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A method to recapitulate early embryonic spatial patterning in human embryonic stem cells

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ABSTRACT

Embryos allocate cells to the three germ layers in a spatially ordered sequence. Human embryonic stem cells (hESCs) can generate the three germ layers in culture, however, differentiation is typically heterogeneous and spatially disordered. Here we show that geometric confinement is sufficient to trigger self-organized patterning in hESCs. In response to BMP4, these colonies reproducibly differentiate to an outer trophectoderm-like ring, an inner ectodermal circle and a ring of mesendoderm expressing primitive-streak markers in between. Fates are defined relative to the boundary with a fixed length scale: small colonies correspond to the outer layers of larger ones. Inhibitory signals limit the range of BMP4 signaling to the colony edge and induce a gradient of Activin/Nodal signaling that patterns mesendodermal fates. These results demonstrate that the intrinsic tendency of stem cells to make patterns can be harnessed by controlling colony geometries, and provide a quantitative assay for studying paracrine signaling.

ATTACHMENTS

dfqxbiqa7.pdf nihms603221.pdf

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MATERIALS TEXT

Primary antibodies:

A	B	C
Antigen	**Antibody**	**Dilution**
pSmad1	Cell signaling 9516	1:100
Smad2	BD Bioscience 610842	1:100
Cdx2	Abcam Ab15258	1:50
Sox17	R&D Systems AF1924	1:200
Eomes	Abcam Ab23345	1:200
Brachyury	R&D systems AF2085	1:300
Sox2	Cell Signaling 3579	1:200
Oct4	BD Biosciences 611203 1	1:400
Nanog	Santa Cruz sc33759	1:100
Nanog	R&D Systems AF1997	1:200
Active β-catenin	Upstate 05-665	1:100
Gata6	Santa Cruz sc9055	1:200
pERK	Cell Signaling 9101	1:200
Snail	R&D Systems SC026	1:10
E-Cadherin	Cell Signaling 3195	1:200
EpCam	R&D Systems AF960	1:100

Purified Mouse Anti-Smad2/3 **BD Biosciences Catalog #610842**

Human SOX17 Antibody **R&D Systems Catalog #AF1924**

Anti-TBR2 / Eomes antibody **Abcam Catalog #ab23345**

Human/Mouse Brachyury Antibody **R&D Systems Catalog #AF2085**

Sox2 (D6D9) XP® Rabbit mAb **Cell Signaling Technology Catalog #3579**

Purified Mouse Anti-Oct-3/4 **BD Biosciences Catalog #611203**

Nanog Antibody (H-155) **Santa Cruz Biotechnology Catalog #sc-33759**

Human Nanog Antibody **R&D Systems Catalog #AF1997**

Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody **Cell Signaling Technology Catalog #9101**

Human EMT 3-Color Immunocytochemistry Kit **R&D Systems Catalog #SC026**

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E-Cadherin (24E10) Rabbit mAb **Cell Signaling Technology Catalog #3195**

Human EpCAM/TROP-1 Antibody **R&D Systems Catalog #AF960**

Cell culture 12h

1

All experiments are performed with the RUES2 hESC line derived in our laboratory and described previously.

For routine culture for maintenance, grow RUES2 cells in HUESM medium that was conditioned by mouse embryonic fibroblasts (MEF-CM) and supplement with **20 ng/ml** bFGF.

2 Test cells for mycoplasma prior to beginning experiments and then again at two-month intervals.

3 Grow cells on tissue culture dishes coated with Matrigel (BD Biosciences 1:40 dilution).

4 1h

Coat dishes in Matrigel **Overnight** at **4 °C** and then incubate at **37 °C** for **01:00:00** immediately prior to seeding the cells on the surface.

5 2h

For micropatterned cell culture, coat micropatterned glass coverslips (CYTOO) with **50 µg/ml** Poly-D-Lysine in H2O (PDL; Millipore) for **02:00:00** .

6 Remove the PDL by serial dilutions without allowing the coverslip to dry (dilution 1:4 in H2O, six times), before performing two complete washes with H2O.

7 2h

Incubate coverslips with Matrigel (1:100 dilution in DMEM-F12) **Overnight** at **4 °C** .

8 Before cell seeding, remove the Matrigel with serial dilutions in ice-cold PBS (dilution 1:4, six times) before 2 complete washes in ice cold PBS.

9 Seed the cells which already resuspended in growth medium onto the coverslips immediately following the removal of the PBS.

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We found it is important to take care to keep the coverslips at **4 °C** at all times when in Matrigel solution and to ensure that the coverslips are not allowed to dry at any time after the application of the Matrigel. Both polymerization and drying of the Matrigel lead to inconsistent cell adhesion with cells more likely to detach from the surface during the experiment.

10 Perform cell seeding onto micropatterned coverslips as follows:

10.1 1h

Pretreat cells growing in MEF-CM and FGF with the Rock-inhibitor Y27632 (Rock-I; 10uM) for **01:00:00** , wash once with PBS, and dissociate with Trypsin.

10.2

Centrifuge cells and resuspend 5×10^5cells in **2.5 mL** growth medium contain Rock-I and place the entire solution over the coverslip in a **35 mm** tissue culture dish.

11 4h

After **02:00:00** , replace the medium with MEF-CM without Rock-I and incubate cells **Overnight** .

12 Test the effects of using the chemically defined mTeSR1 culture medium rather than MEF-CM and obtain similar patterns upon treatment with BMP4 to those presented here (Supplementary Fig. 9).

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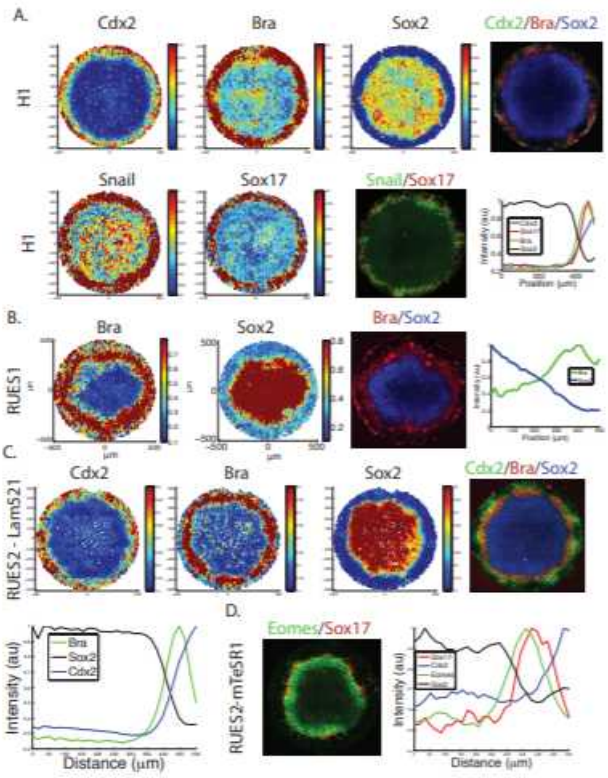
Supplementary Figure 9.



Patterns of differentiation are similar in other hESC lines and for other growth conditions. (**A**) Differentiation patterns of H1 cells. (**B**) Differentiation patterns of RUES1 cells. (**C**) Differentiation patterns of RUES2 cells grown on laminin 521 (**D**) Differentiation patterns of RUES2 cells grown in mTeSR1.

However, we found that MEF-CM better promoted adhesion to the micropatterned surface and it was therefore used in all subsequent experiments.

13 Finally test the effects of growing cells on recombinant Laminin521 (Biolamina) rather than Matrigel, and again obtain similar patterns (Supplementary Fig. 9).

14 Perform Laminin521 coating of coverslips as follows – Coat coverslips with [M]**5 µg/ml** of Laminin521 diluted in PBS with calcium and magnesium for **02:00:00** at **37 °C** . 2h

15 Remove laminin with six serial dilutions in warm PBS (dilution 1:4) before two complete washes in PBS.

16 Seed cells as described above for the PDL-Matrigel coated coverslips.

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siRNA Experiments

17 Passage cells as single cells in Rock-I into **35 mm** dishes at a density of 200000 cells/dish.

18 The next day, transfect cells with siRNA (Ambion Silencer select) using RNAiMax (Invitrogen).

19 Use the final concentration of siRNA **20 Nanomolar (nM)** and **2.5 µl** of RNAiMax for each dish.

20 The following day, seed cells onto micropatterned coverslips and perform differentiation experiments as described above.

PDMS microwells 2m

21 Design molds to create PDMS wells of controlled diameter and depth using a 3D CAD software (Autodesk Inventor) and then 3D printed (3D Systems Projet 3510 HD Plus printer).

> The smallest wells this technique allowed us to make reliably were **250 µm** in diameter and **250 µm** in depth. We found that boiling the 3D printed parts in water containing 1% Triton X100 for four hours was necessary to allow the PDMS to cure on the 3D printed parts.

22 Fill molds with PDMS (10:1 base:reticulant ratio) and degase under vacuum.

23 In order to create opened wells, place the mold between 2 glass slides on which pressure was applied, typically with a large paper clip.

24 After PDMS curing at **80 °C** for several hours, unmold the PDMS wells and boil in distilled water to ensure sterility and that all PDMS was cured.

25 

Wash PDMS wells with ethanol and dry them in a cell culture cabinet to keep them sterile.

26 Stuck wells on the dry cell culture substrate (either glass coverslips, regular tissue culture dish, or optically clear plastic dishes (ibidi)).

27 Coat the wells with cell adhesion promoting proteins either in a one-step protocol (Laminin521) or a two-step protocol (PDL followed by Matrigel).

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28 2m

To remove bubbles trapped in wells, centrifuge the PDMS well filled with the coating solution (**2000 rpm** , **00:02:00**).

Immunofluorescence 2h 30m

29 30m

Rinse coverslips once with PBS, fix with 4% paraformaldehyde, rinse twice with PBS, then block and permiabilize with 3% Donkey Serum, 0.1% Triton X-100 in PBS for **00:30:00** .

30 30m

When performing immunofluorescence for pSmad1, pretreat cells with 1% SDS in PBS for **00:30:00** at **37 °C** before blocking.

31 1h 30m

Incubate coverslips with primary antibodies **Overnight** at **4 °C** (for primary antibodies and dilutions see Supplementary Table 1), wash three times in PBS for **00:30:00** each wash, incubate with secondary antibodies (Alexa488, Alexa555 or Alex647 conjugated (Molecular probes); dilution 1:500) and DAPI nuclear counterstain for **00:30:00** , and then wash twice with PBS.

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Supplementary Table 1.

Antigen	Antibody	Dilution
pSmad1	Cell signaling 9516	1:100
Smad2	BD Bioscience 610842	1:100
Cdx2	Abcam Ab15258	1:50
Sox17	R & D Systems AF1924	1:200
Eomes	Abcam Ab23345	1:200
Brachyury	R & D systems AF2085	1:300
Sox2	Cell Signaling 3579	1:200
Oct4	BD Biosciences 611203	1:400
Nanog	Santa Cruz sc33759	1:100
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Active β-catenin	Upstate 05-665	1:100
Gata6	Santa Cruz sc9055	1:200
pERK	Cell Signaling 9101	1:200
Snail	R & D Systems SC026	1:10
E-Cadherin	Cell Signaling 3195	1:200
EpCam	R & D Systems AF960	1:100

Primary antibodies and dilutions used for immunofluorescence in this study.

32 Mount coverslips on slides using Fluoromount-G mounting medium (Southern Biotech).

Imaging

33

Acquire all widefield images on an Olympus IX71 inverted microscope with a 20X, 0.75 Na lens.

34 Utilize tiled image acquisition to acquire images of the entire coverslip (approximately 2500 stage positions/coverslip) in four channels corresponding to DAPI and Alexa488, Alexa555, and Alexa647 conjugated antibodies.

35 Acquire all confocal images on a Leica SP8 inverted confocal microscope with a 40X, 1.1 Na water immersion objective.

36 Perform three-dimensional visualization and rendering using Imaris software.

Image analysis

37

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Perform all image analysis using custom software written in MATLAB.

Since we imaged the entire coverslip, we utilized the fact that we also imaging the regions without cells to create background and normalization images as follows. We first took the pixel-by-pixel minimum and average across all images:

$m = \min_j(I_j)$; $a = \text{mean}_j(I_j)$

where I_J represents the jth image. We then used m as a background imaged and defined a normalization image according to:

$n = (a - m)/\max(a - m)$

where the max is across all pixels in the image. The mean and normalization images were then smoothed with a Gaussian filter several times the size of a cell diameter and each image was corrected according to:

$I_j \rightarrow (I_j - m)./n$

Where the "./" represents pixel-wise division. In general, subtracting m removes the (typically spatially homogenous) camera noise, while dividing by n corrects for any inhomogeneities in the image due to the illumination or other factors.

38

After each image is corrected in this way, identify and quantify the cells in each image using an algorithm which we described previously.

39 Because larger colonies will span multiple images, perform image alignment and use the resulting overlap between images to put the coordinates of each cell in "coverslip coordinates" resulting in a list of the position of every cell on the coverslip.

40 Separate these cells into colonies by computing the alphavol of the points using the MATLAB function alphavol (http://www.mathworks.com/matlabcentral/fileexchange/28851-alpha-shapes) with a radius (r_V) of 100 pixels.

This function is similar in concept to a convex hull except that it will form separate boundaries for sets A and B if all points in set A are greater than r_V away from all points in set B.

41 Having identified colonies, classify them based on their radius. For quantification of immunofluorescence, normalize the intensity to the DAPI intensity in the same cell, or, for proteins that translocate to the nucleus upon activation, normalize the nuclear intensity to the cytoplasmic intensity in the same cell. Both of these normalizations serve to remove imaging artifacts.

Marker quantification

42

Identify individual cells in images and quantify for markers as described previously.

Normalize all marker intensities to the DAPI intensity in the same cell with the exception of SMAD2 where we normalized

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43 the nuclear SMAD2 intensity to the cytoplasmic intensity in the same cell.

We have found this nuclear to cytoplasmic ratio to be a sensitive metric for signaling activity.

44 Perform radial averages over all colonies on the micropatterned coverslip (n=25 for **1000 µm** colonies, n=144 for **500 µm** colonies, and n=576 for **250 µm** colonies).

45 Perform each marker quantification in at least two different independent experiments.

For **500 µm** and **1000 µm** colonies, we manually excluded those colonies in which cell seeding was uneven (for example, large empty areas within the micropatterned patch).

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