drug screening. They are derived from an aggregate of human Expanded Pluripotent Stem Cells (EPSCs) that undergoes differentiation, self-organization, symmetry breaking, and body axis establishment. However, current models suffer from high morphological variation within and across culture batches, resulting in low reproducibility. Previous study in mouse models demonstrated that introducing extraembryonic endoderm cells (XEN) can maintain anterior structure and enhance neural development.[1] Here, we investigate the development of chimeric human gastruloids by co-aggregating different proportions of pre-differentiated XEN with epiblast(EPI)-lineage human EPSCs. This research serves as a preliminary study to produce more robust human gastruloids for downstream neurodevelopmental disorders research.

Objective

1. To validate 12xCSL-d1EGFP plasmid functionality in HEK293T cells
2. To investigate the effect of varying EPI-to-XEN proportions on gastruloid formation
3. To map Notch and Wnt signaling patterns in the gastruloid model

Method

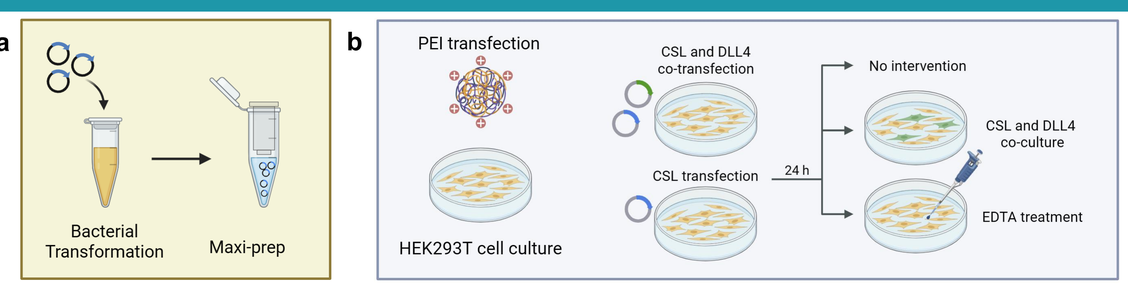


Figure 1. Methods for validating 12xCSL-d1EGFP plasmid in HEK293T cells. (a) Plasmids were amplified in E. coli cultures and isolated by maxi-prep to obtain high yields of purified pDNA. (b) CSL plasmid was introduced to 293T cells using PEI transfection. Four approaches were tested to activate Notch signaling and induce GFP expression.

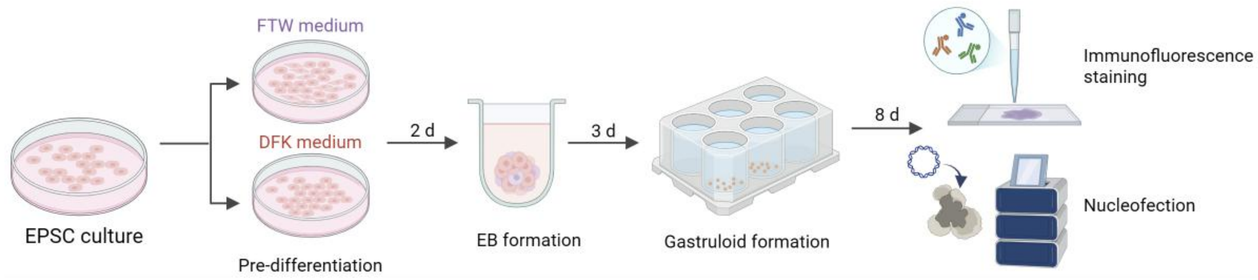
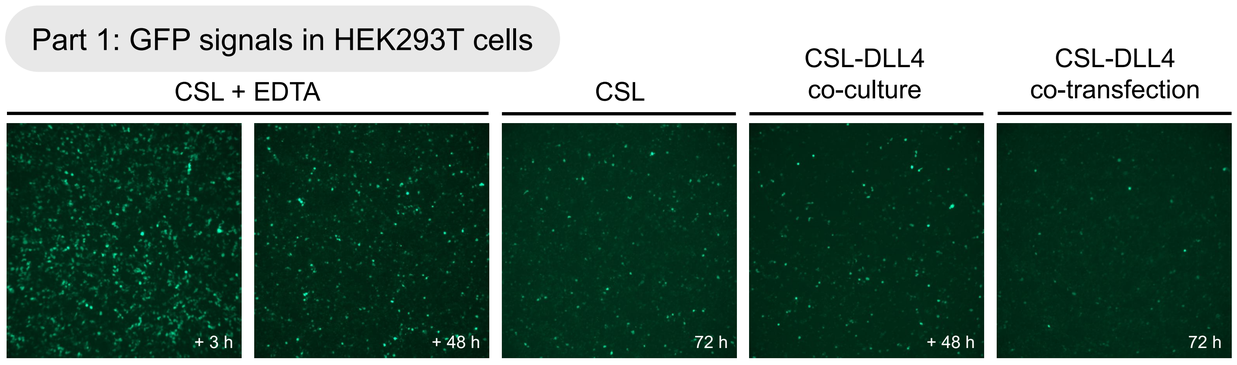


Figure 2. Gastruloid formation protocol. EPSCs were cultured until reaching ~40-60% confluency before *in vitro* differentiation. FTW and DFK media were used to prime EPSCs toward XEN and EPI lineages, respectively. On day 2, cells were dissociated to form mixed aggregates under two conditions (9:1 and 3:1 EPI: XEN). EBs were cultured in tHDM at 37 °C, 5% CO_2. After 3 days, EBs were collected and transferred to STEMdiff hematopoietic kit medium A for 3 days and medium B for 5 days. On day 8, gastruloids with good morphology were collected for IF staining and nucleofection.

Result and Discussion

Part 1: GFP signals in HEK293T cells



- The CSL plasmid is functional and can be used for gastruloid nucleofection.
- The strongest signal was observed at 3 h post-EDTA treatment. Chemical activation by EDTA effectively increased Notch signaling compared to other approaches.
- CSL culture and CSL-DLL4 co-culture showed similar levels of GFP signal, suggesting that inducing DLL4 Notch ligand expression is dispensable for plasmid screening in 293T cells.
- The weakest signal observed under co-transfection condition could be an effect of cis-inhibition.

Figure 3. GFP signal in 293T cells following PEI transfection of 12xCSL-d1EGFP plasmid under four conditions.

Part 2: Gastruloid development

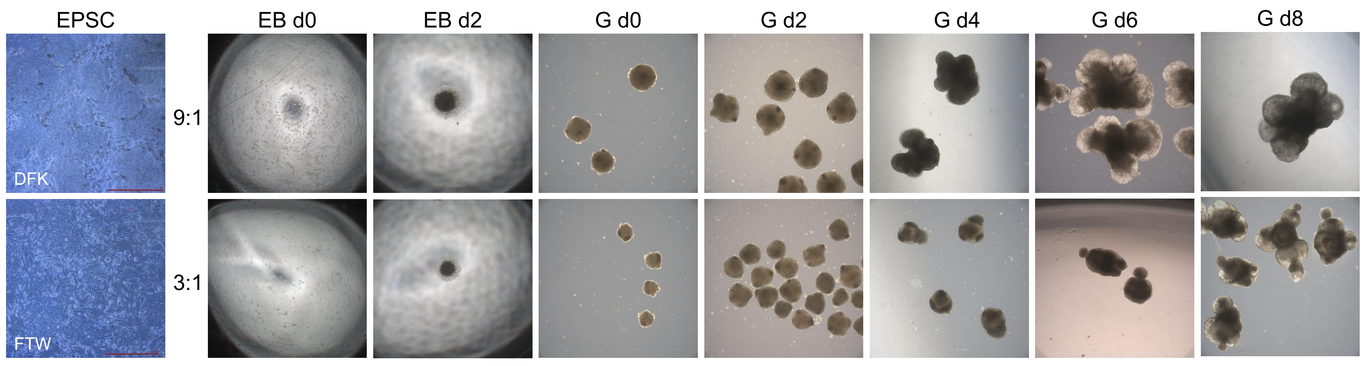


Figure 4. Cell culture under 4x microscope. **a** EPSC colony on day 2 after culturing in DFK and FTW media. **b** Embryoid body (EB) and gastruloid (G) morphology, comparing 9:1 and 3:1 ratios.

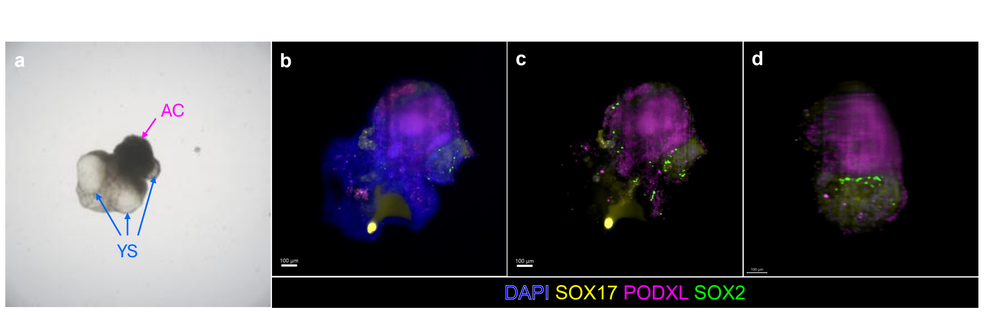


Figure 5. Yolk sac and amniotic cavity of day 8 gastruloid. **a** Bright-field image with blue arrows pointing to the yolk sac (YS) and a pink arrow pointing to the amniotic cavity (AC). Immunofluorescence staining of SOX2 (green), SOX17 (yellow), and PODXL (magenta): **b** 3D image and **c-d** cross-sectional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

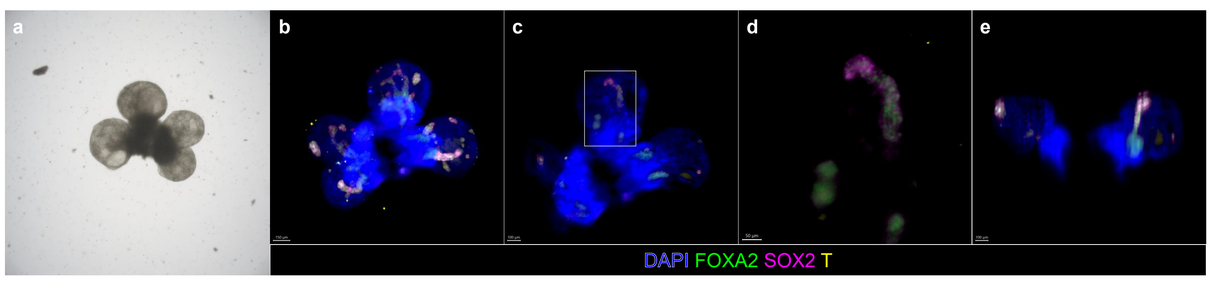


Figure 6. Notochord structure of day 8 gastruloid. **a** Bright-field image shows four yolk sacs. Immunofluorescence staining of FOXA2 (green), SOX2 (pink), and T (yellow): **b** 3D image and **c-e** cross-sectional images: multiple developing notochords in yolk sacs marked by co-localization of T/FOXA2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

Part 3: Gastruloid nucleofection of CSL and TCF plasmids

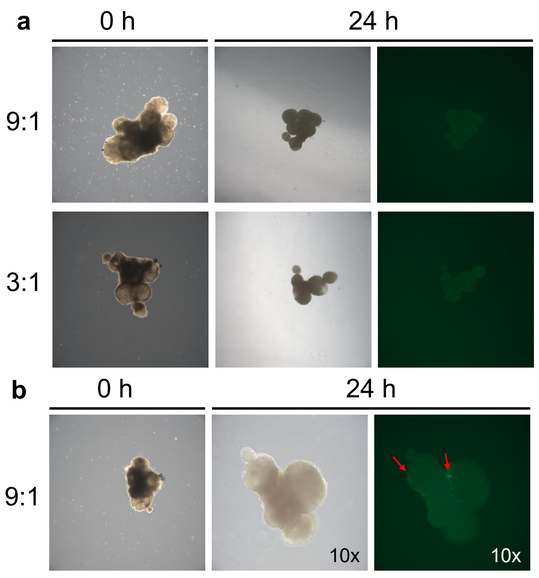


Figure 7. Nucleofection of reporter plasmids at 0 h and 24 h post-transfection. **a** CSL plasmid in 9:1 and 3:1 gastruloids. **b** TCF plasmid in a 9:1 gastruloid.

- No GFP signal was observed in gastruloids transfected with the CSL plasmid. It is possible that the plasmid cannot diffuse deeply enough to reach regions that express Notch signaling.[2]
- The GFP signal of the TCF plasmid was observed, indicating the presence of Wnt/β-catenin signaling in gastruloids.

Conclusion

- Mixed aggregates of XEN and epiblast cells successfully self-organized and developed into day-8 gastruloids, exhibiting key developmental structures including an amniotic cavity, multiple yolk sacs, and developing notochords, coupled with the presence of Wnt/β-catenin signaling. However, comparative analysis of the 9:1 and 3:1 gastruloids was limited by discrepancies in initial cell seeding density, leading to unintended size variations.
- Future research is required to incorporate an epiblast-only control group and standardize cell densities to elucidate the role of XEN cells in gastruloid formation.

Reference

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