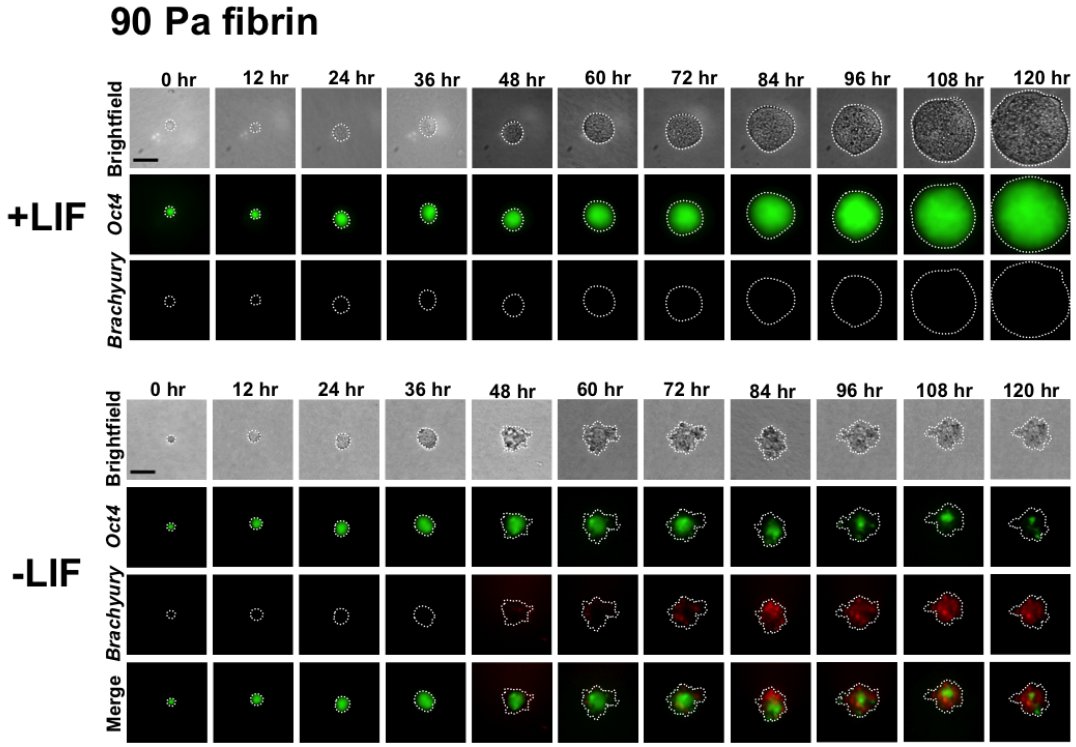
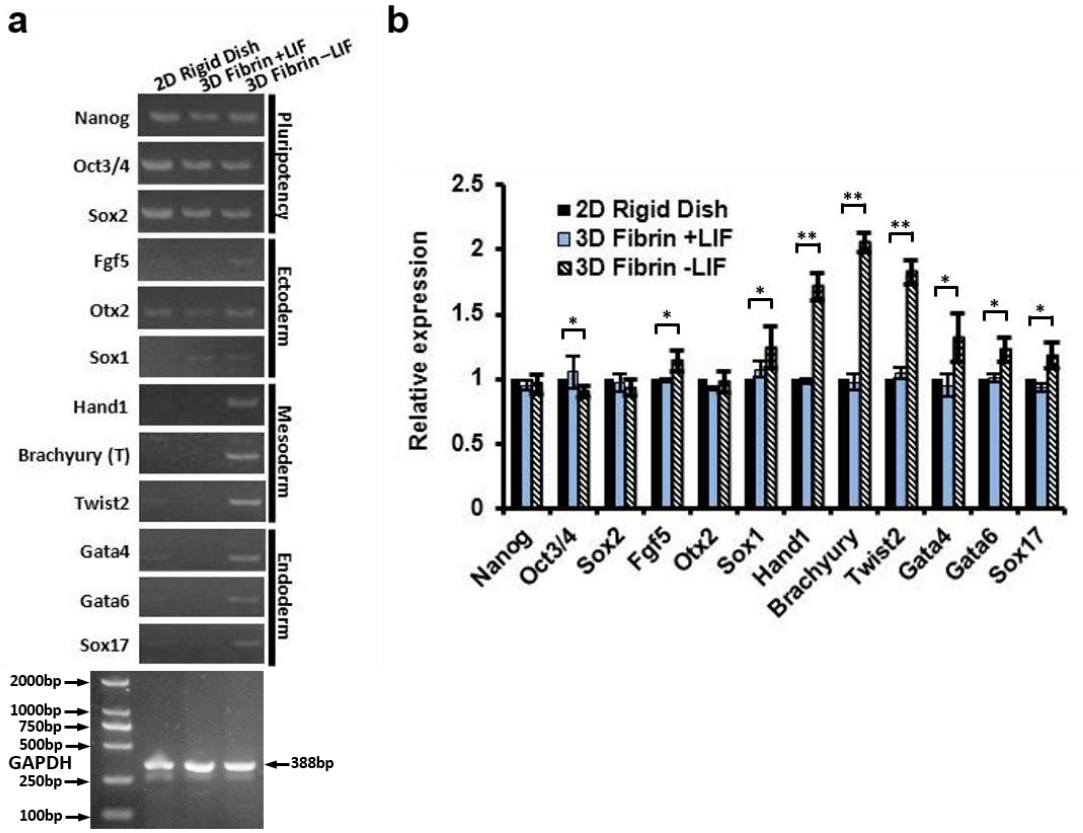
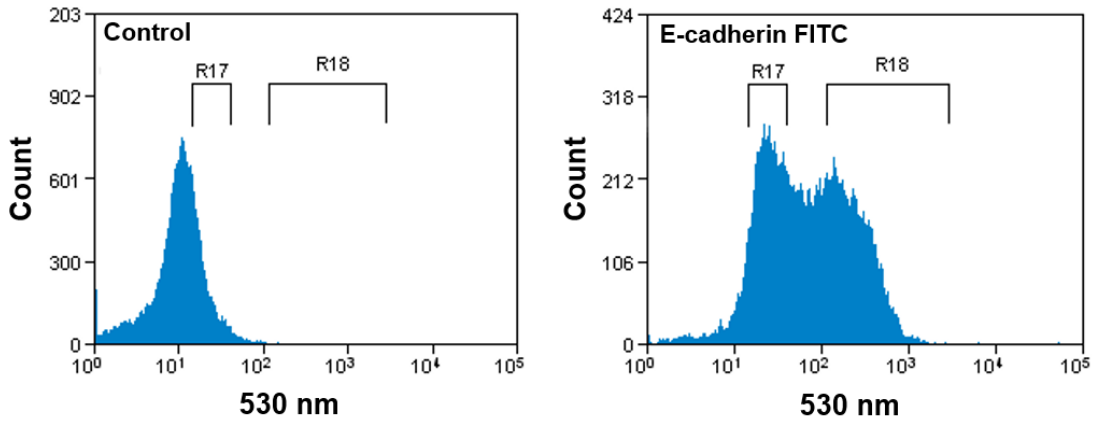


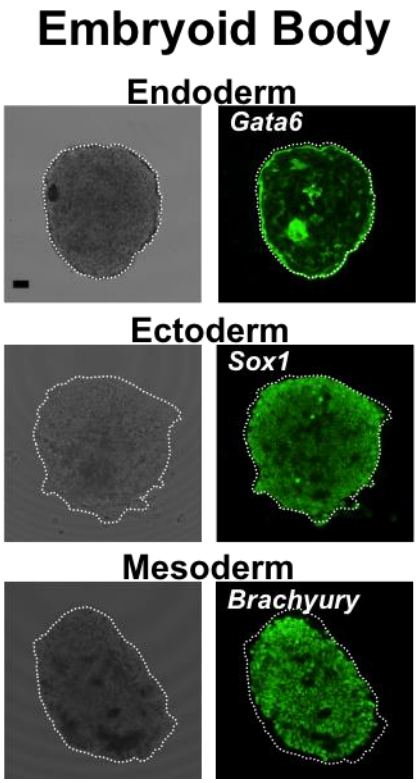

**Supplementary Figure 1. Soft fibrin gels promote growth and organized mesodermal differentiation.** Representative images of single OGTR1 ESCs cultured in 90-Pa 3D fibrin gels for 5 days in the presence of exogenous LIF *(top)*. When the ESC was cultured without LIF, the colony lost its smooth spherical surface and exhibited a Brachyury-positive layer at the outer periphery of the colony *(bottom)*. Scale bars = 50μm.



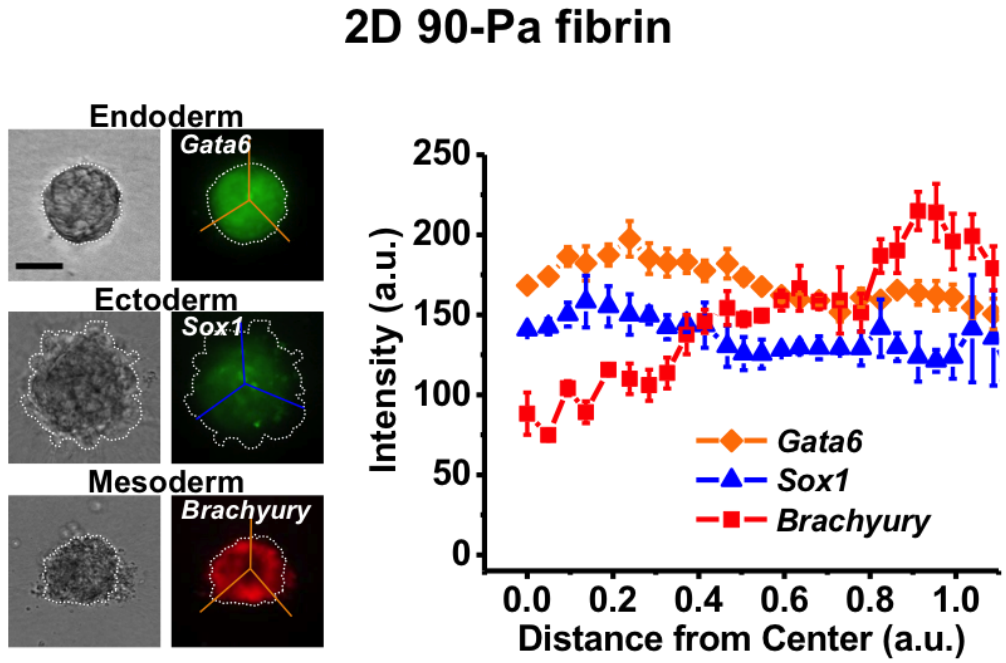

**Supplementary Figure 2. Upregulation of germ layer markers in the absence of LIF. (a)** Total mRNA samples of mESC colonies at day 5 maintained under conditions indicated on the top were extracted and analyzed using RT-PCR for germ layer markers indicated on the left. Cells cultured on regular gelatin coated 2D rigid plastic dishes in the presence of LIF (2D Rigid Dish) were used as a control. Bottom GAPDH gene shows a representative side marker with each size indicated by an arrow. **(b)** Semi-quantitative RT-PCR results shown in (a) were quantified by analyzing the fluorescence intensity of each PCR product on the gel. Markers of mesoderm, endoderm, and ectoderm are all expressed significantly higher in ESC colonies cultured in soft fibrin gel under –LIF conditions than those on 2D rigid dish, whereas *Oct4* expression was lower in ESC colonies maintained under 3D fibrin –LIF conditions. No changes in *Nanog*, *Sox2*, and *Otx2* were observed. Mean+/-s.e.m; n=3 samples for each condition. *p<0.05, **p<0.001, compared with control +LIF 2D rigid dish cells.



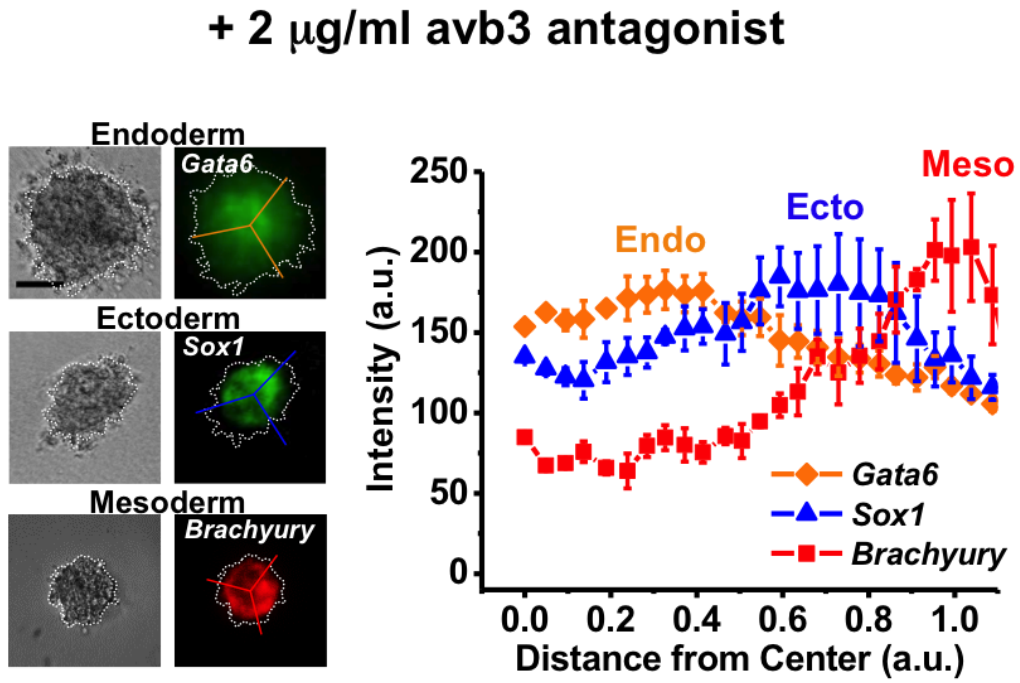

**Supplementary Figure 3. Sorting of ectoderm and endoderm cells based on E-cadherin expression.** (Left) Fluorescence-activated cell sorting (FACS) plot of control wild-type (W4) cells and (Right) cells isolated from –LIF soft fibrin gel cultured embryoid colony stained for E-cadherin. The gating used to identify FITC E-cadherin expression (530nm) is determined according to the result from sorting the W4 control wild-type mESCs that do not have any fluorescence. R17 is the gate used to isolate low E-cadherin expressing endoderm cells. R18 is the gate used to isolate high E-cadherin expressing ectoderm cells. Mid-level E-cadherin FITC labeled cells were not used to avoid potential mixture of ectoderm and endoderm cells. The isolated endoderm cells with low E-cadherin and ectoderm cells with high E-cadherin expression were used for real time quantitative RT-PCR analysis shown in Fig. 2.



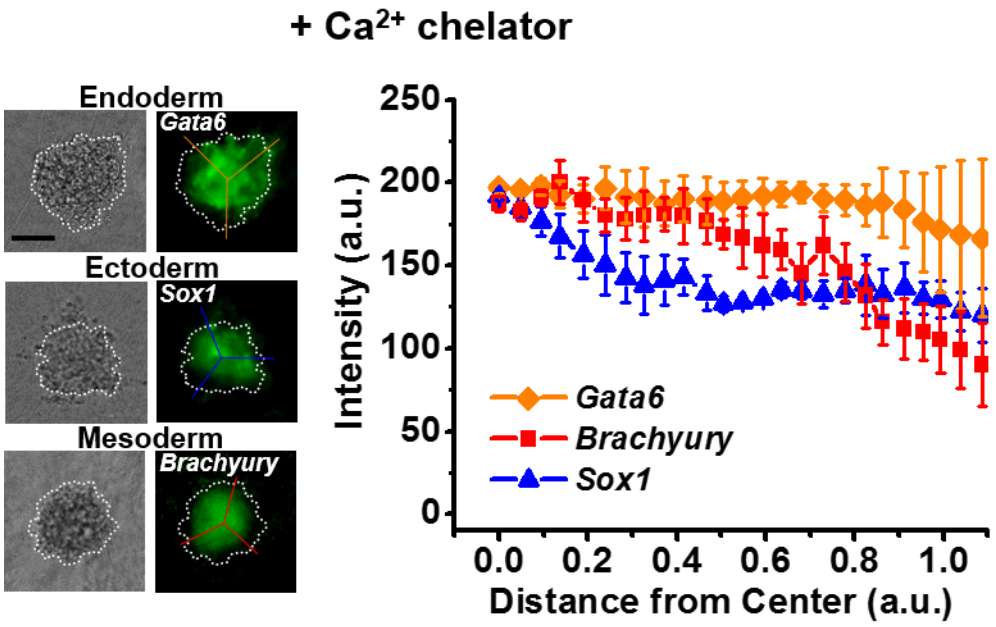

**Supplementary Figure 4. Immunofluorescence staining of embryoid bodies to detect the expression of germ layer markers. Left**: bright field images of colonies. **Right**: representative immunofluorescence images of the embryoid bodies immunostained for Gata6 (endoderm), Sox1 (ectoderm), and Brachyury (mesoderm). Embryoid bodies did not have distinct organization of three germ layers. At least 10 other EBs showed similar results. Scale bar = 50μm.



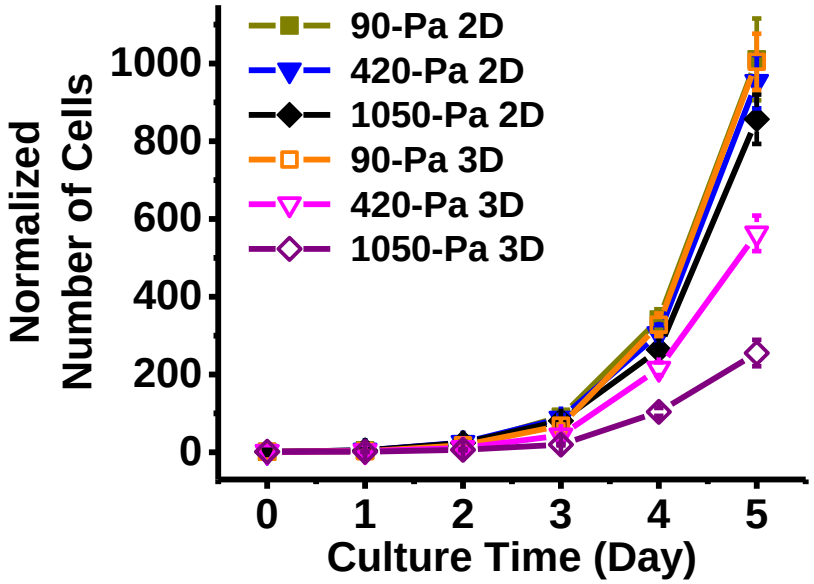

**Supplementary Figure 5. 2D soft fibrin gels do not promote the formation of distinctly organized endoderm and ectoderm layers. Left**: bright field (left) and fluorescence (right) images of colonies cultured for 5 days in –LIF conditions from single ESC on 2D soft fibrin gels. Scale bar = 50μm. **Right**: summarized data from a representative colony showing the localization of Brachyury-positive cells at the outer peripheral layer but no unique or distinct localization of Gata6- and Sox1-positive cells within the colony. Mean+/-s.e.m. Similar immunofluorescence staining results were obtained from at least 3 independent experiments.



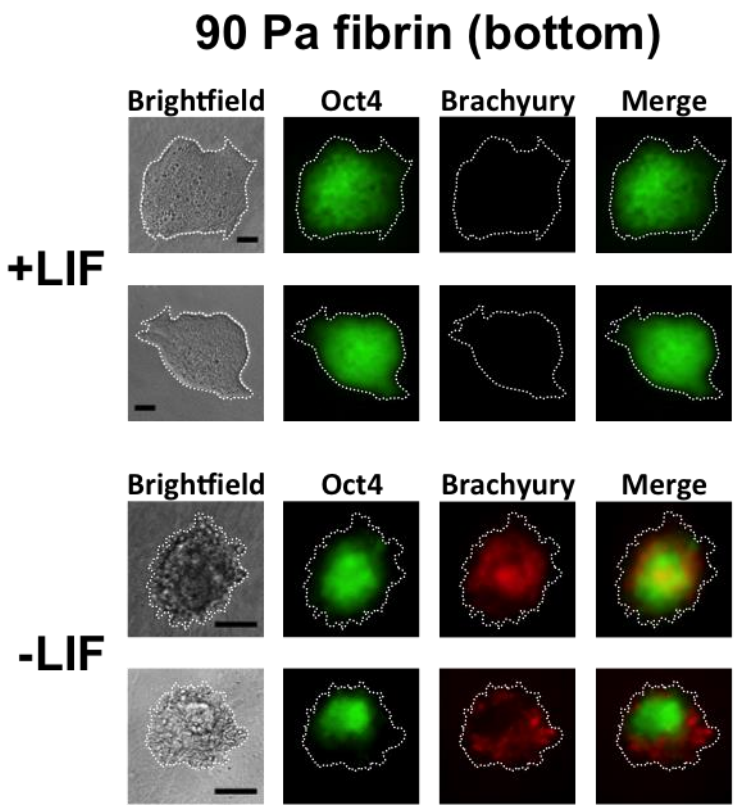

**Supplementary Figure 6. Engagement of fibrin with αvβ3 integrins is important for positioning of the endoderm layer. Left**: brightfield (left) and fluorescence (right) images of ESC colonies 5 days after treatment with 2μg/ml αvβ3 antagonist. Scale bar = 50μm. **Right**: summarized data showing the broad localization of Gata6-positive cells between the inner and the middle layers within the colony. When a 10-fold higher concentration of the antagonist was used, a distinct layer of Gata6-positive cells appeared at the outer periphery of the colonies (see Fig. 1d). Mean+/-s.e.m. Similar results were obtained from at least 2 other independent experiments.



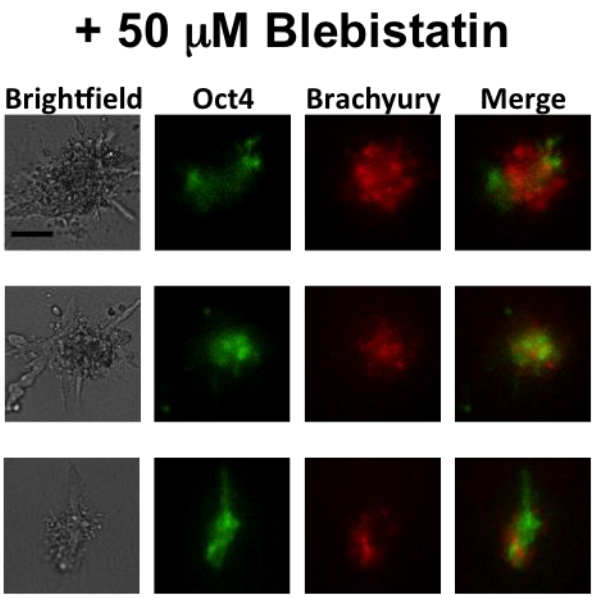

**Supplementary Figure 7. Cell-cell engagement is necessary for the formation of organized germ layers. Left**: Representative bright field (left) and fluorescence (right) images of colonies cultured for 5 days in –LIF conditions from single ESC in 3D soft fibrin gels with 1mM EGTA (calcium chelator), to block the cell adhesion molecule, cadherins. Scale bar = 50µm. **Right**: summarized data from a representative colony showing no distinct organization of all three germ layers. There is no unique localization of Gata6-, Brachyury-, and Sox1-positive cells within the colony. Mean+/-s.e.m. Similar immunofluorescence staining results were obtained from at least 3 independent experiments.



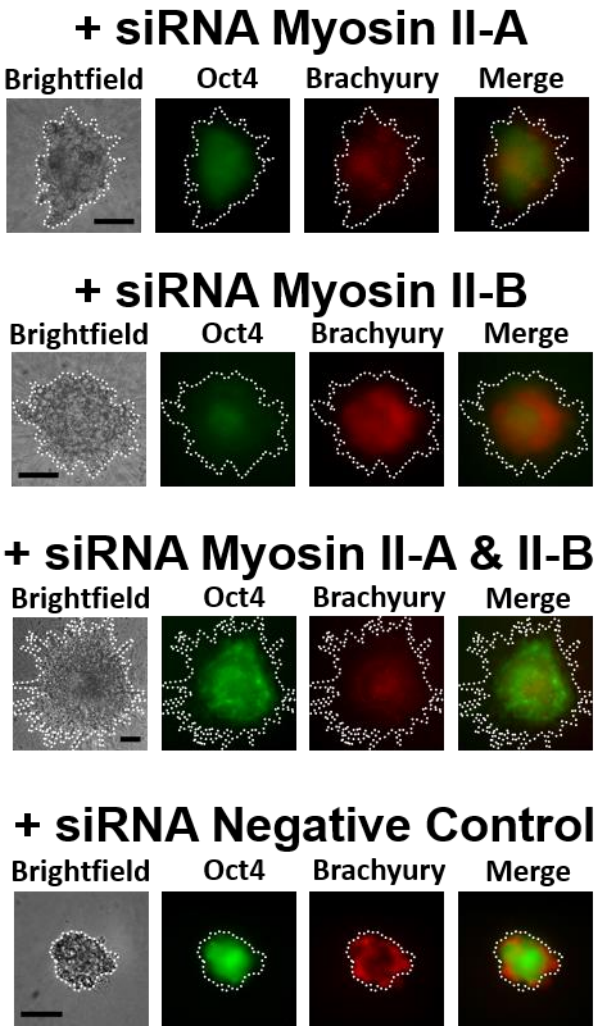

**Supplementary Figure 8. Proliferation of ESCs on 2D and in 3D matrix of varying elasticity.** Cells cultured on 2D fibrin matrix had similar high rate of proliferation as those in 3D soft fibrin gels of 90-Pa. For 3D fibrin gel culture, ESCs proliferation rate decreases as matrix elasticity increases. All cells were cultured in +LIF condition. The number of cells at each day was normalized to that on day 0. Comparing the number of cells at day 5: $p>0.96$ between 2D 90-Pa & 3D 90-Pa; $p>0.76$ between 2D 420-Pa & 3D 90-Pa; $p>0.42$ between 2D 1050-Pa & 3D 90-Pa; $p<0.002$ between 3D 420-Pa & 3D 90-Pa, 3D 1050-Pa & 3D 90-Pa. n=3 experiments for each matrix condition. Mean+/-s.e.m.



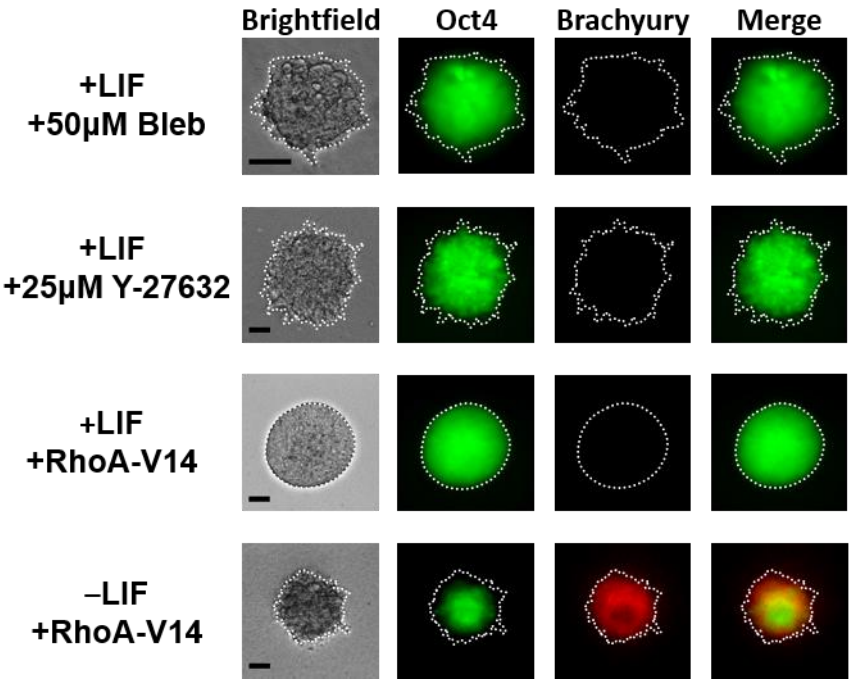

**Supplementary Figure 9. Matrix rigidity dictates colony roundedness.** Representative brightfield (left column) and fluorescence (2nd, 3rd and right columns) images of colonies grown from single OGTR1 ES cell for 5 days at the bottom of the gel near the rigid dish (~2 μm above), under +LIF (top 2 colonies) or –LIF conditions (bottom 2 colonies). **2nd column**: expression of the pluripotency marker Oct4. **3rd column**: expression of the mesodermal marker Brachyury. **Right column**: merged images of 2nd and 3rd columns. Note that these colonies are not round compared with those in the same gel stiffness in Fig. 1a. Scale bars = 50μm. These results are representative of >3 replicates.



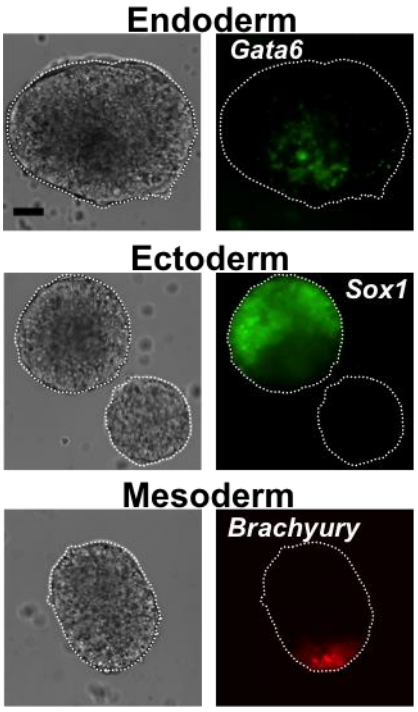

**Supplementary Figure 10. Myosin-II dependent tension is necessary for germ layer organization.** Representative bright field (left column) and fluorescence (2nd, 3rd and right columns) images of 3 different colonies treated with 50 μM Blebistatin cultured under 3D soft fibrin –LIF conditions for 5 days. No distinct organization of Brachyury-positive mesodermal germ layer cells was observed compared to non-treated cells in Fig. 1b. Scale bar = 50μm. These results are representative of >3 experimental replicates.



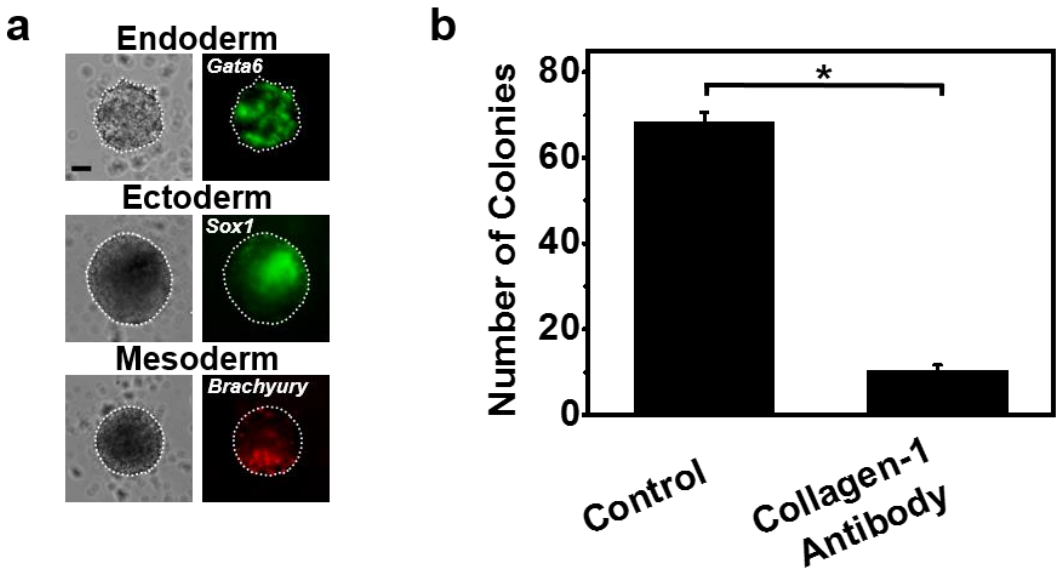

**Supplementary Figure 11. Silencing Myosin-II inhibits organization of germ layers.** Representative bright field (left column) and fluorescence (2nd, 3rd and right columns) images of colonies after siRNA knockdown of Myosin II-A and Myosin II-B under 3D soft fibrin –LIF conditions for 5 days. No distinct organization of Brachyury-positive mesodermal cells was observed when Myosin II-A, Myosin II-B, and Myosin II-A & II-B were silenced. Instead colonies exhibit dendritic morphology similar to ROCK inhibitor Y-27632 treatment as in Fig 7e. Colonies transfected with negative control siRNA formed a mesoderm layer at the outer periphery, same as those in Fig. 1b, c. Scale bar = 50μm. These results are representative of >3 experimental replicates.



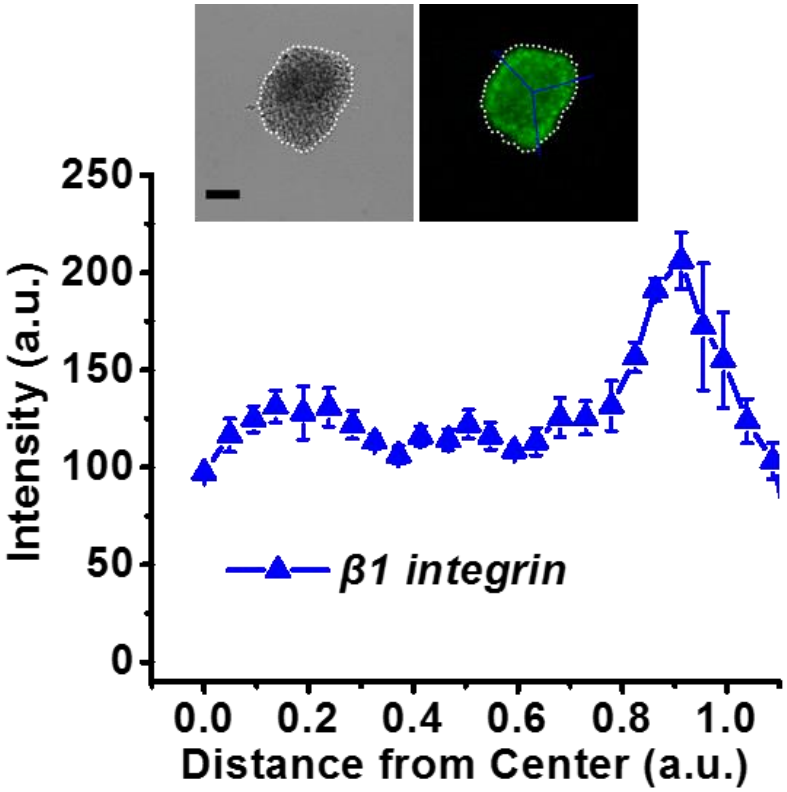

**Supplementary Figure 12. Tension modulation impacts colony morphology.** Representative bright field (left column) and fluorescence (2nd, 3rd and right columns) images of colonies treated with 50 μM Blebistatin **(top row)** to inhibit Myosin-II and 25 μM of Y-27632 **(second row)** to inhibit ROCK cultured under 3D soft fibrin +LIF conditions for 5 days. Despite losing its colony tension and spherical morphology, no Brachyury-positive cells were observed. No differentiation and spreading of ESCs was observed when ESC colony tension was upregulated in the presence of LIF by a constitutive active mutant of RhoA (RhoA-V14) **(third row)**. When ESC colony tension was upregulated in the absence of LIF by a constitutive active mutant of RhoA (RhoA-V14) **(bottom row)**, a distinct Brachyury-positive mesodermal cell layer was observed at the outer periphery, similar to those non-treated, –LIF cells in Fig. 1b. Scale bar = 50μm. Note that the cell in the second row is in +LIF condition whereas the cells in Fig 6e are in –LIF condition. These results are representative of >3 experimental replicates.



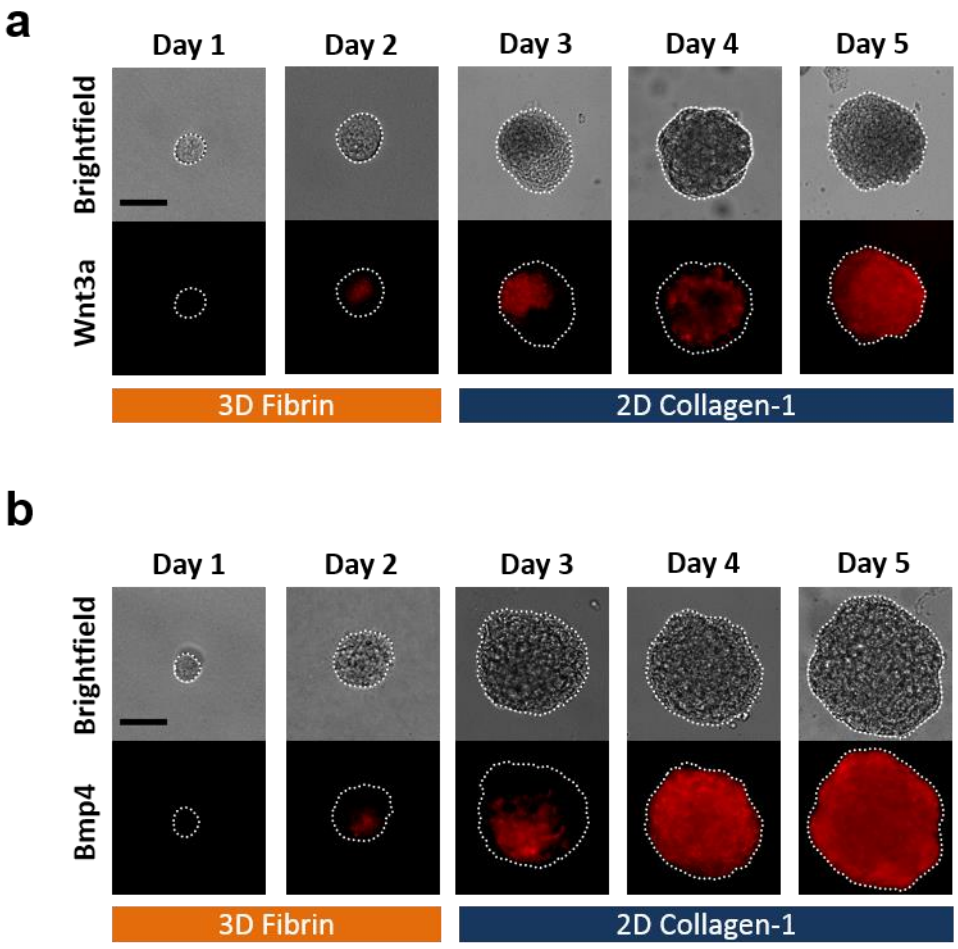

**Supplementary Figure 13. Colony anchorage to fibronectin does not yield proper organization of three germ layers.** Representative brightfield (left) and fluorescence (right) images of colonies that were transferred at Day 2.5 from 3D soft fibrin gels to 1-kPa 2D fibronectin-coated polyacrylamide gels. Before being transferred, cells were cultured in 3D fibrin gels with LIF for the first 11 hours before switching to –LIF condition (as shown in Fig. 7a). At Day 5, despite some segregation of cell populations, colonies showed no proper organization of all three germ layers. There were no distinct organization of Gata6-, Brachyury- and Sox1-positive cells. >1000 colonies exhibited similar disorganized germ layers. Scale bar = 50μm. These results are representative of >3 experimental replicates.



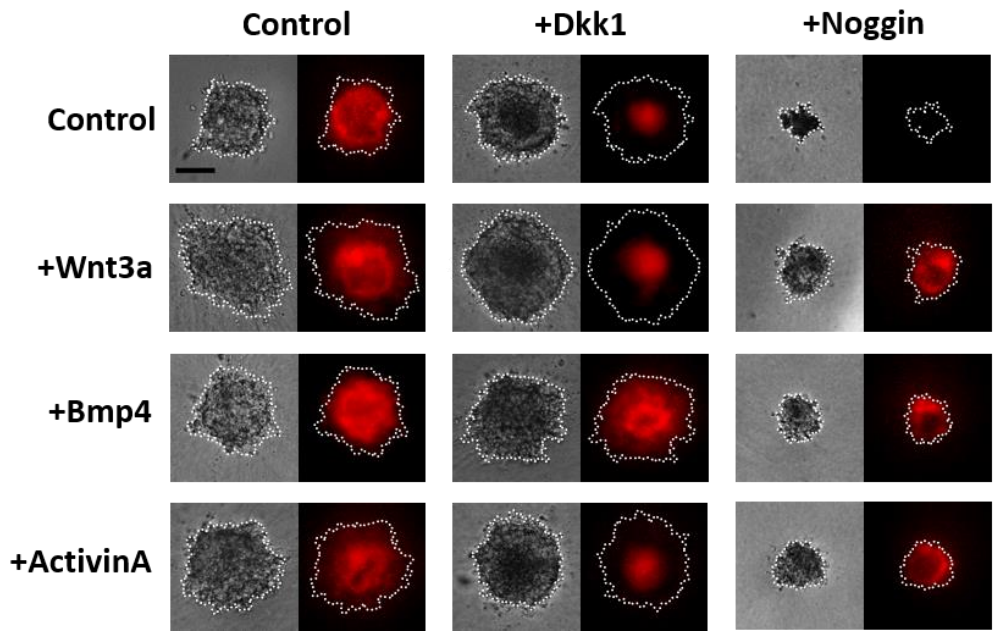

**Supplementary Figure 14. Anchorage of the ESC colony on to Collagen-1 is necessary for proper organization of all three germ layers.** Single OGTR1 ES cells were cultured in 3D fibrin matrix for 2.5 days before transferred to 2D collagen-1-coated polyacrylamide substrates of ~1 kPa stiffness in a –LIF medium. **(a)** Representative brightfield (left) and fluorescence (right) images of colonies that were incubated in a medium containing anti-collagen-1 antibody (5 μg/ml final concentration) before being transferred to the 2D substrate. Despite some segregation of cell populations, no proper organization of all three germ layers were observed (>100 colonies for each layer in separate experiments). Addition of a control antibody (anti-fibronectin antibody, at 25 μg/ml final concentration) did not have any effect on colony attachment on collagen-1 coated PA gels. Scale bar = 50μm. **(b)** Average number of colonies attached to the 2D collagen-1-coated polyacrylamide substrate. About 100 colonies were rinsed with PBS and counted 6 hours after transferred to the collagen-1-coated substrates. A significantly lower number of colonies were found adhered to the substrate after being incubated in the medium containing anti-collagen-1 antibody. The control indicates colonies that were not incubated with anti-collagen-1 antibody before the transfer. Mean+/-s.e.m; n=3 dishes for each condition; *$p<0.01$.



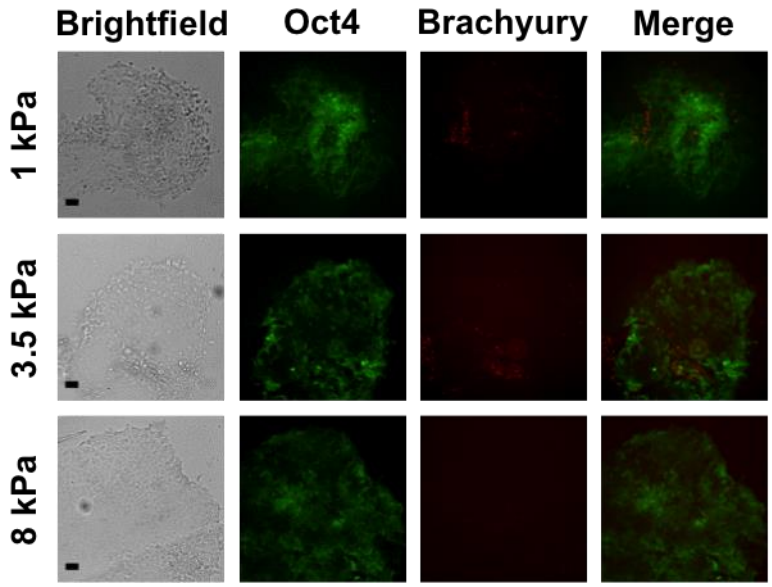

**Supplementary Figure 15. β1 integrin protein expression is higher in cells at the outer layer.** Representative bright field and immunofluorescence image of a colony stained for β1 integrin (top). Scale bar = 50μm. Summarized data from the representative colony show a distinct localization of β1 integrin at the outer layer (bottom), similar to the location of the ectoderm layer in Fig 7c & 7d. Cells were cultured as described in Fig. 7a. Mean+/-s.e.m. Similar immunofluorescence staining results were obtained from >3 independent experiments.



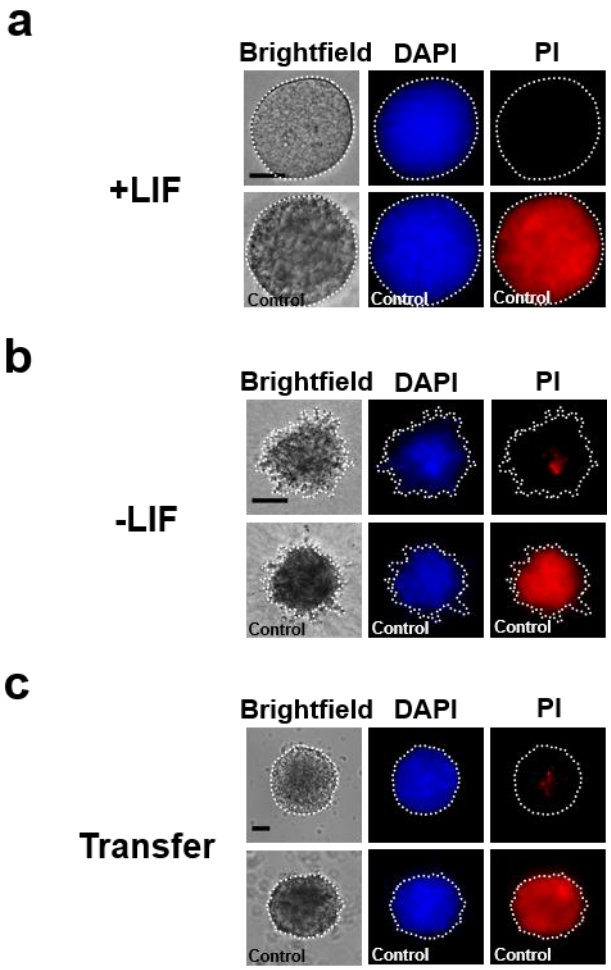

**Supplementary Figure 16. Time course immunofluorescence staining of Wnt3a and Bmp4.** ESCs were cultured as described in Fig. 7a and stained at different days. Immunofluorescence staining shows localized **(a)** Wnt3a and **(b)** Bmp4 segregate at one end of the colony at Day 3, before being dispersed throughout the whole colony at Day 4 and 5. ~70% out of >200 colonies showed similar results. These results are representative of >3 experimental replicates. Scale bars = 50μm.



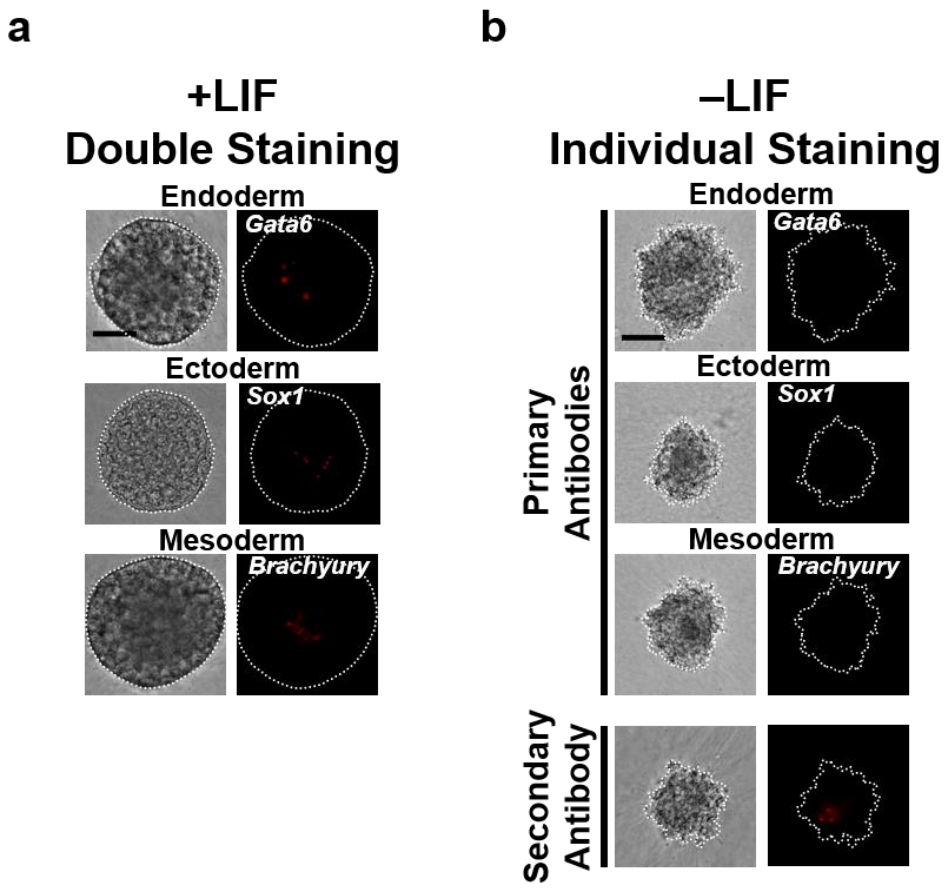

**Supplementary Figure 17. Wnt signaling influences germ layer organization.** Single ESC was cultured with the respective proteins in –LIF condition for 5 days. Brachyury expression was at the outer layer when cells were not treated (>90% of 50 colonies). Addition of Wnt3a (10 ng/ml), Bmp4 (12.5 ng/ml), or ActivinA (12.5 ng/ml) caused the mesoderm cells to form the middle layer (90%, 83%, 70%, respectively, n=40 colonies). In the presence of the Wnt antagonist, Dkk1 protein (60 ng/ml), Brachyury expressing cells were at the center core of colony (83% of 40 colonies), even when Wnt3a (67% of 40 colonies) or ActivinA (100%, of 40 colonies) were added with Dkk1. Cells that were concurrently treated with Dkk1 and Bmp4 formed a middle mesoderm layer (73% of 40 colonies). The Bmp antagonist, Noggin (12.5 μg/ml)[1] treatment with Wnt3a, Bmp4, or ActivinA (70%, 75%, 65%, respectively of 40 colonies each) gave rise to colonies that were smaller with Brachyury expression at the outer peripheral layer. Noggin treatment alone for 5 days caused cells to undergo apoptosis (100% of 40 colonies), as verified with Propedium Iodide staining (not shown). Results were pooled from >4 independent experiments. Scale bar = 50μm.




**Supplementary Figure 18. LIF at the early stage of colony growth in 3D is necessary for germ layer organization on 2D.** Representative brightfield (left column) and fluorescence (2nd, 3rd and right columns) images of colonies grown from single OGTR1 ES cell maintained under conditions described in Fig. 7a. **2nd column**: expression of the pluripotency marker Oct4. **3rd column**: expression of the mesodermal marker Brachyury. **Right column**: merged images of 2nd and 3rd columns. Colonies cultured in 3D Fibrin without LIF for 2.5 days spread within 24 hours after re-plated onto 2D substrates and did not generate proper mesoderm layers. Scale bars = 50μm. These results are representative of >3 experimental replicates.




**Supplementary Figure 19. Apoptosis staining.** Brightfield (left) and fluorescence (middle and right) images of single ES cells cultured under conditions indicated on the left for 5 days are shown. Cell nuclei were stained with DAPI and Propedium Iodide (PI) to distinguish apoptotic cells that are labeled with PI. Controls indicate colonies that have been fixed with methanol. **(a)** A representative colony cultured in 3D fibrin gel +LIF conditions for 5 days, similar to those presented in Fig 1a. **(b)** A representative colony cultured in 3D fibrin gel –LIF conditions for 5 days, similar to those presented in Fig 1b. **(c)** A representative colony cultured in 3D fibrin gels for 2.5 days before transferred to 2D collagen-1 coated polyacrylamide gels, similar to those presented in Fig 7. Little to no staining of PI indicates that almost all of the cells examined are healthy and alive. Fixed colonies are completely stained by PI. Scale bars = 50μm. These results are representative of >3 experimental replicates.




**Supplementary Figure 20. Negative control immunofluorescence.** Brightfield (left) and fluorescence (right) images of single ES cells cultured under conditions indicated at the top for 5 days are shown. **(a)** Representative colonies cultured in 3D fibrin gel +LIF conditions for 5 days were stained for endoderm, ectoderm, and mesoderm. Little to no staining of germ layers indicate that cells were mostly undifferentiated. Note that there are some negligible fluorescent spots. **(b)** Representative colonies cultured in 3D fibrin gel –LIF conditions for 5 days were treated with primary or secondary antibodies alone. No fluorescence is observed in primary antibody staining alone. Small fluorescent spots were observed in colonies stained with secondary antibody alone, similar to those in (a). This indicates that there are some small fluorescent spots due to nonspecific staining of unwashed out secondary antibodies. Scale bars = 50μm. W4 cells were used in all figures. These results are representative of >3 experimental replicates.

**Supplementary Table 1. Primer sequences used for Reverse Transcription PCR analysis of ESCs.**

| Reverse Transcription PCR | | |
|---|---|---|
| Mus Nanog | 5' primer | GTCTGCTACTGAGATGCTCTG |
| | 3' primer | CTTGCACTTCATCCTTTGG |
| Mus Oct3/4 | 5' primer | CATACTGTGGACCTCAGGTT |
| | 3' primer | CTCACACGGTTCTCAATGCT |
| Mus Sox2 | 5' primer | CAAGACGCTCATGAAGAAGG |
| | 3' primer | AGTGGGAGGAAGAGGTAACC |
| Mus Fgf5 | 5' primer | ATGAGCCTGTCCTTGCTCTT |
| | 3' primer | TCGTGGGAGCCATTGACTTT |
| Mus Otx2 | 5' primer | GACGTTCTGGAAGCTCTGTT |
| | 3' primer | ATGGTTGGGACTGAGGTACT |
| Mus Sox1* | 5' primer | CCAAGAGACTGCGCGCGCTG |
| | 3' primer | GGGTGCGCCGGGTGTGCGTG |
| Mus Hand1 | 5' primer | GCTACGCACATCATCACCAT |
| | 3' primer | GATCTTGGAGAGCTTGGTGT |
| Mus Brachyury (T) | 5' primer | CTGCCTACCAGAATGAGGAG |
| | 3' primer | GAGAACCAGAAGACGAGGAC |
| Mus Twist2 | 5' primer | AGAGCGACGAGATGGACAAT |
| | 3' primer | TGTTCTGAGAGCCTTGGTC |
| Mus Gata4 | 5' primer | CAGAAGGCAGAGAGTGTGTC |
| | 3' primer | AGTGGCATTGCTGGAGTTAC |
| Mus Gata6 | 5' primer | CCTCATCAAGCCACAGAAGC |
| | 3' primer | CCAGAGCACACCAAGAATCC |
| Mus Sox17 | 5' primer | ATGGCACGGAATCCAACCAG |
| | 3' primer | GAGCAAGTCCCTCAAGGCAT |
| Mus GAPDH | 5' primer | AATTCAACGGCACAGTCAAGG |
| | 3' primer | GTGGCAGTGATGGCATGGA |

(*, Sox1 sequence was obtained from Abranches et. al.[2])

**Supplementary Table 2. Primer sequences used for Real Time quantitative RT-PCR analysis of ESCs.**

| Real-Time PCR | | |
|---|---|---|
| Mus Fgf5 | 5' primer | GCCTGTCCTTGCTCTTCCTCAT |
| | 3' primer | GGAGAAGCTGCGACTGGTGA |
| Mus Otx2 | 5' primer | GCGAAGGGAGAGGACGACTTT |
| | 3' primer | CTGCTGTTGGCGGCACTTAG |
| Mus Sox1 | 5' primer | GCCGAGTGGAAGGTCATGTC |
| | 3' primer | TTGAGCAGCGTCTTGGTCTTG |
| Mus Pax6 | 5' primer | ACCAGTGTCTACCAGCCAATCC |
| | 3' primer | GCACGAGTATGAGGAGGTCTGA |
| Mus Hand1 | 5' primer | CACCACCTACCACCGCAGTA |
| | 3' primer | CCTTCTTGGGTCCTGAGCCTTT |
| Mus Brachyury (T) | 5' primer | TCTCTGGTCTGTGAGCAATGGT |
| | 3' primer | TGCGTCAGTGGTGTGTAATGTG |
| Mus Twist2 | 5' primer | CAGTGAGGAAGAGCTGGAGAGG |
| | 3' primer | CTGGATCTTGCTGAGCTTGTCA |
| Mus FoxA2 | 5' primer | AGCACCATTACGCCTTCAAC |
| | 3' primer | CCTTGAGGTCCATTTTGTGG |
| Mus Mixl1 | 5' primer | TCCTCCATTGCCCTGCTCCT |
| | 3' primer | ACGCCTCCTCCAGTCATGCT |
| Mus Gata4 | 5' primer | CAGCAGCAGCAGTGAAGAGATG |
| | 3' primer | ACCAGGCTGTTCCAAGAGTCC |
| Mus Gata6 | 5' primer | TCTACACAAGCGACCACCTCAG |
| | 3' primer | GCCAGAGCACACCAAGAATCC |
| Mus Sox17 | 5' primer | ATACGCCAGTGACGACCAGAG |
| | 3' primer | ACCACCTCGCCTTTCACCTTTA |
| Mus α1 | 5' primer | CCACCAAGATGAACGAGCCTCT |
| | 3' primer | GATCCGATCTGCTCTCCACTGA |
| Mus α2 | 5' primer | CTCCTGCTGCGGCTGCTAAT |
| | 3' primer | GCCACTCCACGGTGAACCAA |
| Mus α5 | 5' primer | CTTGCTGGACTGTGGTGAAGAC |
| | 3' primer | GTCAGGTTCAGTGCGTTCTTGT |
| Mus αv | 5' primer | GCCATCCGACGAGCACTGTT |
| | 3' primer | CCGTTCTCTGGTCCAACCGATA |
| Mus β1 | 5' primer | GGCGTGGTTGCTGGAATTGTT |
| | 3' primer | CACAGTTGTCACGGCACTCTTG |
| Mus β3 | 5' primer | ATATCCTGGTGGTCCTGCTGTC |
| | 3' primer | ACTTGGCTCTGGCTCGTTCTT |
| Mus Wnt3 | 5' primer | GAACCGTCACAACAATGAGGCT |
| | 3' primer | CACCATCTCCGAGGCACTGT |
| Mus E-cad | 5' primer | ACCAGCAGTTCGTTGTTGTCAC |
| | 3' primer | GGTTCCTCGTTCTCCACTCTCA |
| Mus GAPDH | 5' primer | AGGTCGGTGTGAACGGATTTG |
| | 3' primer | TGTAGACCATGTAGTTGAGGTCA |

## References


1. ten Berge, D. *et al*. Wnt Signaling Mediates Self-Organization and Axis Formation in Embryoid Bodies. *Cell Stem Cell* **3**, 508-518 (2008).

2. Abranches, E. *et al*. Neural Differentiation of Embryonic Stem Cells In Vitro: A Road Map to Neurogenesis in the Embryo. *Plos One* **4**, e6286 (2009).