Self-organization of human embryonic stem cells on micropatterns

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Fate allocation in the gastrulating embryo is spatially organized as cells differentiate into specialized cell types depending on their positions with respect to the body axes. There is a need for *in vitro* protocols that allow the study of spatial organization associated with this developmental transition. Although embryoid bodies and organoids can exhibit some spatial organization of differentiated cells, methods that generate embryoid bodies or organoids do not yield consistent and fully reproducible results. Here, we describe a micropatterning approach in which human embryonic stem cells are confined to disk-shaped, submillimeter colonies. After 42 h of BMP4 stimulation, cells form self-organized differentiation patterns in concentric radial domains, which express specific markers associated with the embryonic germ layers, reminiscent of gastrulating embryos. Our protocol takes 3 d; it uses commercial microfabricated slides (from CYTOO), human laminin-521 (LN-521) as extracellular matrix coating, and either conditioned or chemically defined medium (mTeSR). Differentiation patterns within individual colonies can be determined by immunofluorescence and analyzed with cellular resolution. Both the size of the micropattern and the type of medium affect the patterning outcome. The protocol is appropriate for personnel with basic stem cell culture training. This protocol describes a robust platform for quantitative analysis of the mechanisms associated with pattern formation at the onset of gastrulation.

INTRODUCTION

Within the developing embryo, pluripotent cells of the epiblast undergo a series of cell-fate decisions, first differentiating into the three germ layers and eventually to all the cell fates that make up the adult animal. These decisions are under the control of developmental signaling pathways that follow complex spatiotemporal sequences[1]. Although extensive research on model organisms has elucidated the identities and components of these signaling pathways, quantitative, systems-level understanding remains elusive because of the difficulty involved in observing and perturbing embryonic development *in vivo*. Mammalian development is particularly inaccessible because it takes place *in utero*, and much of our knowledge of human development is extrapolated from studies on model organisms such as the mouse, despite the substantial differences that are known to exist between species[2–4]. Many of these issues can be overcome through the use of human embryonic stem cells (hESCs) as a complement to *in vivo* studies. A challenge of using hESCs to study development is that the differentiation must be made to resemble *in vivo* development as closely as possible, and, in particular, systems need to be developed in which hESCs differentiate in spatial patterns akin to those in the early embryo. Here we present a method for controlling the spatial organization of hESC differentiation patterns that are associated with embryonic gastrulation.

Development of the method

We initially analyzed the relationship between the transforming growth factor (TGF)-β superfamily and cell fate in a murine myoblast cell line, and showed that TGF-β signaling displays adaptive dynamics. We then explored the consequences of this mechanism in cell-fate decisions[5,6]. We sought to extend similar methods to study signaling and fate decisions in hESCs, but were hampered by the inherent variability between cells. The response of cells to applied ligands varied within a colony, and every colony had a different spatial pattern of signaling. As regular hESC cultures present colonies of different sizes and shapes, we reasoned that variations in colony geometries likely underlie these variable colony-level responses. We therefore sought to control colony geometries. Methods to control the shape of single cells had previously been used to study the biophysics of cell shape, adhesion, and division[7,8]. Micropatterning technologies aimed at spatial control of extracellular matrix deposition—and thus colony geometries on 2D surfaces—had also been applied to hESCs, where it was observed that colonies of different sizes gave rise to different proportions of cell fates upon differentiation[9,10]; however, spatial differentiation patterns were not observed. In our experiments, we found that micropatterned colonies treated with bone morphogenetic protein 4 (BMP4) responded with particular spatial patterns of signaling that translated into cell-fate patterns. These patterns of signaling involved both differential responses to the initial BMP4 stimulus and patterns of endogenous Nodal signaling that were shaped by the production of both the Nodal ligand and its feedback inhibitor, Lefty[11]. Wnt signaling probably serves as a required intermediate between BMP4 and Nodal, as Wnt ligands are targets of BMP4 signaling both *in vivo* in the mouse[1] and in hESCs differentiated with BMP4 (ref. 12).

Comparison with other methods

Here, we describe a protocol that takes advantage of commercially available micropatterned coverslips (from CYTOO). These are produced by first covering the culture surface with a cell-repellant substrate such as lysine-grafted PEG[13], and then selectively removing it using UV light or a plasma etch in a pattern defined by a mask. Homemade solutions using this technique can also yield satisfactory cell confinement[14]. An alternative method for

producing the same results is microcontact printing, which is performed with an embossed stamp coated with an extracellular matrix of interest capable of mediating cellular attachment. When the stamp is pressed onto a slide, it deposits a cell-adherent coating in the desired pattern[9,15]. The uncoated areas may be backfilled with a passivating material to interfere with nonspecific attachment of cells. The stamps are made by spin-coating a negative mold—made using standard photolithography or silicon etching—with polydimethylsiloxane elastomer. As commercial chips offer only a limited number of designs, it may be necessary to use homemade micropatterned chips for special applications—for example, when alternative colony shapes are required or when one wants to use a substrate softer than glass.

The main alternative to using 2D micropatterned culture surfaces is to grow a defined number of cells in a 3D aggregate. Various groups have recently reported a degree of self-organization in aggregates of mouse ESCs[16–18]. These methods have the advantage of allowing cell movement in three dimensions, and, in some protocols, the aggregates elongate in a process that mimics gastrulation and convergent extension in the embryo. The cells begin in an approximately spherical orientation and spontaneously break symmetry to position particular germ layers in separate locations. Thus, these systems may represent a promising arena for the investigation of symmetry breaking and the events that lead to the formation of the body axis from a symmetric embryo. These methods follow from earlier studies showing polarized signaling and differentiation within aggregates of mouse ESCs[18] and are similar to methods for growing self-organized organoids[19–21], applied to the early embryo as a whole. On the other hand, the initial state of a ball of cells is quite different from that of the epiblastic disk, and the confined 2D disk of cells in micropatterned colonies may be a better representation of the epiblast that is a disk-shaped epithelium at the onset of gastrulation[22]. 2D cultures are also more amenable to imaging. Most importantly, 2D micropatterning allows for reproducible colony geometries that lead to quantitatively reproducible differentiation patterns[11]. By contrast, quantification of the variability in spatial differentiation patterns has not been performed for 3D aggregate cultures.

Thus, 2D micropatterning is the current method of choice for studying signaling and spatial patterning in stem cell colonies, particularly in applications in which quantitative reproducibility is essential. 3D aggregates are a more suitable system for studying the cellular movements involved in gastrulation, and may also allow the study of the mechanisms of spontaneous symmetry breaking. In the future, hybrid methods, such as patterning the surface of a gel that the cells can invade upon differentiation, may combine the advantages of the two methods and also allow for the investigation of mechanical aspects of differentiation that have been shown to play a role in other stem cell systems[23].

Limitations

As discussed above, this protocol allows for the quantitative observation of early embryonic signaling and cell-fate patterns directly using human cells. It is the only system for examining mammalian patterning *in vitro* with quantitative reproducibility. The restriction to two dimensions limits cell movements and does not allow the cells to assume an organization identical to that of the embryo *in vivo*. In particular, gastrulation results in the three germ layers assuming a trilaminar structure with the mesoderm between the ectoderm and endoderm, whereas in micropatterned cultures these layers are positioned in the same order, one next to the other. Embryos have a well-defined anterior–posterior axis, but micropatterned colonies do not, and their organization may more closely reflect that of embryos in which the distal visceral endoderm has failed to migrate anteriorly[24,25]. Finally, although many aspects of *in vivo* patterning are recapitulated in micropatterned culture, careful comparison with *in vivo* systems is always required to validate new discoveries.

Experimental design

A single-cell suspension of hESCs in Rho-associated protein kinase (ROCK) inhibitor (RI) is used to seed micropatterned colonies at or near confluence. Cells are differentiated by application of BMP4 ligand for the desired period of time. 48 h of differentiation is sufficient to generate patterns consisting of all germ layers and extra-embryonic tissue. Cells can be imaged live during this time or they can be fixed at the conclusion of the protocol. Patterns can be visualized with immunofluorescence staining. Large amounts of imaging data are generated by acquiring tiled images of the entire coverslip. Quantitative analysis of these images provides information on cell-fate patterns and the signaling pathways that generate them with single-cell resolution in hundreds to thousands of colonies on a single coverslip. The following details should be considered when planning an experiment with micropatterned hESCs.

Cells. The data that we show were obtained using the hESC RUES2 and ESI017 lines. The protocol has also been used successfully with other hESC lines (RUES1, H1) and iPSCs[11]. Therefore, this protocol may be suitable for induction of self-organized differentiation patterns from any high-quality hPSCs.

Culture media. The protocol was originally developed with mouse embryonic fibroblast conditioned medium (MEF-CM), and this provides the most robust adhesion to the culture surface. In cases in which a defined culture medium is essential or for continuity with culturing conditions in laboratories that used defined media, the protocol can be successfully performed in mTeSR1 culture media as well (see procedure in **Box 1** and **Supplementary Fig. 1**, unpublished results). In MEF-CM, cells have a more spread morphology and tighter adhesion to the culture surface as compared with mTeSR1, and the use of MEF-CM may require less optimization in other aspects of the protocol to ensure robust adhesion to the culture surface. Because of the differences in surface adhesion and morphology, the main challenge with using mTeSR1 is that the cells have a tendency to retract when RI is removed following seeding, and this tendency is more pronounced when using mTeSR1 than when using MEF-CM. The retraction can lead to suboptimal filling of the micropatterned area and colonies lifting off the cell surface. These issues can be avoided by initiating differentiation soon after RI removal, as described in the alternative protocol below.

Surface coating. The original protocol involves a two-layer coating of poly-D-lysine and Matrigel; however, the two-step coating protocol increases the complexity and time requirements of the protocol. Although standard laminin coating did not yield

Box 1 | Alternative protocol using mTeSR1 ● TIMING 6 h

1. Perform procedure Steps 5–12, but replace MEF-CM medium containing bFGF and Y-27632 with mTeSR1 containing Y-27632 (10 µM). In Step 10, use 1–1.5 × 10^6 cells.
2. Warm the mTeSR1 medium without Y-27632 to 37 °C. Aspirate the medium and add 2 ml of prewarmed mTeSR1 without Y-27632.
3. Incubate for 3 h at 37 °C and 5% CO_2.
4. Prepare mTeSR1 medium with BMP4 (50 ng/ml) and warm it to 37 °C. Aspirate the medium from the chip and add 2 ml of the fresh mTeSR1 medium containing BMP4.
5. Continue the remainder of the protocol beginning with Step 17.

▲ **CRITICAL** We have observed some retraction of the cells the day following BMP4 treatment so that at ~12–24 h, the cell colony may not reach to the edge of the micropatterned circle; however, the cells then recover to cover the entire circle and form extremely reproducible patterns 2 d after seeding.

reproducible adhesion, recombinant laminin-521 (Biolamina) allows for a simpler coating protocol with robust results[11]. We have observed some batch-to-batch variability that requires optimization of the LN521 concentration for each batch.

Cell density. The most important requirement is that the micropatterns be grown to confluence before initiating differentiation. Holes in the colonies create additional borders and can lead to irregular patterns of differentiation that depend on the precise configuration of the gaps. A large range of cell densities is compatible with this requirement, and the length scale of differentiation will depend on density. Denser configurations yield smaller rings of the outer layers, with a larger inner ectodermal layer. Some optimization of cell density to give the desired outcome is typically necessary.

Immunoflourescence and imaging. Imaging of three antibodies along with a nuclear counterstain is straightforward and follows standard immunofluorescence protocols. Typically, DAPI and Alexa Fluor 488, 555, and 647 are imaged with filters designed for DAPI, GFP, Cy3, and Cy5, although many other permutations are possible. Imaging the entire coverslip greatly enhances the statistical power of the approach, and most microscope controller software has the ability to create tiled montages—for example, the Tile explorer function for the open-source micromanager software. For some software, such as the multiposition solution for Olympus cellSens software, additional modules may be required and may require a separate purchase. Analysis of tiled image sets is discussed in detail below ('Image acquisition and analysis of immunostained micropatterns' section).

Micropattern design. The protocol uses Arena CYTOO chips. These glass coverslips have 19 mm × 19 mm dimensions. Disk-shaped micropatterns of 1,000-, 500-, 225-, 140-, and 80-µm diameters are regularly dispersed over the full surface. There are 25, 144, 576, 900, and 1,296 colonies, respectively, associated with the different sizes. Alternatively, one can purchase CYTOO chips composed of colonies of a single size. These different types of chips can be used interchangeably in the protocol, and from here on, we refer to the type being used only as 'CYTOO'.

MATERIALS

REAGENTS

- We have used hESCs, RUES2 cells (WiCell) or ESI017 cells (ESIBIO)
  ! **CAUTION** It is essential to regularly test cells for potential *Mycoplasma* contamination, as it can generate inconsistent results. The hESC lines used in this study repeatedly tested negative for *Mycoplasma* contamination.
  ! **CAUTION** Experiments using hESCs must conform to all relevant governmental and institutional regulations. This work was approved by the Tri-Institutional Stem Cell Initiative Embryonic Stem Cell Oversight Committee (Tri-SCI ESCRO) **!** **CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic.
- ▲ **CRITICAL** Working with hESCs does not require special safety conditions (Biosafety 2 level)
- GlutaMAX (Life Technologies, cat. no. 35050-061)
- Knockout serum replacement (Life Technologies, cat. no. 10828-028)
- MEM non-essential amino acid solution, 100× (Life Technologies, cat. no. 11140-050)
- Basic fibroblast growth factor (bFGF; aa 1–155), recombinant human protein (Life Technologies, cat. no. PHG0263)
- β-Mercaptoethanol, 55 mM (Life Technologies, cat. no. 21985-023)
- B-27 Supplement (50×), minus vitamin A (Life Technologies, cat. no. 12587-010)
- MEM non-essential amino acids (NEAA) solution (100×; Thermo Fisher Scientific, cat. no. 11140050)
- DMEM, high glucose, pyruvate (Life Technologies, cat. no. 11995-065)
- CF-1 mouse embryonic fibroblasts (MEF) feeder cells, irradiated, high density (GlobalStem, cat. no. GSC-6101G)
- Falcon 875 cm^2 rectangular straight-neck cell culture multi-flask, 5-layer with vented cap (Corning, cat. no. 353144)
- 35-mm Tissue culture dishes for chip coating and culture (Falcon, cat. no 353001)
- 15-ml Conical centrifuge tubes (Corning, cat. no. 352097)
- 2-ml Serological pipettes (Falcon, cat. no. 357507)
- 5-ml Serological pipettes (Corning, cat. no. 4487)
- 10-ml Serological pipettes (Corning, cat. no. 4488)
- 25-ml Serological pipettes (Corning, cat. no. 4489)
- 10-µl Barrier pipette tips (Denville Scientific, cat. no. P1096-FR)
- 20-µl Barrier pipette tips (Thermo Fisher Scientific, cat. no. 2149)
- 200-µl Barrier pipette tips (Thermo Fisher Scientific, cat. no. 2069GPK)
- 1000-µl Barrier pipette tips (Thermo Fisher Scientific, cat. no. 2079GPK)
- Gelatin, 0.1% in water (StemCell Technologies, cat. no. 07903)
- Dulbecco's PBS, calcium, magnesium ($DPBS^{++}$, Life Technologies, cat. no. 14040-133)
- Dulbecco's PBS, no calcium, no magnesium ($DPBS^{--}$, Life Technologies, cat. no. 14190-094)
- Sterile filter units, 500 ml, 0.2-µm (Thermo Fisher Scientific, cat. no. 569-0020)
- 0.2-µm Syringe filters (Pall Corporation, cat. no. 4612)
- 3-ml Syringes (BD, cat. no. 309657)
- Laminin-521 (BioLamina, cat. no. LN521-04)
- CYTOOchips, Arena 500 A (CYTOO, cat. no. 10-024-00-18)
- CYTOOchips Arena A (CYTOO, cat. no. 10-020-00-18)
- Accutase (StemCell Technologies, cat. no. 07920)
- mTeSR1 (StemCell Technologies, cat. no. 05857)
- RI, Y-27632 dihydrochloride (Abcam, cat. no. ab120129)
- Penicillin–streptomycin (Life Technologies, cat. no. 15140-148)

- Trypan blue (Thermo Fisher Scientific, cat. no. 15250061)
- Recombinant human BMP-4 protein (R&D Systems, cat. no. 314-BP-050)
- Hydrochloric acid (Fisher Scientific, cat. no. A144S-500) **! CAUTION** Hydrochloric acid is an irritant. Use it in a fume hood.
- Paraformaldehyde 4% (wt/vol) in PBS, pH 7.4 (Poly Scientific R&D, cat. no. S2303) **! CAUTION** Paraformaldehyde is an irritant. Use it in a fume hood.
- Triton X-100 detergent (Bio-Rad, cat. no. 161-0407)
- Fluoromount-G mounting medium (Southern Biotech, cat. no. 0100-01)
- BSA (Sigma-Aldrich, cat. no. A4503-100g)
- Normal donkey serum (Jackson ImmunoResearch, cat. no. 017-000-121)
- Tween-20 (Sigma-Aldrich, cat. no. P1379-500ML)
- DAPI (Cell Signaling Technologies, cat. no. 4083S)
- **Primary antibodies:**

Antigen	Commercial information	Dilution
POU5F1	BD Biosciences, cat. no. 611203	1:400
SOX2	Cell Signaling, cat. no. 3579	1:200
NANOG	R&D Systems, cat. no. AF1997	1:200
BRA	R&D Systems, cat. no. AF2085	1:200
SOX17	R&D Systems, cat. no. AF1924	1:300
CDX2	Abcam, cat. no. Ab15258	1:50

- **Secondary antibodies:**

Antibody	Commercial information	Dilution
Donkey anti-Mouse Alexa Fluor 488	Thermo Fisher Scientific, cat. no. A-21202	1:500
Donkey anti-Goat Alexa Fluor 555	Thermo Fisher Scientific, cat. no. A-21432	1:500
Donkey anti-Rabbit Alexa Fluor 647	Thermo Fisher Scientific, cat. no. A-31573	1:500

EQUIPMENT

- Inverted fluorescence microscope (e.g., Olympus IX83) with an X-Y motorized stage for tiling and digital imaging capture system (e.g., Andor Zyla 4.2 C-Mos camera)
- Inverted laser scanning confocal microscope (e.g., Zeiss LSM780) for high-resolution optical slicing
- Inverted tissue culture microscope with phase contrast (e.g., Olympus CKX41)
- Biosafety cabinet for cell culture (e.g., SterilGuard III Advance SG403, The Baker Company)
- CO_2 incubator with controlling and monitoring system for CO_2, humidity and temperature (e.g., HeraCell 150i; Thermo Fisher Scientific, cat. no. 51026282)
- Cell culture centrifuge (e.g., Sorvall Legend X1R, Thermo Fisher Scientific, cat. no. 75004261)
- Glass hemocytometer (e.g., Electron Microscopy Sciences, cat. no. 63511-11)
- Cell culture disposables: Petri dishes, multiwell plates, centrifuge tubes, pipettes, pipette tips, filter units and so on
- Pipette controller (accu-jet pro, BrandTech, cat. no. 26333)
- 1,000-µl Pipette (Pipetman Classic, Gilson, cat. no. F123602)
- 200-µl Pipette (Pipetman Classic, Gilson, cat. no. F123601)
- 20-µl Pipette (Pipetman Classic, Gilson, cat. no. F123600)
- 10-µl Pipette (Pipetman Classic, Gilson, cat. no. F144802)
- 2-µl Pipette (Pipetman Classic, Gilson, cat. no. F144801)
- Coverslip forceps (Fine Science Tools, cat. no. 11251-33)

REAGENT SETUP

FM10 medium Prepare 500 ml of FM10 medium by mixing 439 ml of DMEM, 50 ml of FBS, 10 ml of GlutaMAX, and 1 ml of β-mercaptoethanol. Filter the medium with a 0.22-µm filter unit, and store it for up to 4 weeks at 4 °C.

HUESM medium Prepare 500 ml of HUESM medium by mixing 379 ml of DMEM medium with 100 ml of knockout serum replacement, 5 ml of GlutaMAX, 5 ml of NEAA, 1 ml of β-mercaptoethanol, and 10 ml of B27 supplement without vitamin A. Filter the medium with a 0.22-µm filter unit, and store it for up to 4 weeks at 4 °C.

MEF-CM Coat a 5-layered flask (875 cm^2) with 0.1% gelatin for 20 min at 37 °C. Thaw 9 vials of irradiated MEFs (60×10^6 cells), and resuspend them in 125 ml of FM10 medium. Aspirate the gelatin from the flask and add the MEF/FM10 mixture. Incubate it overnight. The next day, remove the medium and replace it with 150 ml of HUESM. Incubate overnight to condition the medium. After 24 h, harvest the conditioned medium in 50-ml conical flasks, and replace with fresh HUESM. Repeat HUESM collection for up to 9 additional days. Freeze MEF-CM aliquots at −80 °C after collection and store them for up to 6 months. When they are ready to use, add fresh bFGF at a concentration of 20 ng/ml.

BMP4 solution Prepare a 4 mM HCl solution containing 0.1% (wt/vol) BSA in a sterile tube and use it to dissolve the lyophylized BMP4 to a final concentration of 50 µg/ml. Prepare 20-µl aliquots in microcentrifuge tubes, and store them at −80 °C for up to 6 months. Thawed aliquots can be stored at 4 °C for 2 weeks.

bFGF solution Resuspend the lyophylized bFGF in PBS containing 0.1% (wt/vol) BSA to a final concentration of 20 µg/ml. Prepare 100-µl aliquots in microcentrifuge tubes, and store them at −80 °C for up to 6 months. Thawed aliquots can be stored at 4 °C for 2 weeks.

Blocking solution Add 10 µl of Triton X-100 and 300 µl of normal donkey serum to 10 ml of PBS. Gently mix by inversion. In order to avoid foaming, do not vortex the solution. The solution can be stored at 4 °C for 1 week.

Washing solution Add 20 µl of Tween-20 to 20 ml of PBS. Gently mix by inversion. In order to avoid foaming, do not vortex the solution. The solution can be stored at room temperature (18–25 °C) for 6 months.

DAPI stock Reconstitute 1 mg of DAPI in 10 ml of deionized water to obtain a 0.1 mg/ml solution. Make 100-ul aliquots and freeze them at −20 °C for up to 2 years.

LN-521 solution Thaw an aliquot of LN-521 at 4 °C. Dilute 100 µg/ml LN-521 solution to the required concentration in $DPBS^{++}$ (containing Ca^{++} and Mg^{++}). LN-521 solution can be stored at 4 °C for 1 month.

▲ **CRITICAL** Avoid repeated freeze–thaw cycles of LN-521.

PROCEDURE

Coating the CYTOO chip with LN-521 ● TIMING ~2 h or overnight

▲ **CRITICAL** There is some batch-to-batch variability in the quality of LN521, and optimal concentrations (in the range of 5–20 µg/ml) may need to be determined empirically. We typically use a final concentration of 5 µg/ml. For one CYTOO chip, use 2 ml of LN-521 solution.

1| Use tweezers to place the CYTOO chip face-up in a 35-mm tissue culture dish. Pipette 2 ml of LN-521 onto the chip.

▲ **CRITICAL STEP** The side of the CYTOO chip on which the CYTOO label is written in the forward direction is the patterned surface.

▲ **CRITICAL STEP** The chip should remain submerged during the coating procedure. If necessary, press the borders of the chip with the tweezers to keep it at the bottom of the dish. Be careful to touch only the borders, and to avoid scratching the internal patterned surface when using tweezers.

2| Incubate the chip at 37 °C for 2 h or overnight at 4 °C

3| Prewarm 34 ml of DPBS^{++} to 37 °C. Pipette 4 ml of DPBS^{++} into the dish. Remove 3 ml of the DPBS^{++} from the dish.
▲ **CRITICAL STEP** Minimize the time the chip is exposed to air during transfer to prevent drying of the chip and damage to the laminin matrix.

Washing the chip ● TIMING 15 min
4| Wash the chip five times by adding 6 ml of DPBS to the dish and removing 6 ml from the dish.
▲ **CRITICAL STEP** The chip should remain submerged under DPBS throughout all wash cycles to avoid drying the surface. If necessary, press the borders of the chip with the tweezers to keep it submerged during the washes. Be careful to touch only the borders, and to avoid scratching the internal patterned surface when using tweezers.
■ **PAUSE POINT** The coated chip can be stored under DPBS^{++} for at least 1 week at 4 °C.

Single-cell passage and seeding of hESCs onto LN-521-coated CYTOO chips ● TIMING 30 min
▲ **CRITICAL** One CYTOO chip requires 5 × 10^5 to 1 × 10^6 cells grown in MEF-CM. Cells should be passaged from a dish that is between 60% and 80% confluent. A 35-mm dish that is between 60% and 80% confluent should contain 1–2 × 10^6 cells. The volumes referred to in Steps 5–14 will be those that are required when using one 35-mm dish.
5| Before passaging, prepare the medium by adding bFGF (20 ng/ml), Y-27632 (10 μM), and Penicillin–Streptomycin (Pen/Strep; 1%) to MEF-CM. Warm the medium to room temperature. Prepare 2 ml of medium for each 35-mm dish to be passaged and 2 ml of medium for each CYTOO chip to be seeded.

6| Rinse the dish containing hESCs with DPBS^{-}. Add enough volume of Accutase to the dish to cover the cell layer. For one 35-mm dish, use 1 ml of Accutase. Incubate at room temperature for 5–7 min or until the cells detach from the culture surface.

7| Gently break up the colonies into single cells by pipetting with a 1-ml tip.

8| Add the cell suspension to the same volume of medium. Centrifuge the suspension at 300*g* for 4 min at room temperature, and discard the supernatant. Resuspend the pellet in a volume of medium that brings the concentration of cells to 1–3 × 10^6 per ml (~1 ml). Pipette the suspension gently with a 1-ml tip to break up any aggregates that are formed during centrifugation.

9| Mix 5–10 μl of the cell suspension in a 1:1 ratio with Trypan blue, and count the cells using a hemocytometer.

10| Add the volume of the cell suspension containing 5 × 10^5 to 1 × 10^6 cells to additional medium to bring the total volume to 2.5 ml.

11| Aspirate the DPBS^{++} from the dish containing the CYTOO chip, and add the cell suspension.
▲ **CRITICAL STEP** Minimize the amount of time that the coated chip is exposed to air. Drying can damage the laminin matrix.

Incubation and washing ● TIMING 2 h
12| Incubate the chip at 37 °C and 5% CO_2 for 2 h.

13| Prepare MEF-CM with bFGF (20 ng/ml) and Pen/Strep (1%) without Y-27632, and warm it to 37 °C.

14| Aspirate the medium and wash the chip once with 2 ml of prewarmed DPBS^{++}. Add 2 ml of MEF-CM medium without Y-27632, prepared in Step 13.
? TROUBLESHOOTING

Differentiation of hESCs into organized germ layers on LN-521-coated CYTOO chips ● TIMING ~2–3 d
15| Incubate the seeded chip overnight at 37 °C and 5% CO_2 for 12–18 h.

16| Prepare MEF-CM with bFGF (20 ng/ml), BMP4 (50 ng/ml), and Pen/Strep (1%), and warm it to 37 °C. Aspirate the medium from the chip and add 2 ml of fresh medium containing BMP4.

17| Incubate the chip at 37 °C and 5% CO_2 for 48 h.
? TROUBLESHOOTING

Immunofluorescence staining of hESCs on CYTOO chips ● TIMING ~2 d

18| Wash the chip once with 2 ml of DPBS, and move the chip to a new 35-mm dish with the patterned surface facing up.

19| Fix with 2 ml of 4% (wt/vol) paraformaldehyde for 20 min at room temperature.
! CAUTION The pipettes that contact paraformaldehyde should be discarded as chemical waste.

20| Remove the paraformaldehyde solution, and wash the chip twice with 2 ml of DPBS.
! CAUTION The paraformaldehyde solution should be discarded as chemical waste.
■ PAUSE POINT The chip can be stored under 2 ml of PBS for 1 week.

21| Prepare the blocking solution and filter it using a 0.2-μm pore-size filter. Aspirate the DPBS from the dish containing the chip, and add 2 ml of blocking solution. Incubate the chip for 30 min at room temperature.

22| Remove the blocking solution and add primary antibodies in blocking solution. Use 500 μl of blocking solution with antibodies for one chip in a 35-mm dish. Keep the dish covered to prevent drying.
! CAUTION Different primary antibodies may require specific immunostaining conditions

23| Incubate the chip at room temperature for 2 h or at 4 °C for at least 8 h

24| Remove the blocking solution with primary antibodies and wash the chip three times with 1 ml of washing solution.

25| Dilute the secondary antibodies 1:500 in blocking solution and add 1 μg/ml DAPI. Remove the washing solution and add 500 μl of blocking solution with secondary antibodies and DAPI. Cover and incubate for 30 min at room temperature.

26| Remove the blocking solution with secondary antibodies and DAPI. Wash the chip twice with 1 ml of wash solution followed by one wash with $DPBS^{--}$.
■ PAUSE POINT The chip can be stored under 2 ml of $DPBS^{--}$ for 1 week.

27| To mount the chip on a microscope slide, dab the edge of the chip on a paper towel to remove excess PBS. Apply 30–50 μl of mounting medium to the patterned surface, and lay the chip on a clean microscope slide with the patterned surface facing down.
▲ CRITICAL STEP Lower the chip slowly onto the microscope slide to avoid trapping bubbles in the sample.

28| Allow the sample to dry overnight protected from light.

Image acquisition and analysis of immunostained micropatterns ● TIMING ~2–3 d

▲ CRITICAL All steps can be accomplished using the open-source software ImageJ[26], or they can be integrated into a more dedicated custom framework.

29| *Acquisition*. Acquire images and correct them for uneven illumination using flatfield correction. Subtract background intensity from the images.

30| *Stitching*. Stitch the individual images to obtain a larger field of view with several colonies.
▲ CRITICAL STEP Combining all the individual tiles into a large one will result in very large image sizes. It is therefore advisable to store only a down-sampled stitched image and the positions of the individual tiles relative to each other.

31| *Colony identification*. Using the down-sampled stitched image from Step 30, apply morphological closing to remove small-scale noise. Optionally, apply a Gaussian filter with a large standard deviation to further smoothen the image. Then, obtain the individual colonies by segmentation of the image—e.g., using Otsu's thresholding—to decide whether pixels belong to the background or a colony. Classify the connected components obtained after thresholding as colonies, and inspect visually for correct identification.

32| *Segmentation of nuclei in individual colonies*. Use a nuclear marker (such as DAPI) to segment the individual cell nuclei. Examples of standard methods for this are graph-cut algorithms such as FARsight[27], seeded watershed transformations such as ImageJ's 3D Watershed[28], machine learning such as Ilastik (http://www.ilastik.org/), and line-of-sight decomposition[29]. Combine the resulting pixel values for each segmented nucleus in an array, and store the list of arrays corresponding to each

nucleus. If you are performing the analysis using ImageJ, launch the 'Trainable Weka Segmentation' plugin. Define 3 classes—background, nuclei, and cell–cell contacts—and train the classifier. After segmentation, get the probability maps and select the image corresponding to the 'nuclei' class. Convert this image to a binary mask, and fill holes, erode, dilate, and watershed. Use the 'Analyze particles' class to identify individual nuclei.

33| *Application of nuclear data to other channels.* Having obtained the pixel information of the nuclei in Step 32, apply this to the other channels to obtain expression levels in the respective channels. Subtract background and treat the channels for unequal illumination, if necessary. If a 3D reconstruction of the nuclear data is available from optical sectioning using a confocal microscope, sum all pixels that belong to a nucleus in the respective channels. If only 2D data are available, correct for nuclei having different sizes and being in different positions relative to the focal plane by normalizing the integrated intensity of the nuclear marker. If using ImageJ, apply the pixel information from the segmented DAPI image by using the 'Set Measurements' function and selecting an open image to 'Redirect to'. Save the resulting list of intensities.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
14	Cells attach outside of the micropatterned colonies	Concentration of LN-521 is too high	Find the working dilution of LN-521 for each batch; test in the range of 1:5 to 1:20
		Substrate dried up during coating/washing steps	Ensure that the chips are kept immersed in liquid
		Substrate was not washed properly	Properly wash the substrate according to the protocol
		Cells were left in ROCK inhibitor too long	Ensure that cells are exposed to Y-27632 for the appropriate time
		Too many cells were seeded	Adjust the number of cells used in Step 8 such that surface coverage of the colonies is 95–100% 2 h after seeding
	Uneven seeding	Poor mixing	Gently mix the cells when seeding the chip, taking care not to swirl, as this will concentrate cells in the center of the dish.
		Cells were not reduced to a single-cell suspension	Single cells are critical for accurate counting and seeding; if colonies are difficult to break up into single colonies, incubate them longer with Accutase
		Too many or too few cells were seeded	Adjust the number of cells used in Step 8 such that surface coverage of the colonies is 95–100% 2 h after seeding
17	Holes form in colonies upon removal of RI	Poor seeding	Adjust the number of cells used in Step 8 such that surface coverage of the colonies is 95–100% 2 h after seeding
	Cells or colonies detach from the chip	Problems with coating	Try a higher concentration of LN-521 or a longer coating time
		Cell density is too high	Try lowering the cell concentration

● TIMING

Steps 1–3, coating the chip with LN-521: ~2 h or overnight
Step 4, washing the chip: 15 min
Steps 5–11, single-cell passage and seeding of hESCs onto the chip: 30 min
Steps 12–14, incubation and washing: 2 h
Steps 15–17, differentiation of hESCs into organized germ layers: ~2–3 d
Steps 18–28, immunostaining of hESCs: ~2 d
Steps 29–33, image acquisition and analysis: ~2–3 d
Box 1, alternative protocol using mTeSR1: 6 h

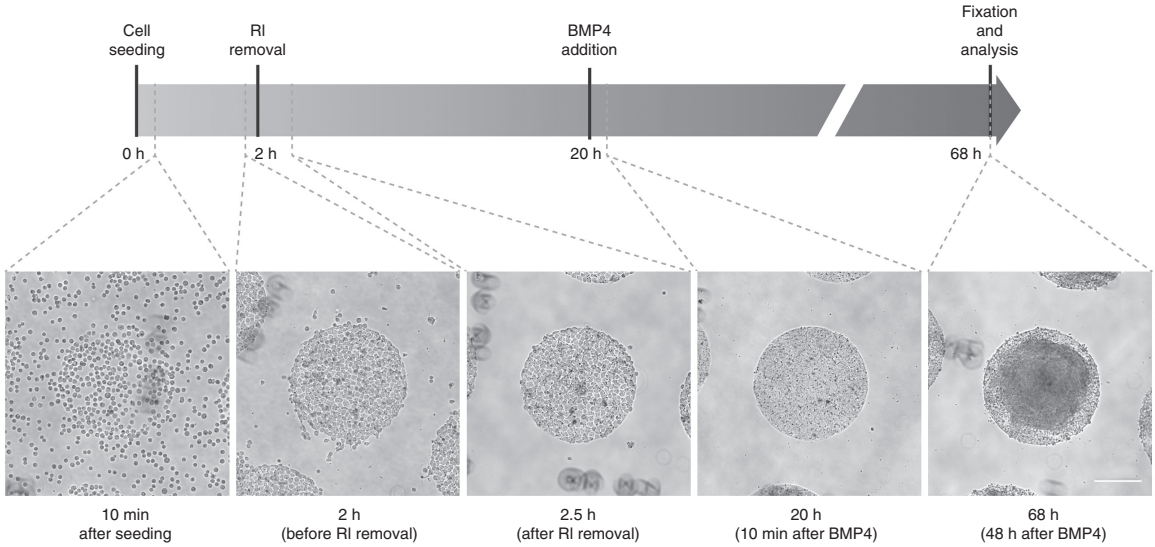

Figure 1 | Time line of the morphology of micropatterned colonies. The images show the morphology of the micropatterned hESC colonies under phase microscopy 10 min after seeding (Step 11), before removal of ROCK inhibitor (Step 13), after removal of ROCK inhibitor (Step 14), 10 min after BMP4 addition (Step 16), and 48 h after BMP4 addition (Step 17). RI, ROCK inhibitor. ESCRO institutional regulatory board permission was obtained to perform these experiments. Scale bar, 200 μm (applies to all panels).

ANTICIPATED RESULTS

Upon seeding, hESCs will attach to only the permissive areas of the CYTOO chip (**Fig. 1**) and will form circular colonies with tightly packed cells within a couple of hours (**Fig. 1**).

In the absence of added morphogens and when cultured under pluripotency conditions, hESCs on patterns maintain expression of the pluripotency markers SOX2, NANOG, and OCT4 for at least 24 h (**Fig. 2a**). Although all cells appear pluripotent, in colonies with diameters of 500 μm or larger, the immunofluorescence intensity of these markers increases from the center of the colony to the edge. This effect may be interpreted as a consequence of edge-sensing in the signaling pathways, as the levels of signal transducers (e.g., SMAD 1/5/8 in the BMP pathway) are also elevated at the colony borders[11]. Colonies that are 250 μm in diameter or smaller may therefore be considered equivalent to the edges of large colonies.

After a 48-h stimulation of colonies with 50 ng/ml BMP4, hESCs differentiate into organized and radially symmetric rings resembling embryonic patterning (**Fig. 2b**,**c**). The cells at the center of the colonies express SOX2, marking the prospective

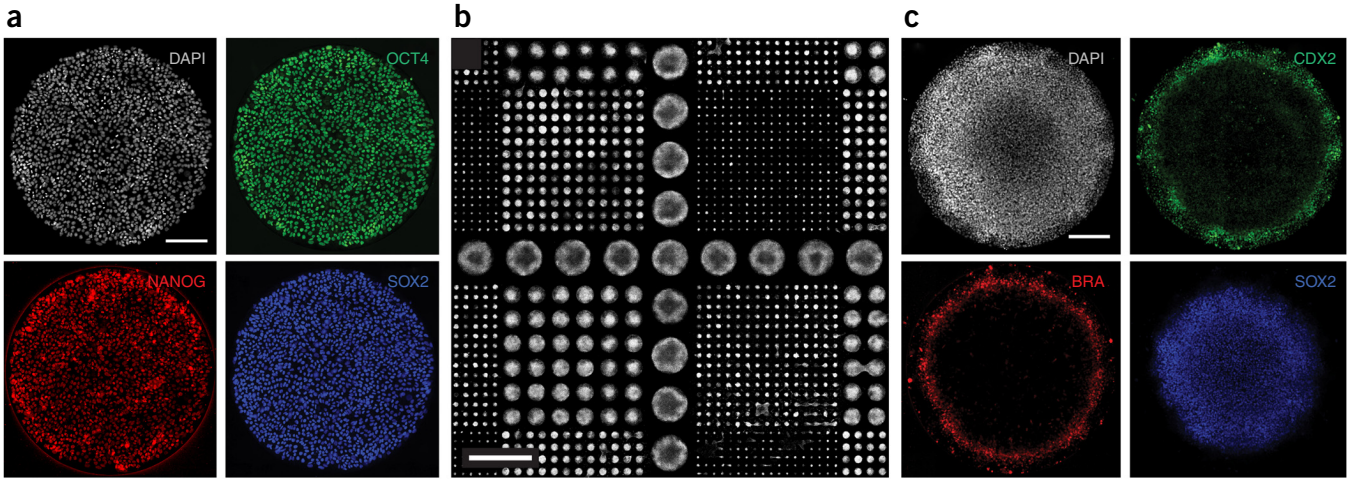

Figure 2 | Immunostaining of micropatterns: pluripotency and differentiation. (**a**) Immunofluorescence staining of hESCs grown on a 1,000-μm micropattern 24 h after seeding on a CYTOO chip. Pluripotency markers are expressed in all cells of the colony. DAPI (top left, gray); OCT4 (top right, green); NANOG (bottom left, red); SOX2 (bottom right, blue). Scale bar, 200 μm. (**b**) The central portion of a CYTOO chip following 24 h of BMP4 (50 ng/ml) differentiation. Nuclei are marked by DAPI staining. Scale bar, 2 mm. Colonies of different sizes (1,000, 500, 250, 125, and 80 μm) can be identified. (**c**) Immunofluorescence staining of hESCs grown on a 1,000-μm micropattern 48 h after BMP4 (50 ng/ml) treatment. DAPI (top left, gray); CDX2 (top right, green); BRA (bottom left, red); SOX2 (bottom right, blue). Scale bar, 200 μm. ESCRO institutional regulatory board permission was obtained to perform these experiments.

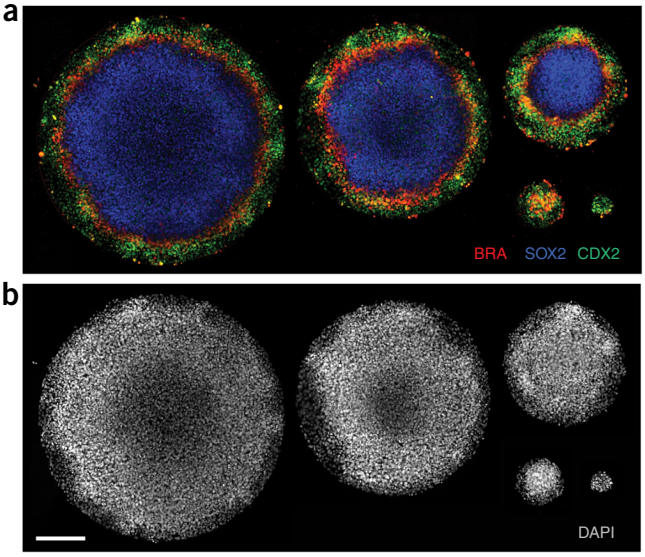

Figure 3 | Effect of colony size on cell fate. (**a**) As the colony radius decreases from 1,000 µm to 80 µm, the central fate is gradually lost. Radius length of the colonies shown are 1,000, 800, 500, 200, and 80 µm. Immunofluorescence staining of hESCs 48 h after BMP4 (50 ng/ml) treatment. CDX2 (green); BRA (red); SOX2 (blue). (**b**) DAPI image for colonies in **a**. Scale bar, 200 µm (applies to both panels). ESCRO institutional regulatory board permission was obtained to perform these experiments.

ectoderm, with rings at progressively larger radii expressing BRA, SOX17, and CDX2, marking the emergence of, respectively, mesoderm, endoderm, and extra-embryonic trophoblast[11] (**Fig. 2c**). Patterned differentiation is also evident in a morphological change of the colony, marked by a dense build-up of cells at the center and an enlargement and wider spreading of cells at the border (**Fig. 1**).

The spatial organization of fates is affected by the size of colonies, such that the smaller colonies do not exhibit central fates (**Fig. 3**). This observation indicates that, in the case of BMP4, fates are acquired from the edges of the colonies inward. Another factor that affects the outcome of patterning with BMP4 is the initial seeding density of cells. Namely, the spatial organization arises only if the cell density is sufficiently high. Therefore, if attempting alternative differentiation strategies, the size of confined colonies and the initial seeding density should be carefully considered.

Similar results are obtained using the chemically defined medium mTeSR instead of conditioned medium (unpublished results, **Supplementary Fig. 1**). Furthermore, in this article, we have used two different cell lines, RUES2 and ESI017, and we have previously shown[11] that RUES1 and H1 cells can be differentiated into spatially organized patterns using the protocol described here.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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1. Arnold, S.J. & Robertson, E.J. Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. *Nat. Rev. Mol. Cell Biol.* **10**, 91–103 (2009).
2. Behringer, R.R., Wakamiya, M., Tsang, T.E. & Tam, P.P. A flattened mouse embryo: leveling the playing field. *Genesis* **28**, 23–30 (2000).
3. Dobreva, M.P., Pereira, P.N., Deprest, J. & Zwijsen, A. On the origin of amniotic stem cells: of mice and men. *Int. J. Dev. Biol.* **54**, 761–777 (2010).
4. Rossant, J. Mouse and human blastocyst-derived stem cells: vive les differences. *Development* **142**, 9–12 (2015).
5. Warmflash, A. *et al.* Dynamics of TGF-β signaling reveal adaptive and pulsatile behaviors reflected in the nuclear localization of transcription factor Smad4. *Proc. Natl. Acad. Sci. USA* **109**, E1947–E1956 (2012).
6. Sorre, B., Warmflash, A., Brivanlou, A.H. & Siggia, E.D. Encoding of temporal signals by the TGF-β pathway and implications for embryonic patterning.. *Dev. Cell* **30**, 334–342 (2014).
7. Thery, M. *et al.* Anisotropy of cell adhesive microenvironment governs cell internal organization and orientation of polarity. *Proc. Natl. Acad. Sci. USA* **103**, 19771–19776 (2006).
8. Thery, M., Jimenez-Dalmaroni, A., Racine, V., Bornens, M. & Julicher, F. Experimental and theoretical study of mitotic spindle orientation. *Nature* **447**, 493–496 (2007).
9. Peerani, R., Bauwens, C., Kumacheva, E. & Zandstra, P.W. Patterning mouse and human embryonic stem cells using micro-contact printing. *Methods Mol. Biol.* **482**, 21–33 (2009).
10. Lee, L.H. *et al.* Micropatterning of human embryonic stem cells dissects the mesoderm and endoderm lineages. *Stem Cell Res.* **2**, 155–162 (2009).
11. Warmflash, A., Sorre, B., Etoc, F., Siggia, E.D. & Brivanlou, A.H. A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. *Nat. Methods* **11**, 847–854 (2014).
12. Kurek, D. *et al.* Endogenous WNT signals mediate BMP-induced and spontaneous differentiation of epiblast stem cells and human embryonic stem cells. *Stem Cell Rep.* **4**, 114–128 (2015).
13. Azioune, A., Storch, M., Bornens, M., Thery, M. & Piel, M. Simple and rapid process for single cell micro-patterning. *Lab. Chip* **9**, 1640–1642 (2009).
14. Ma, Z. *et al.* Self-organizing human cardiac microchambers mediated by geometric confinement. *Nat. Commun.* **6**, 7413 (2015).
15. Thery, M. & Piel, M. Adhesive micropatterns for cells: a microcontact printing protocol. *Cold Spring Harb. Protoc.* **2009** http://dx.doi.org/10.1101/pdb.prot5255 (2009).
16. van den Brink, S.C. *et al.* Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells. *Development* **141**, 4231–4242 (2014).
17. Poh, Y.C. *et al.* Generation of organized germ layers from a single mouse embryonic stem cell. *Nat. Commun.* **5**, 4000 (2014).
18. ten Berge, D. *et al.* Wnt signaling mediates self-organization and axis formation in embryoid bodies. *Cell Stem Cell* **3**, 508–518 (2008).
19. Eiraku, M. *et al.* Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* **472**, 51–56 (2011).
20. Lancaster, M.A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013).
21. Sato, T. & Clevers, H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science* **340**, 1190–1194 (2013).

22. Deglincerti, A. *et al.* Self-organization of the *in vitro* attached human embryo. *Nature* **533**, 251–254 (2016).
23. Engler, A.J., Sen, S., Sweeney, H.L. & Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677–689 (2006).
24. Migeotte, I., Omelchenko, T., Hall, A. & Anderson, K.V. Rac1-dependent collective cell migration is required for specification of the anterior-posterior body axis of the mouse. *PLoS Biol.* **8**, e1000442 (2010).
25. Nowotschin, S. *et al.* The T-box transcription factor eomesodermin is essential for AVE induction in the mouse embryo. *Genes Dev.* **27**, 997–1002 (2013).
26. Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
27. Al-Kofahi, Y., Lassoued, W., Lee, W. & Roysam, B. Improved automatic detection and segmentation of cell nuclei in histopathology images. *IEEE Trans. Biomed. Eng.* **57**, 841–852 (2010).
28. Gniadek, T.J. & Warren, G. WatershedCounting3D: a new method for segmenting and counting punctate structures from confocal image data. *Traffic* **8**, 339–346 (2007).
29. Mathew, B. *et al.* Robust and automated three-dimensional segmentation of densely packed cell nuclei in different biological specimens with lines-of-sight decomposition. *BMC Bioinformatics* **16**, 187 (2015).