Formation of EpiTS embryoids

Mehmet Ugur Girgin
EPFL https://orcid.org/0000-0001-6191-3071
Matthias Lutolf (matthias.lutolf@epfl.ch)
EPFL

Method Article

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Abstract

The difficulty of studying post-implantation development in mammals has sparked a flurry of activity to develop *in vitro* models, termed embryoids, based on self-organizing pluripotent stem cells. Previous approaches to derive embryoids either lack the physiological morphology and signaling interactions, or are unconducive to model post-gastrulation development. Here, we report a bioengineering-inspired approach aimed at addressing this gap. We employ a high-throughput cell aggregation approach to simultaneously coax mouse embryonic stem cells into hundreds of uniform epiblast-like aggregates in a solid matrix-free manner. When co-cultured with mouse trophoblast stem cell aggregates, the resulting hybrid structures initiate gastrulation-like events and undergo axial morphogenesis to yield structures, termed *EpiTS embryoids*, with a pronounced anterior development, including brain-like regions. We identify the presence of an epithelium in EPI aggregates as the major determinant for the axial morphogenesis and anterior development seen in *EpiTS embryoids*. Our results demonstrate the potential of *EpiTS embryoids* to study peri-gastrulation development *in vitro*.

Procedure

Cell culture

Mouse embryonic stem cells (SBr line[55]) were cultured at 37^0C in 5% CO_2 in medium composed of DMEM+Glutamax (#61965-026), 10% ES cell-qualified FBS (#16141-079), 1mM sodium pyruvate (#11360-070), 1x MEM non-essential aminoacids (#11140-035), 0.1mM 2-mercaptoethanol (#31350-010) and 1000u/ml Pen/Strep (#15140-122) supplemented with 3µm GSK3i (#361559), 2µm MEKi (#S1036) and 0.1µg/ml LIF (in house preparation). Cells were routinely passaged every 2-3 days by seeding 8000-9000 cells/cm^2 and every 20 passages a fresh vial was thawed. Cells were tested and confirmed free of mycoplasma. Mouse trophoblast stem cells (TS:GFP line[45]) were cultured at 37^0C in 5% CO_2 in TS medium composed of RPMI 1640+Glutamax (#61870-010), 20% ES cell-qualified FBS (#16141-079), 1mM sodium pyruvate (#11360-070), 0.1mM 2-mercaptoethanol (#31350-010) and 1000u/ml Pen/Strep (#15140-122). TS medium was conditioned on irradiated MEFs for 3 days and stored at -20^0C. This was repeated three times for one batch of irradiated MEFs. Aliquots of TS conditioned medium (TSCM) were thawed and mixed 3:1 with fresh TS medium before cell passaging. 50ng/ml Fgf4 (#100-31) and 1µg/ml Heparin (#H3149) were added to make final TS medium. TS cells were routinely passaged every 2-3 days by seeding 5000-6000 cells/cm^2 and every 20 passages a fresh vial was thawed. MEFs were prepared in house and stocks were prepared at passage 1. MEFs were cultured in TS medium with no additional growth factors. For the co-culture experiments, MEFs from passages 4-7 were used. Cells were tested and confirmed free of mycoplasma.

Preparing EPI and TS differentiation medium

N2B27 medium was prepared by 1:1 mixing of DMEM/F12+Glutamax (#31331-028) and Neurobasal (#21103-049) with the addition of 0.5x N2 supplement (#17502001), 0.5x B27 supplement (#17504001), 0.5x Glutamax (#35050-038), 1mM sodium pyruvate (#11360-070), 1x MEM non-essential aminocacids (#11140-035), 0.1mM 2-mercaptoethanol (#31350-010) and 1000u/ml Pen/Strep (#15140-122). 12ng/ml Fgf2 (#PMG0035), 20ng/ml Activin-A (#338-AC) and 1% KSR (#10828-010) were added to make final EPI differentiation medium (EPIdiff). TS differentiation medium (TSdiff) was prepared by 1:1 mixing of N2B27 and TS medium supplemented with 25ng/ml Fgf4 (#100-31) and 500ng/ml Heparin (#H3149).

Preparing EPI and TSC Aggregates on PEG microwells

Poly(ethylene glycol) (PEG) microwells with 400μm well diameter (121 wells per array) were prepared on 24-well plates as previously described[56]. Microwells were equilibrated with 50μl of either EPIdiff (for ES cells) or TSdiff (for TS cells) for at least 30 minutes at 37^0C. Mouse ES and TS cells and were dissociated to single cells with Accutase (#A11105-01) or TrypLE (#12605-028), respectively. Cells were then centrifuged at 1000 rpm for 5 minutes and washed twice with 10 ml PBS at 4^0C. Cells were resuspended in cold EPIdiff (for ES cells) or TSdiff (for TS cells) and suspension of 484.000 cells/ml was prepared. 35μl of the suspension was added dropwise on microwell arrays to have 100-150 cells/well. Seeding was done at 37^0C for 15 minutes. Growth factor reduced Matrigel (#356231) was diluted in cold EPIdiff or TSdiff to 3% or 2% (v/v), respectively. The medium was vortexed and 1ml was slowly added from the side of the well, avoiding direct addition from the top of the microwell arrays. Plates were kept at 37^0C in 5% CO_2 for at least 72 hours before further processing.

Forming EpiTS embryoids

At 72-75 hours of culture, aggregates on microwell arrays were flushed out and transferred to non-tissue culture treated 10cm plates in 10ml warm N2B27 medium. Single EPI and TSC aggregates were picked in 10μl and transferred to low adherent U-bottom 96 well plates (#COR-7007). 170μl of N2B27 medium was added on top. At 96 and 120 hours, 150μl of medium was replaced with fresh N2B27 and *EpiTS embryoids* were kept until 168h. Protein inhibitors Lefty (#746-LF-025), Noggin (in house preparation), Dkk1 (#5897-DK-010) were added at indicated timepoints at 200ng/ml final concentration. Small molecule inhibitors; SB431542 (#S4317), XAV939 (#S1180), LDN193189 (#SML0559) were added at indicated timepoints at 10μm, 10μm and 1μm final concentrations, respectively.