Supplemental Material

Supplemental Figures

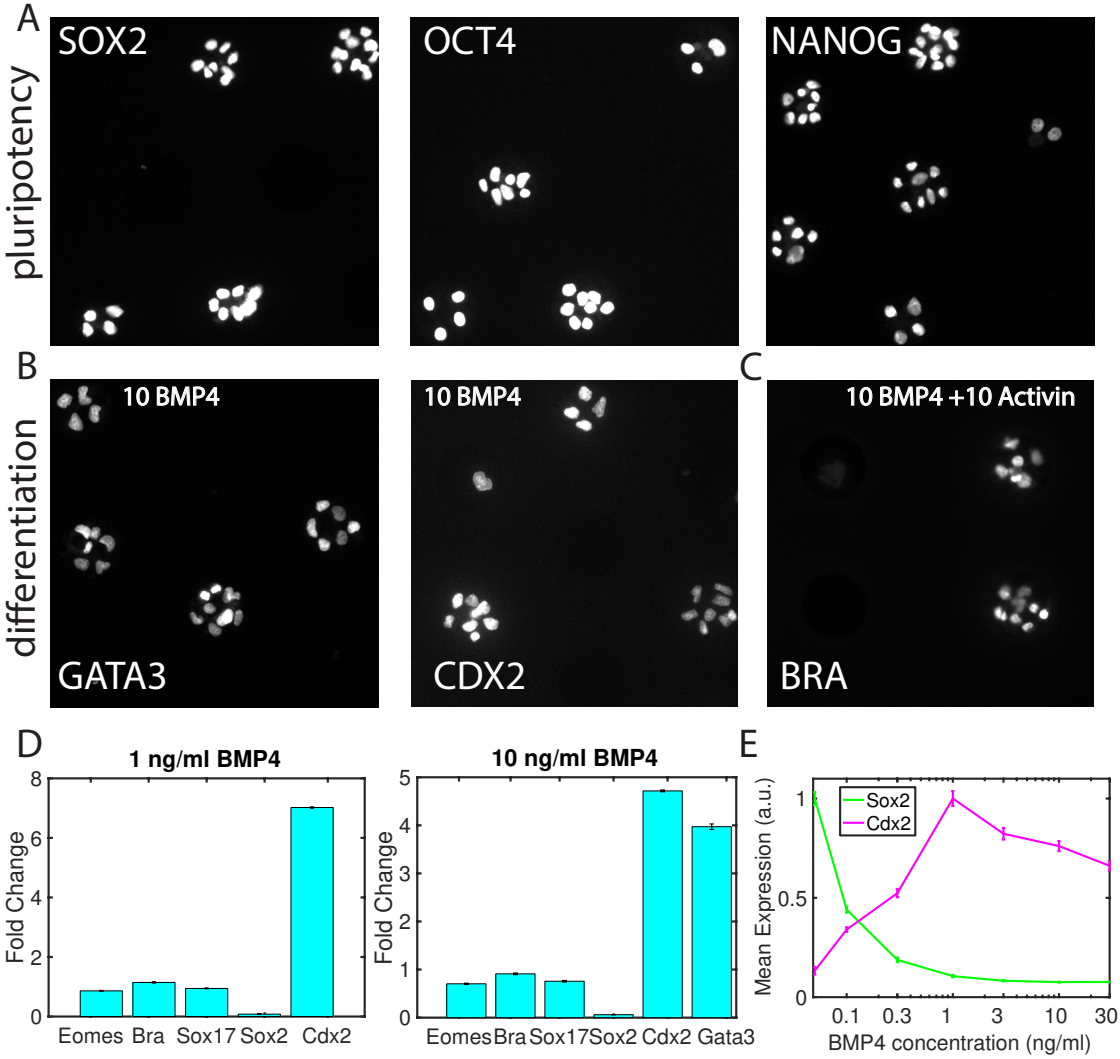


FIGURE S1

Figure S1. Stem cells in µColonies express pluripotency markers and differentiate to a single fate in response to BMP4 stimulation. (A) Representative immunofluorescence images for SOX2, OCT4 and NANOG in pluripotent conditions. (B-C) Representative immunofluorescence images for (B) GATA3 and CDX2 upon differentiation with 10 ng/ml BMP4 and (C) BRA upon differentiation with 10 ng/ml BMP4 and 10 ng/ml Activin. Scale bar 50µm. (D) Mean fold change in the indicated genes upon differentiation with either 1 ng/ml or 10 ng/ml BMP4. (E) Mean levels of SOX2 and CDX2 as a function of BMP4 dose. The levels were normalized to the maximum. Each data point represents the average over thousands of cells on a separate micropattern. Here and in all data below, SOX2 and CDX2 intensities are normalized to DAPI signal in each cell. Error bars represent standard error of the mean.

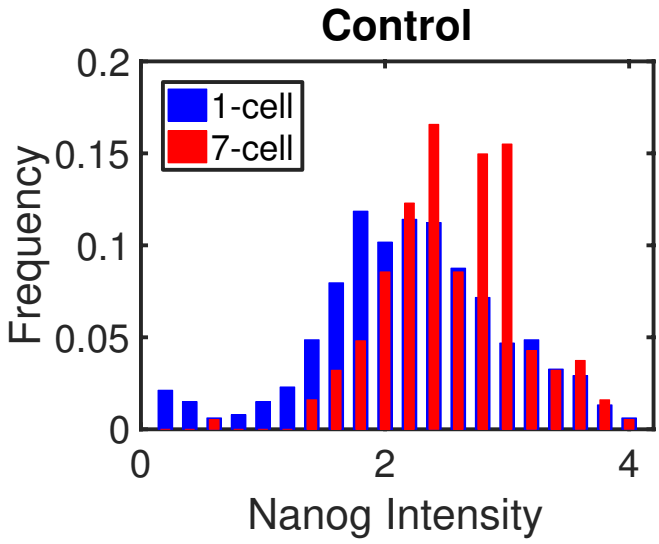


Figure S2

Figure S2. NANOG marker shows community effect in pluripotent conditions. Distributions of NANOG intensity in one- and seven-cell colonies for cells grown in pluripotent conditions.

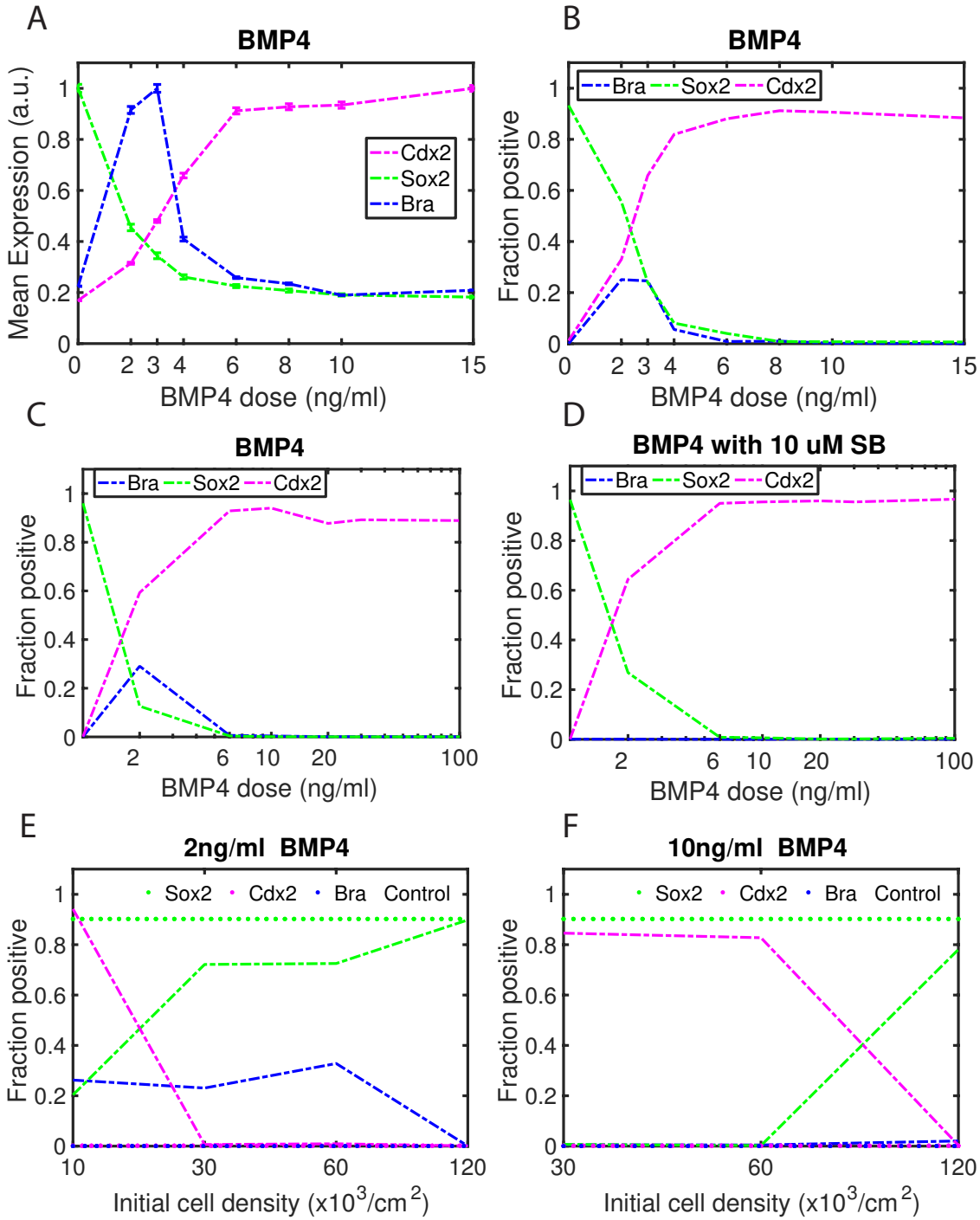


FIGURE S3

Figure S3. In standard culture, expression of BRA in response to BMP4 requires Nodal signaling and specific cell densities. (A) Mean levels of SOX2, BRA and CDX2 and (B) fractions of SOX2, BRA and CDX2 positive cells upon treatment with BMP4 in the range of 0-15 ng/ml. (C)-(F) Fraction of cells expressing SOX2, CDX2 or BRA under the indicated conditions. These represent the same experiments for which the mean expression of each marker is shown in Figure 2.

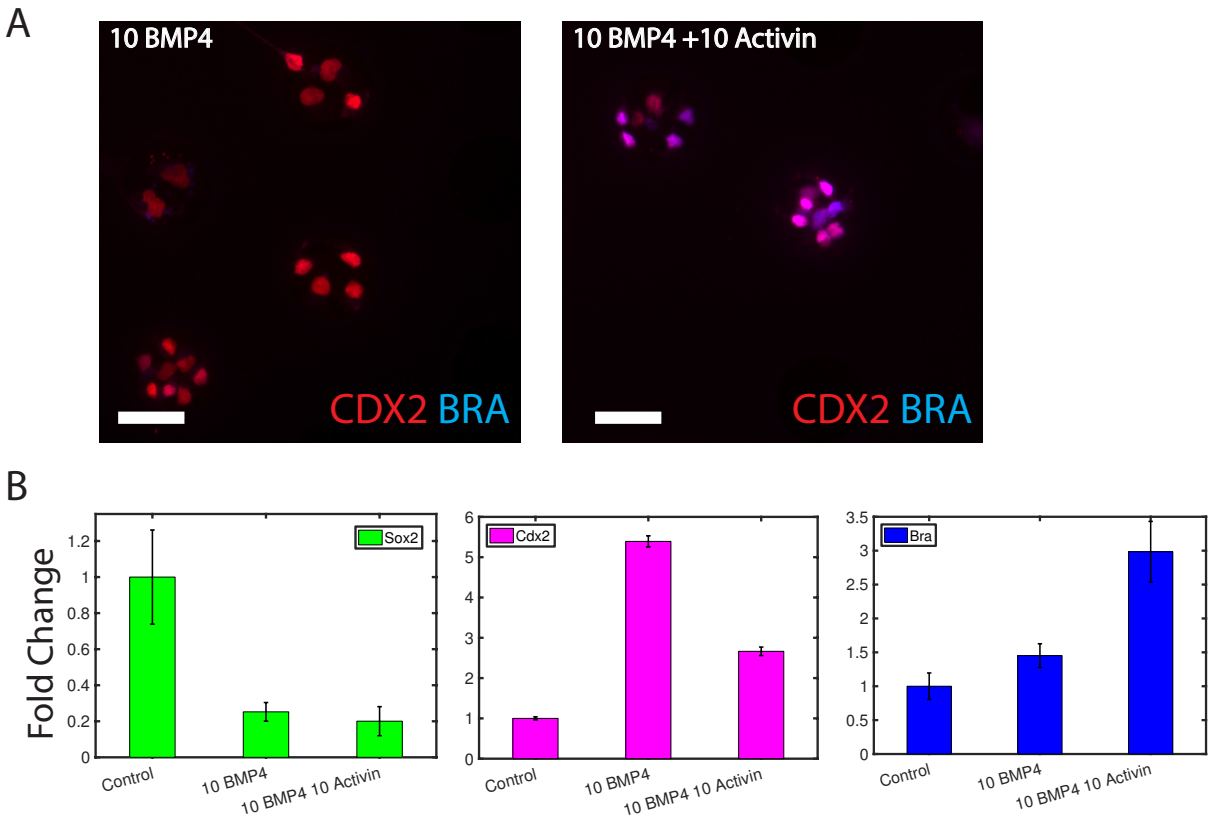


FIGURE S4

Figure S4. Activin treatment during BMP4-mediated differentiation induces BRA expression in µColonies. (A) Representative images of CDX2 and BRA expression in µColonies treated either with 10 ng/ml of BMP4 alone or 10 ng/ml BMP4 with 10 ng/ml Activin. Scale bar 50 µm. (B) Fold changes in expression of SOX2, CDX2 and BRA in these conditions. Error bars represent standard error of the mean.

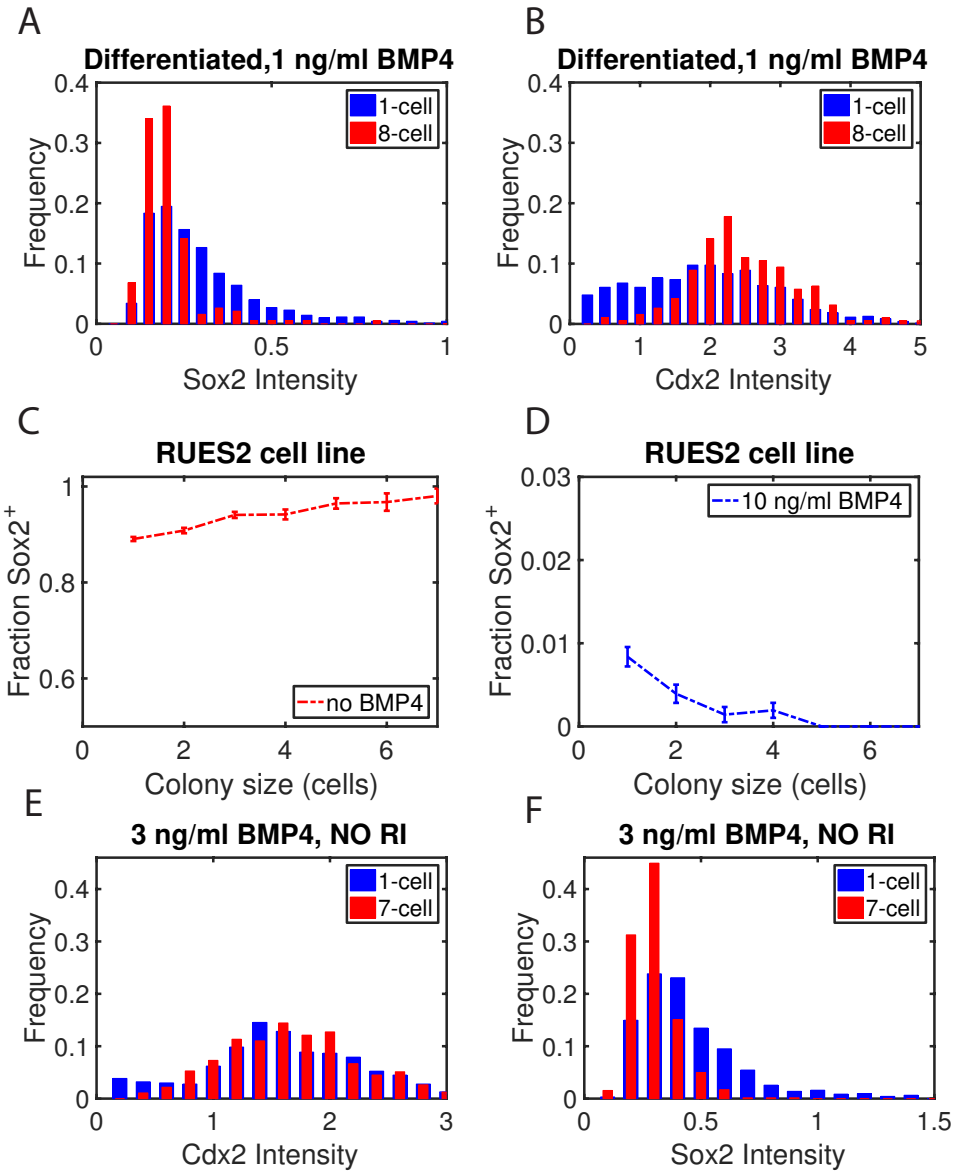


FIGURE S5

Figure S5. Cells show the community effect in the differentiated conditions, in a different cell line, and independently of presence of Rock-inhibitor. (A)-(B) Distributions of SOX2 and CDX2 markers in μColonies upon BMP4 differentiation are long-tailed. Distributions of SOX2 (A) and CDX2 (B) in cells treated with 1 ng/ml BMP4 for the indicated colony sizes. (C)-(D) Community effect in RUES2 cells. Fraction of SOX2 expressing cells in pluripotency (C) or differentiation (D) conditions. Error bars represent standard error of the mean. (E)-(F) Differentiated cells in μColonies retain community effect regardless of Rock-inhibitor (RI) in the media. Distributions of CDX2 (E) and SOX2 (F) markers in one- and seven-cell colonies upon differentiation with 3 ng/ml BMP4 but without Rock-inhibitor (Y27632) in the media. Compare to the distributions in Figure 3B and Figure S2.

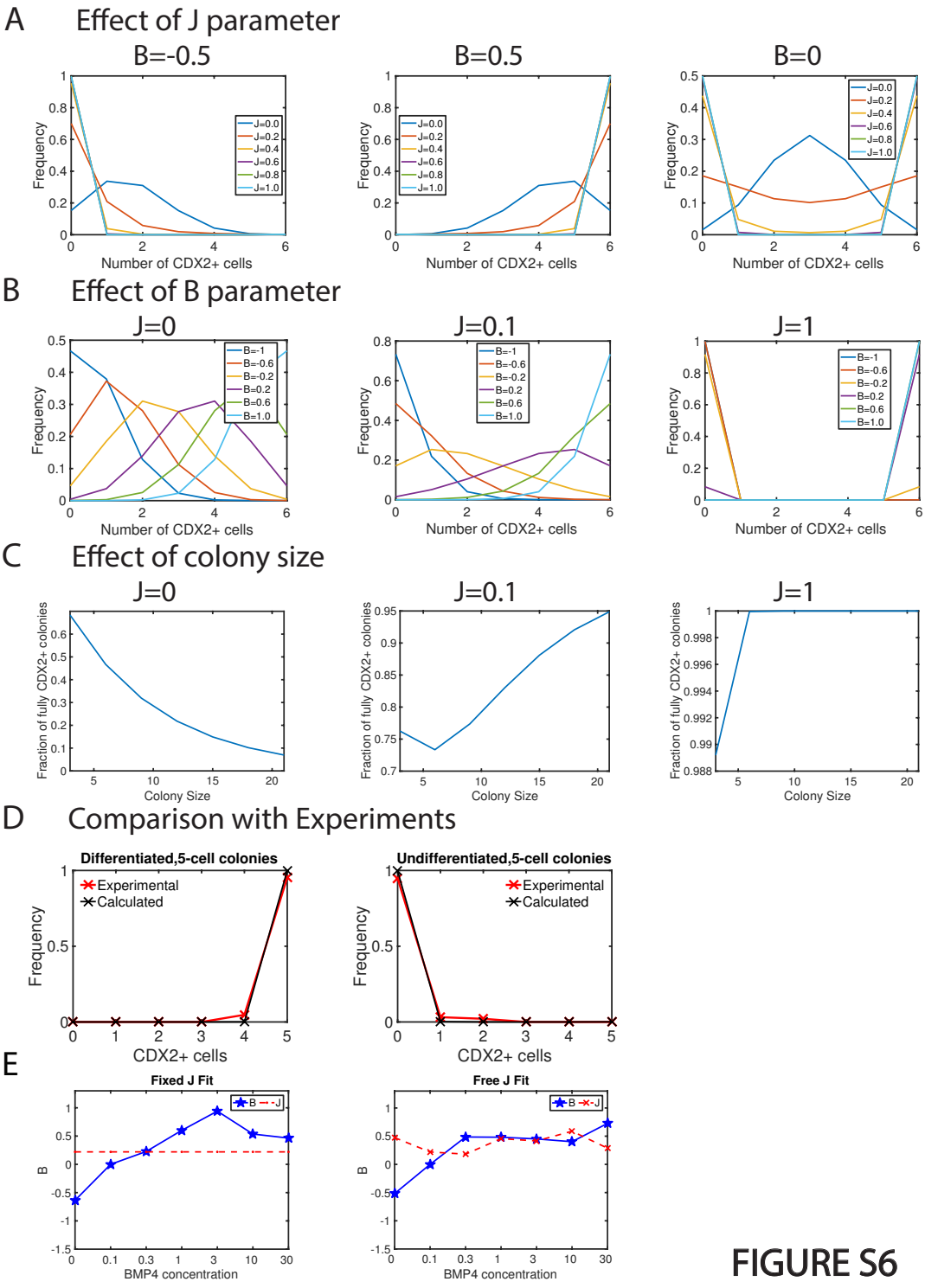


FIGURE S6

Figure S6. Response of the model to variations in model parameters and comparison with experimental data (A) For the indicated value of the B parameter, the J parameter was varied and the distribution of the number of CDX2+ cells in 6 cell colonies was plotted. (B) For the indicated value of the J parameter, the B parameter was varied and the distribution of the number of CDX2+ cells in 6 cell colonies was plotted (C) For the value B = 0.1 and the indicated value of J, the colony size was varied and the fraction of colonies that were entirely CDX2+ was plotted. (D) Comparison between experimental data and calculated distributions of CDX2+ cells for 5-cell μColonies. Parameters were those used to fit the data in Figure 3C without adjustment. (E) The fitted values of B and J are plotted as a function BMP concentration. In the left panel, the value of J was held fixed while in the right it was allowed to vary. χ^2 statistic was 1.40 for the fixed J case and 1.16 for the variable J case.

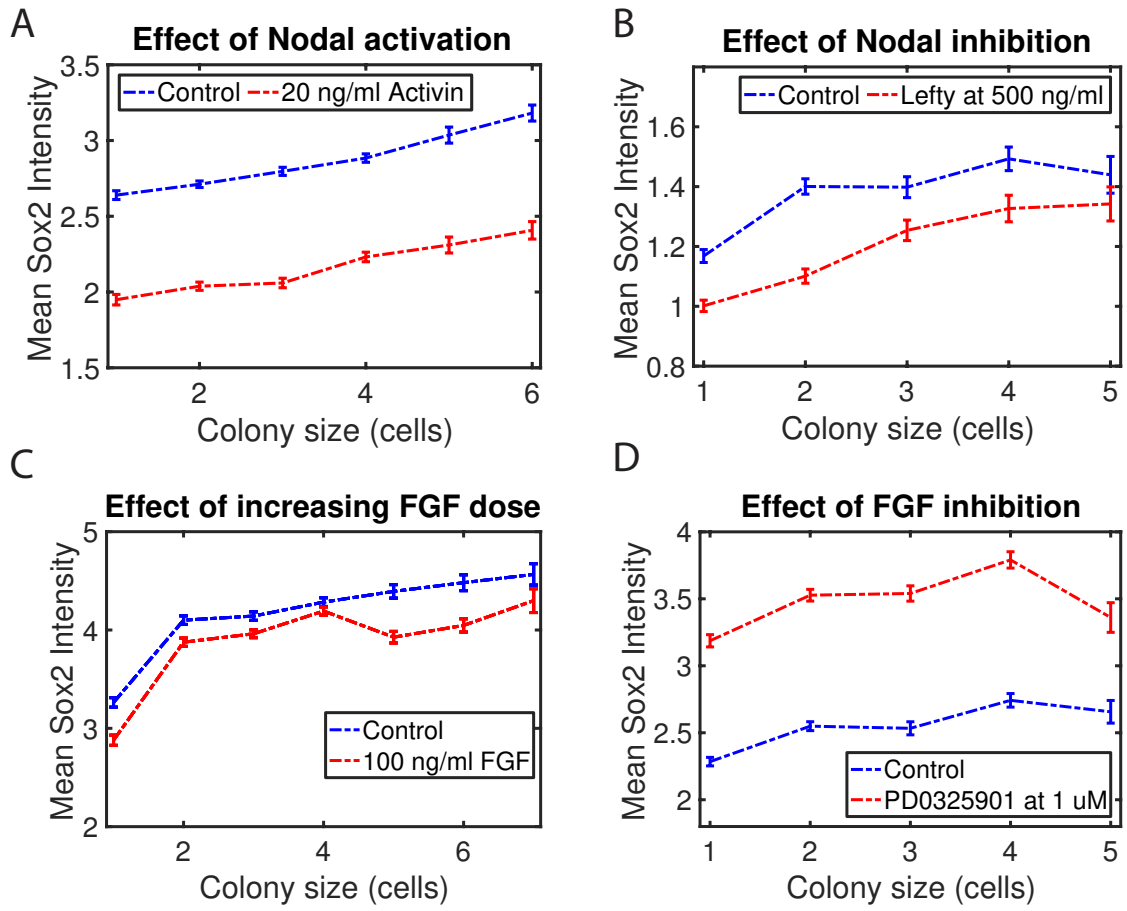


FIGURE S7

Figure S7. Manipulation of pluripotency supporting pathways in µColonies (A) Mean expression of SOX2 marker as a function of colony size in µColonies upon additional Nodal activation with 20 ng/ml Activin. (B) Mean expression of SOX2 marker as a function of colony size in µColonies upon inhibition of Nodal with Lefty (500 ng/ml). (C) Mean expression of SOX2 marker as a function of colony size upon activation of the FGF pathway with additional bFGF (100 ng/ml). (D) Mean expression of SOX2 marker as a function of colony size in undifferentiated conditions upon FGF pathway inhibition (via MEK1/2 inhibition using 1µM of PD0325901). In all panels the error bars represent standard error of the mean for each colony size. Colony sizes that contributed less than 100 cells were not considered.

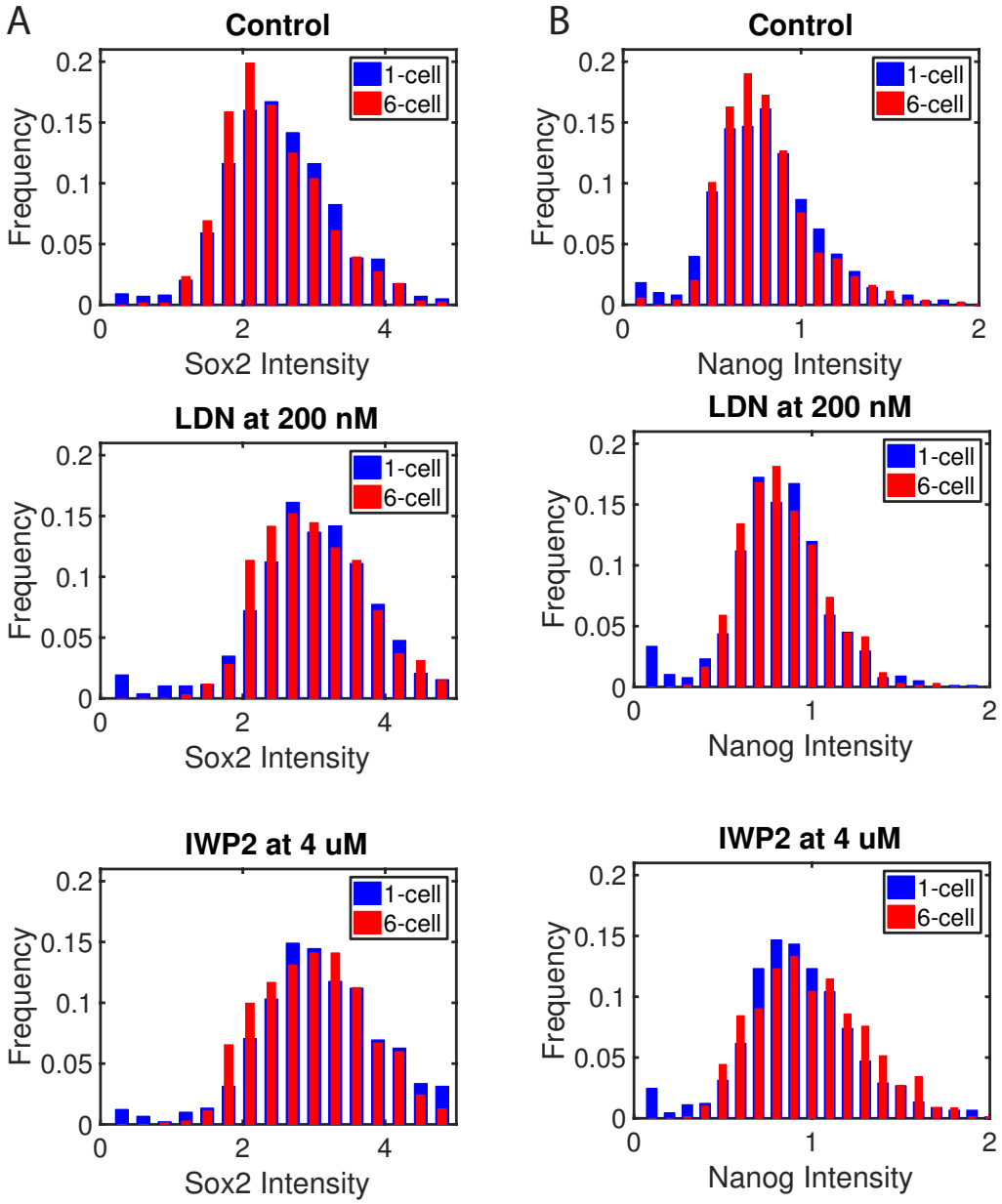
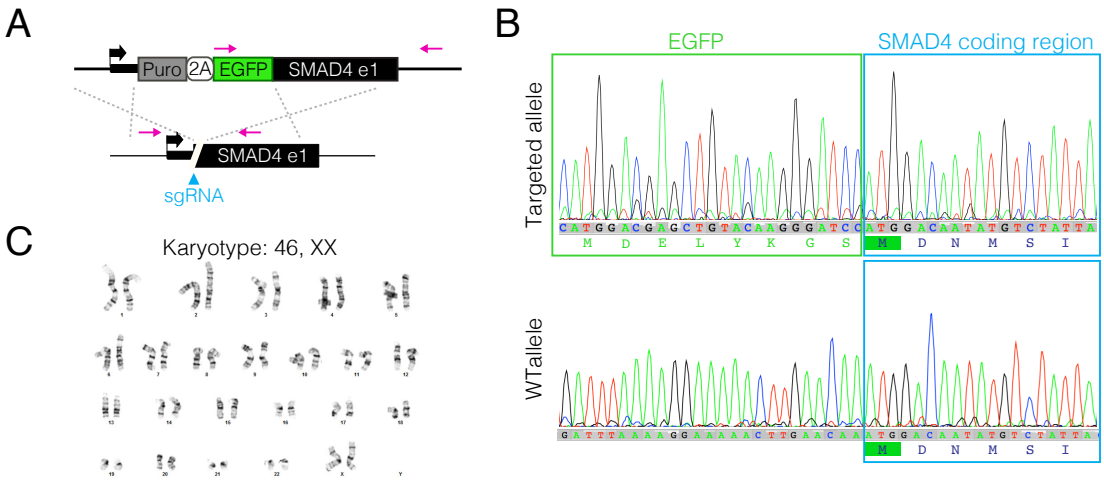
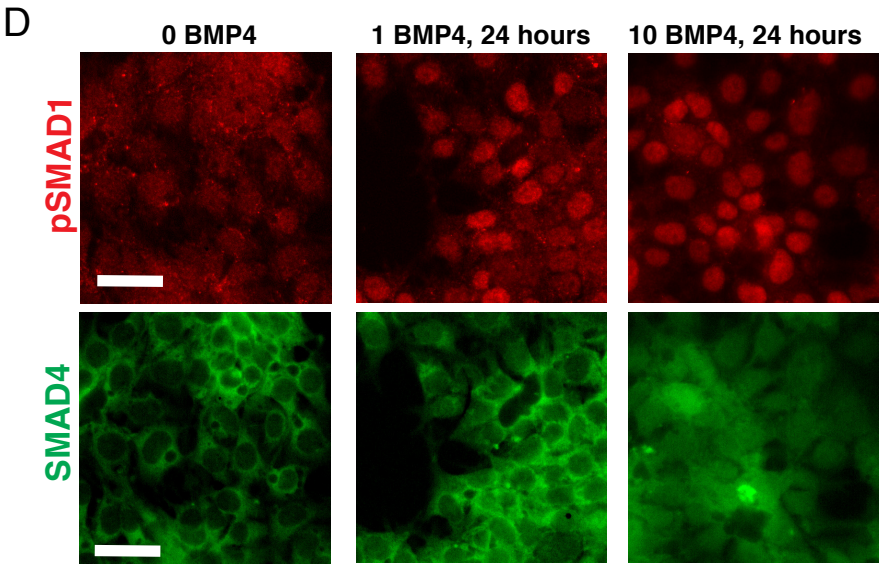


FIGURE S8

Figure S8. Inhibition of BMP4 or Wnt pathways does not influence the community effect in pluripotent conditions. Distributions for cells expressing SOX2 (column A) or NANOG (column B) in control cells or with inhibition of BMP4 signaling via LDN193189 (200nM) or Wnt signaling via IWP2 (4μM). All treatments show a subpopulation of cells that spontaneously differentiate in 1 cell colonies but not in 6-cell colonies.





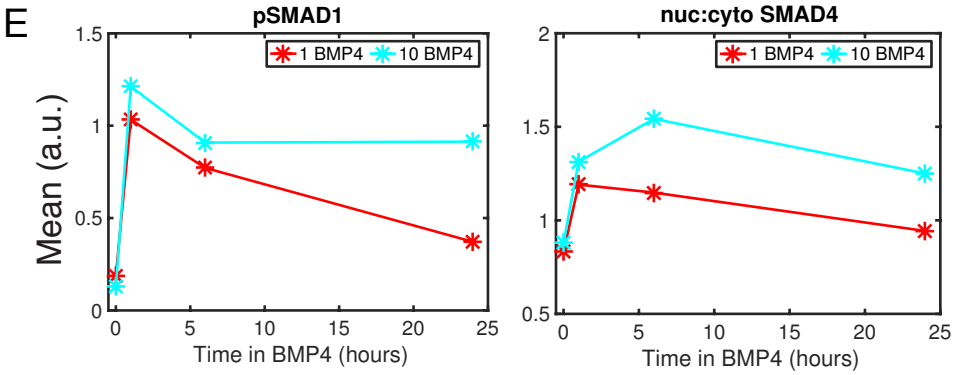
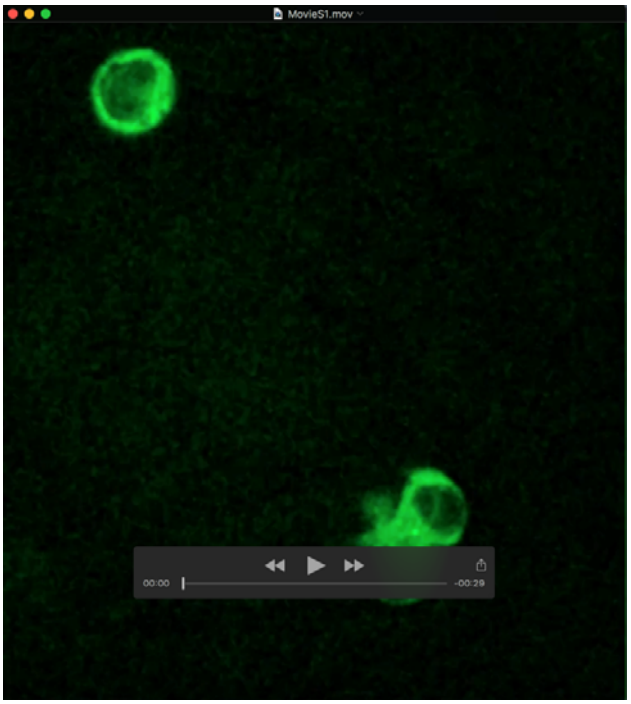


FIGURE S9

Figure S9. Characterization of the GFP-SMAD4 reporter cell line. (A) Schematic of the modification of the endogenous Smad4 locus used to create the RUES2-GFP-SMAD4 cell line via CRISPR/Cas9 genome engineering. (B) Sequencing revealed correct integration of EGFP into one allele and an unmodified second allele. (C) The resulting line possessed a normal karyotype. (D) Representative images for pSMAD1 and SMAD4 staining at various time points during differentiation with low (1 ng/ml) or high (10 ng/ml) BMP4 doses. Scale bar 50 μm. (E) The mean intensity of pSMAD1 staining and the GFP-SMAD4 nuclear to cytoplasmic ratios were determined at the indicated times.

Table S1. Primary antibodies and dilutions

Antibody	Vendor	Catalogue number	Dilution
SOX2	Cell Signaling Technologies	Cat# 5024S	1:200
CDX2	BioGenex	Cat# MU392A	1:100
CDX2	Abcam	Cat# ab15258	1:50
NANOG	BDBiosciences	Cat# 560482	1:400
OCT4	BDBiosciences	Cat# 611203	1:400
phosphoSMAD1	Cell Signaling Technologies	Cat# 13820	1:200
BRACHYURY	R&D Systems	Cat# AF2085	1:300
EOMES	Abcam	Cat# ab23345	1:200
GATA3	Thermofisher	Cat# PA1-101	1:500



Movie S1. Smad4 signaling in one- and two-cell µColonies. BMP4 is added in frame 10. Time interval is 12 minutes. Total movie length is 17.4 hours (2 hours in MEF-CM alone and the remaining 15.4 hours - in MEF-CM + 10 ng/ml BMP4).