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SHORT REPORT

Micropatterning of human embryonic stem cells dissects the mesoderm and endoderm lineages

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Abstract Human pluripotent cells such as human embryonic stem cells (hESC) are a great potential source of cells for cell-based therapies; however, directing their differentiation into the desired cell types with high purity remains a challenge. The stem cell microenvironment plays a vital role in directing hESC fate and we have previously shown that manipulation of colony size in a serum- and cytokine-free environment controls self-renewal and differentiation toward the extraembryonic endoderm lineage. Here we show that, in the presence of bone morphogenetic protein 2 and activin A, control of colony size using a microcontact printing technology is able to direct hESC fate to either the mesoderm or the endoderm lineage. Large, 1200-μm-diameter colonies give rise to mesoderm, while small 200-μm colonies give rise to definitive endoderm. This study links, for the first time, cellular organization to pluripotent cell differentiation along the mesoderm and endoderm lineages.

Introduction

For pluripotent human cells such as embryonic stem cells (hESC) to be used as a source for cell-based therapies, protocols need to be developed that can guide their differentiation into specific cell types with high purity. In particular, transplantable tissues derived from the mesoderm (e.g., hematopoietic cells) and endoderm (e.g., insulin-secreting β cells) are of immediate interest for treating blood disorders and diabetes. We have previously shown that controlling hESC colony size using microcontact printing is able to direct hESC fate in a serum- and cytokine-free environment in which large colonies show increased self-renewal and small colonies differentiate into extraembryonic endoderm (Peerani et al., 2007). Moreover, previous studies have shown that bone morphogenetic proteins (BMP) and activin/nodal are able to induce primitive streak-like differentiation of ESC (Ng et al., 2005a; Nostro et al., 2008). These studies, however, demonstrate that under these conditions a mixture of mesoderm- and endoderm-fated cells emerges when, ideally, a pure population is desired for cell-based therapies (Shiraki et al., 2008; Tada et al., 2005; Yang et al., 2008).

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Here we show that activin A and BMP2 act synergistically to induce the expression of the primitive streak transcription factors Brachyury (Bry) and Mix 1 homeobox-like 1 (Mixl1). However, the expression of mesoderm-specific and endoderm-specific genes is also upregulated, indicating the appearance of both mesoderm- and endoderm-fated cells. Having shown that colony size is able to regulate hESC differentiation in a serum- and cytokine-free environment, we hypothesized that controlling colony size might also be able to selectively guide these primitive streak-like cells to either mesoderm or definitive endoderm lineages. In the presence of activin A and BMP2 we found that smaller colonies direct hESC differentiation toward definitive endoderm and larger colonies lead to mesoderm differentiation. The functional relevance of controlling colony size and early mesendoderm differentiation was confirmed through hematopoietic and primitive gut differentiation assays, in which it was found that the larger colonies led to a 10-fold increase in hematopoietic colony-forming units (CFU), whereas the smaller colonies gave rise to FoxA2 (forkhead homeobox A2)-, HNF1β (hepatocyte nuclear factor 1β)-, and HNF4α-expressing primitive gut tube cells and the larger colonies did not. These results demonstrate the ability of colony size in the presence of inductive factors to selectively guide hESC differentiation to either endoderm or mesoderm in a serum-free environment.

Results

Activin A and BMP2 synergistically induce primitive streak-like development from hESC

To determine the optimal conditions for primitive streak-like differentiation of hESC, we treated hESC with varying concentrations of activin A and BMP2 for 48 h and measured the frequency of cells expressing Bry, a transcription factor expressed in the primitive streak of developing mouse embryos. Using quantitative immunofluorescent microscopy (see Supplementary Methods) we found that Bry expression increases with increasing BMP2 and activin A concentrations, with the greatest frequency of Bry expression (25.6 ± 0.6%) obtained at 50 ng/ml activin A and BMP2 (Figs. 1a and 1b). Quantitative gene expression analysis confirms the upregulation of Bry as well as Mixl1 expression. Mixl1 is another marker of primitive streak development (Fig. 1c). In addition

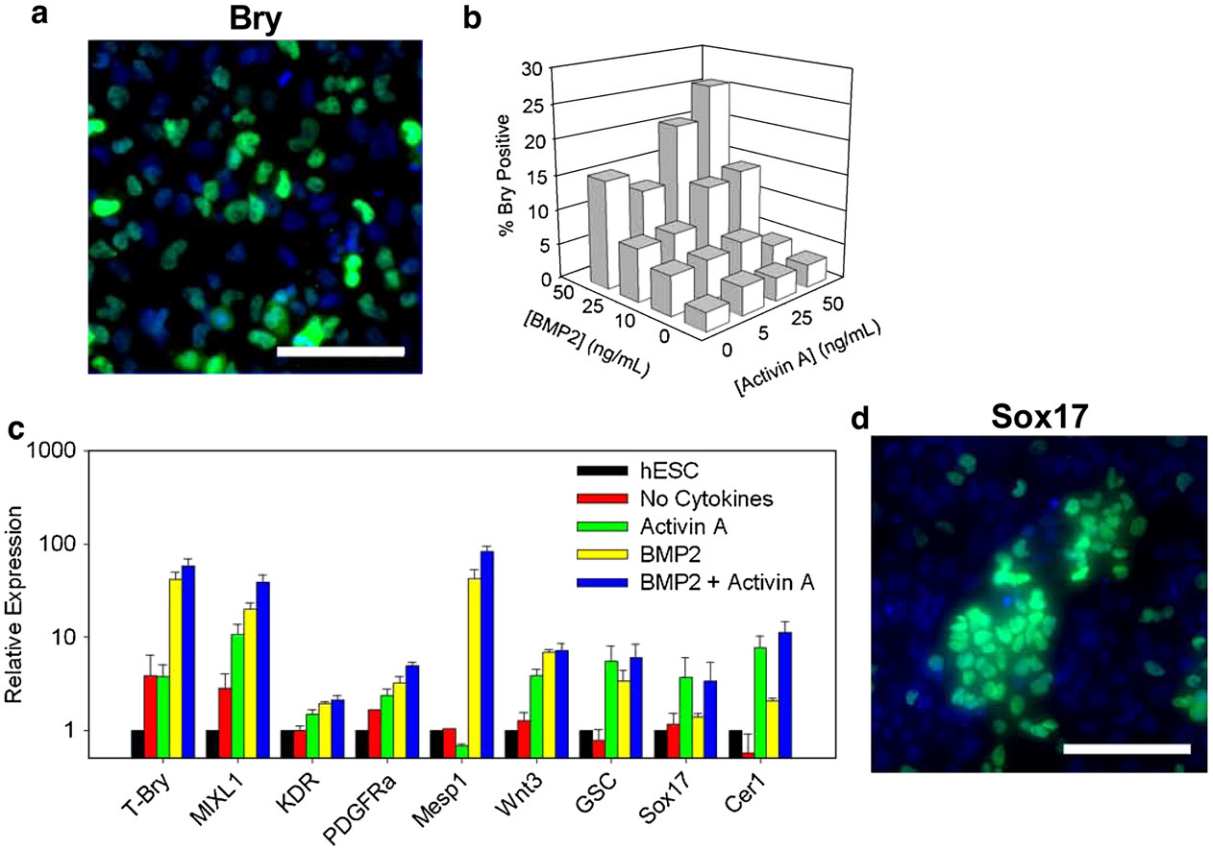

Figure 1 BMP2 and activin A interact to elicit the expression of both mesoderm- and endoderm-associated genes. (a) Immunofluorescence image of Bry expression following 48 h of treatment with 50 ng/ml BMP2 and activin A. (b) Frequency of Bry expression as a function of varying BMP2 and activin A concentrations; values represent the mean of three independent experiments. (c) Quantitative gene expression analysis showing upregulated expression of primitive streak markers as well as mesoderm- and endoderm-associated transcripts; values and error bars represent the mean and standard error of three independent experiments. (d) Immunofluorescence image showing Sox17 expression following 48 h of treatment with 50 ng/ml BMP2 and activin A. Original magnification: 40×. Scale bar, 100 μm. Images of negative control samples can be found in Supplementary Fig. 1.

to primitive-streak genes, the expression of mesoderm- (*KDR, Mesp1, Wnt3,* and *PDGFRa*) (Davis et al., 2008; Kennedy et al., 2007; Rivera-Perez and Magnuson, 2005; Saga et al., 1996) and endoderm-specific genes (*Sox17, GSC, Cer1*) (Izumi et al., 2007; Perea-Gomez et al., 1999) is also upregulated (Fig. 1c) and is confirmed through immunofluorescence showing protein expression of Sox17 (Fig. 1d). This demonstrates that both mesoderm and endoderm precursors arise in the presence of BMP2 and activin A and that further control of the ESC microenvironment is required to dissect the two populations.

Manipulating colony size controls mesoderm and endoderm differentiation of primitive streak-like cells

We previously showed that manipulating hESC colony size in the absence of exogenous inductive signals using microcontact printing can control hESC self-renewal and differentiation (Peerani et al., 2007). Here we used a similar technique to manipulate hESC colony size in the presence of BMP2 and activin A. hESC were dissociated into single cells and seeded onto islands of Matrigel with tightly controlled geometry to form colonies of defined shape and size. We examined colonies 200, 400, 800, and 1200 μm in diameter (Fig. 2a) and found that in the presence of BMP2 and activin A expression of endoderm-associated genes, *Sox17, GSC,* and *Cer1,* increases with decreasing colony size. Conversely, the expression of *Bry* and *KDR* is greatest in larger colony sizes and, together with low expression of endoderm markers, indicates mesoderm differentiation (Gadue et al., 2006; Nostro et al., 2008) in large colonies (Fig. 2b). Gene expression is further confirmed through protein level analysis using flow cytometry (Supplementary Methods), showing a 1.3- and 1.8-fold increases in Bry and KDR, respectively, as well as a 2.5-fold decrease in Sox17 expression frequency in large 1200-μm colonies compared to small 200-μm colonies (Fig. 2c). Surface expression of the mesoderm-associated protein PDGFRa (platelet-derived growth factor receptor-α, also known as CD140a) (Davis et al., 2008) was also analyzed by flow cytometry; however, greater expression was found in the small 200-μm colonies, contrary to the observed trend in Bry and KDR expression (Fig. 2c). Although

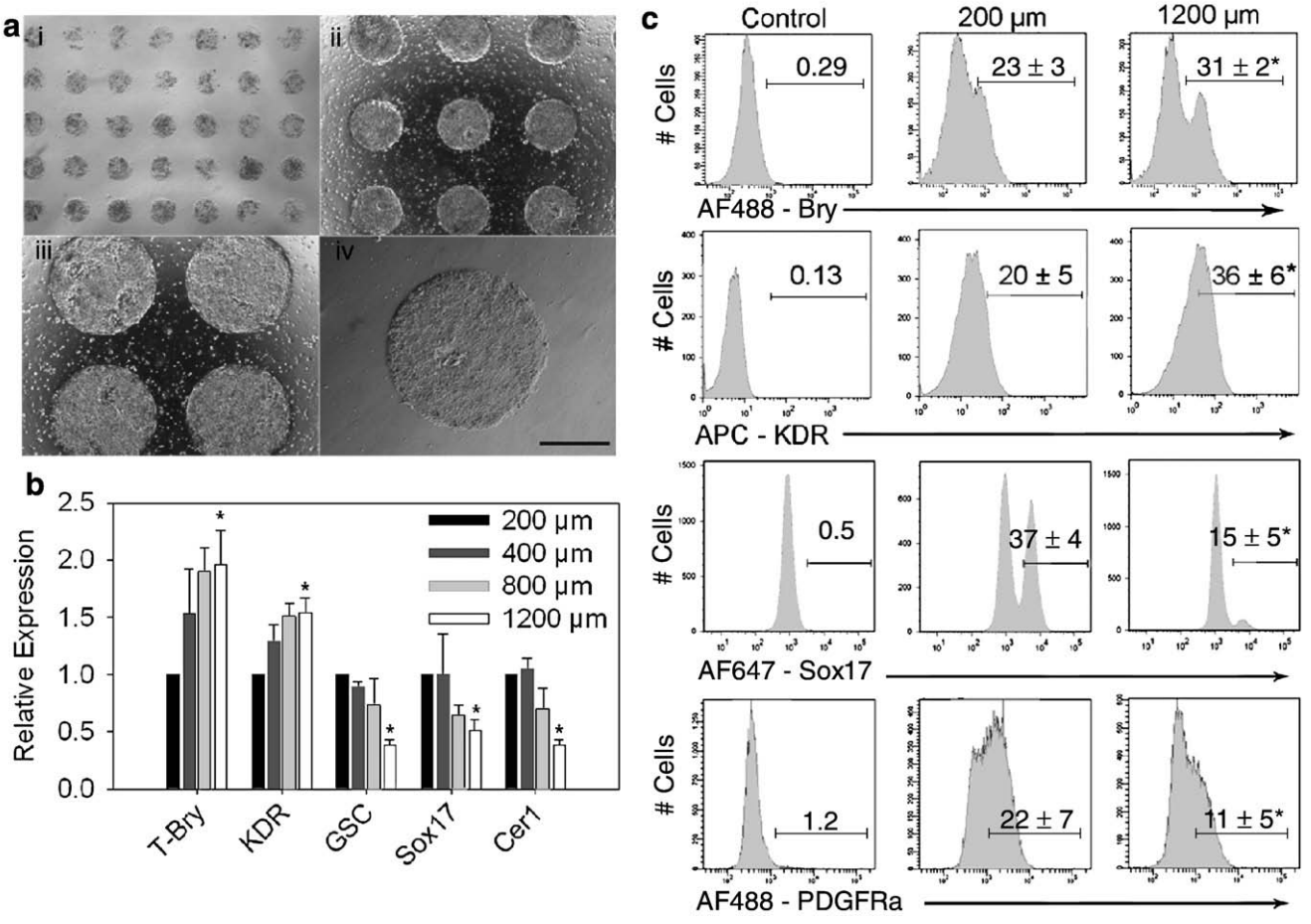

Figure 2 Control of colony size regulates mesoderm and endoderm differentiation of hESC. (a) Bright-field images of micropatterned hESC colonies ((i) 200, (ii) 400, (iii) 800, and (iv) 1200 μm in diameter). (b) Quantitative gene expression analysis of mesoderm- and endoderm-associated transcripts; values and error bars represent the mean and standard error of nine independent experiments. (c) Flow cytometry analysis of Bry, KDR, Sox17, and PDGFRa protein expression in 200- and 1200-μm colonies. Representative histograms are shown and numerical values represent the mean and standard error of at least three independent experiments. Original magnification: 4×. Scale bar, 500 μm. *Significance ($P<0.05$) of 1200- compared to 200-μm colonies.

the reason for this apparent discrepancy is not clear, together our data suggest that, while mesoderm differentiation is favored in large colonies, endoderm differentiation is favored in small colonies.

Larger, mesoderm-enriched colonies generate greater numbers of hematopoietic progenitors

We employed a serum-free hematopoietic differentiation assay to determine if the larger, mesoderm-enriched colonies possess greater capacity for hematopoietic differentiation. We used forced-aggregation embryoid body (EB) formation (Ng et al., 2005b; Ungrin et al., 2008) to initiate hematopoietic differentiation because it allows us to uniformly control the differentiation conditions under which the only parameter that is varied is the colony size during the initial 48-h primitive streak-like induction step. Following 9 days of suspension culture in serum-free medium supplemented with hemogenic cytokines, embryoid bodies derived from 200- and 1200-μm colonies were assessed for hematopoietic differentiation. Flow-cytometry analysis of hematoendothelial surface markers showed a 2.5-fold increase ($P=0.01$) in the frequency of cells coexpressing CD34, CD31, and KDR in embryoid bodies derived from larger 1200-μm colonies compared to the smaller 200-μm colonies (Fig. 3a). Moreover, colony-forming assays reveal a 10-fold increase ($P=0.04$) in the frequencies of hematopoietic CFU found in embryoid bodies formed from larger colonies compared to smaller colonies (Figs. 3b and 3c). Morphological analysis of EB-derived hematopoietic colonies using Wright–Giemsa staining confirms the presence of mixed myeloid lineage cells in selected colonies (Fig. 3d).

Smaller, endoderm-enriched colonies lead to greater primitive gut endoderm differentiation

To confirm that the larger colonies are deficient in endoderm precursors, while the smaller colonies are enriched, we employed a previously established endoderm differentiation protocol (D'Amour et al., 2006; Kroon et al., 2008). Following 2 days of treatment in activin A, cultures originating from 200-μm colonies express CXCR4 (chemokine (C-X-C motif) receptor 4), a marker of definitive endoderm (D'Amour et al., 2006; Yasunaga et al., 2005), at significantly greater

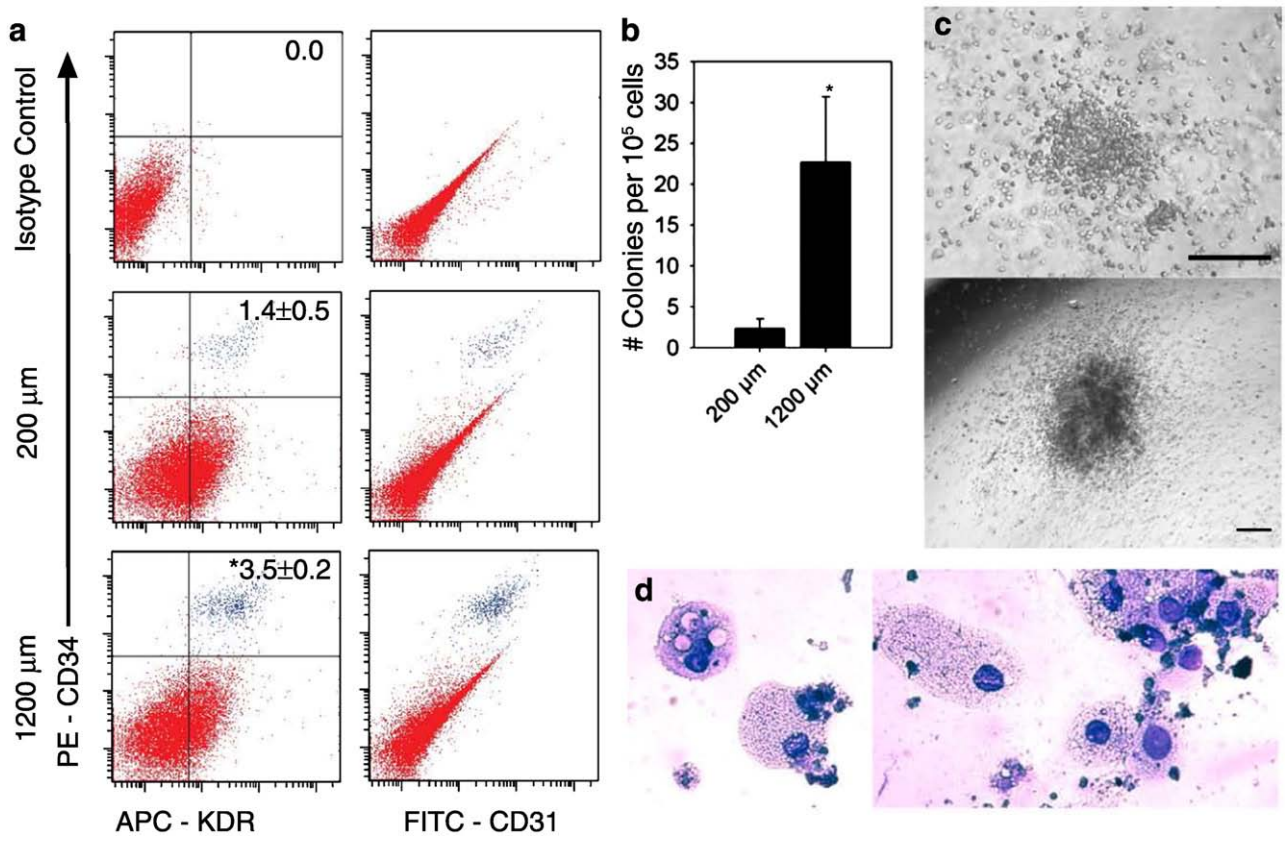

Figure 3 Larger, mesoderm-enriched colonies show greater hematopoietic differentiation potential. (a) Flow-cytometry analysis of EB derived from 200- and 1200-μm colonies following 9 days of suspension culture in hemogenic cytokines; values represent mean ± standard error, of five independent experiments, of the percentage coexpressing all three hematoendothelial surface markers, CD34, CD31, and KDR. (b) Hematopoietic colony-forming frequency of EB derived from 200- and 1200-μm colonies; values and error bars represent mean and standard error of three independent experiments. (c) Representative images of hematopoietic colonies. Original magnification: 4× and 10×. Scale bar, 200 μm. (d) Morphological analysis of cytospin preparations of EB-derived hematopoietic cells using Wright–Giemsa staining showing monocytes and macrophages. Original magnification: 40×. *Significance ($P<0.05$) of 1200- compared to 200-μm colonies.

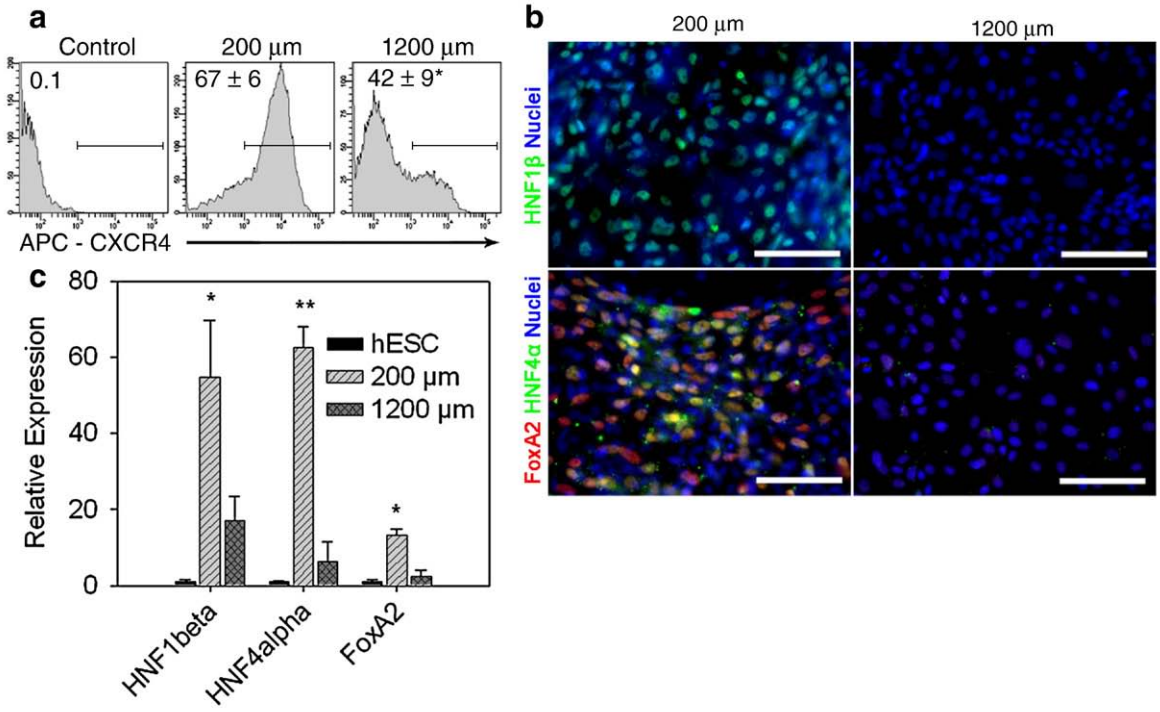

Figure 4 Smaller, endoderm-enriched colonies show greater primitive gut endoderm-forming potential. (a) Flow-cytometry analysis of CXCR4 expression following reseeding of micropatterned colonies and 2 days of culture in activin A. Representative histograms are shown and values represent mean and standard error of four independent experiments. (b) Immunofluorescence images of HNF1β, FoxA2, and HNF4α expression after further differentiation with FGF7 followed by Noggin, cyclopamine, and retinoic acid. (c) Quantitative gene expression analysis following endoderm differentiation assay. RNA isolated from hESC is shown as a negative control. Original magnification: 40× . Scale bar, 100 μm. Significance (*$P<0.05$ and **$P<0.01$) of 1200- compared to 200-μm colonies is indicated.

frequencies compared to the 1200-μm colonies as measured by flow cytometry (Fig. 4a). Moreover, after treatment with FGF7 followed by Noggin, cyclopamine, and retinoic acid, cultures originating from the 200-μm colonies express markers of primitive gut endoderm, including HNF1β (Coffinier et al., 1999), FoxA2 (Sasaki and Hogan, 1993), and HNF4α (Duncan et al., 1994), while cultures originating from the 1200-μm colony cultures do not (Fig. 4b). Protein expression was further confirmed through quantitative gene expression analysis showing greater expression of *Foxa2*, *HNF1β*, and *HNF4α* in cultures originating from the smaller 200-μm colonies compared to the larger 1200-μm colonies (Fig. 4c).

Discussion

The emergence of a primitive streak-like phenotype has been described from mouse and human ESC (Davis et al., 2008; Gadue et al., 2006; Nostro et al., 2008; Yang et al., 2008); however, the regulation of the further commitment of these cells remains challenging. Although fluorescent reporter cell lines combined with cell sorting are powerful tools that have been used to isolate target cell types from mixed cell populations (Davis et al., 2008; Yang et al., 2008), better technologies for the generation of specific mesoderm- and endoderm-derived cells are needed. Here we show that by precisely controlling colony size we are able to manipulate hESC fate to either mesoderm or endoderm lineages. Interestingly, this size-dependent control of cell fate occurs even in the presence of high concentrations of inductive factors (activin A and BMP2), suggesting an important role for endogenous signaling in pluripotent cell specification.

We have used the a blood progenitor cell assay (CFC) to determine if the apparent changes in mesoderm versus endoderm differentiation result in differences in the output of derivative tissues. We demonstrate that larger mesoderm-enriched colonies give rise to greater frequencies of CFC, while smaller endoderm-enriched colonies more effectively differentiate into primitive gut precursors. Although the hematopoietic CFC frequency reported here is lower than in previous reports (Chadwick et al., 2003; Ng et al., 2005a), this is probably due to suboptimal conditions for generating hematopoietic progenitors from mesoderm precursors under defined serum-free conditions. It is also likely that other mesoderm lineages, such as cardiac or somitic mesoderm, are being generated. Moreover, the markers used here to delineate mesoderm from endoderm lineages (Bry, KDR, GSC, Sox17, and Cer1) are not strictly confined to a particular germ layer, as reports have shown their expression in mesoderm, endoderm, and undifferentiated hESC (Kubo et al., 2004; Tada et al., 2005; Yang et al., 2008). Additionally, the observed trend in PDGFRa expression is seemingly contrary to what might be anticipated from previous reports demonstrating that PDGFRa marks mesoderm and hematopoietic precursors from hESC (Davis et al., 2008). However,

the report by Davis and colleagues found that the PDGFRa-negative population is still able to give rise to a significant PDGFRa-expressing population following further differentiation and that a significant number of hematopoietic colonies do arise from the initially PDGFRa-negative population. This suggests that PDGFRa expression is not an accurate predictor of hESC lineage commitment at the early time points examined here. In the experiments performed here, PDGFRa expression was lower in the early mesoderm-enriched population.

Although only transforming growth factor β (TGFβ) pathway activators were used to induce endoderm and mesoderm precursors, it is possible that other signaling pathways are also responsible for the colony-size-mediated effect described here. Wnt signaling is required for the *in vivo* primitive streak development of mouse embryos (Liu et al., 1999) and has been implicated in early lineage specification of hESC (Sumi et al., 2008); however, in the presence of activin A and BMP2 the addition of soluble Wnt ligand (Wnt3a, 50 ng/ml) did not increase Bry expression (data not shown). This is explained by the upregulation of Wnt3 expression (Fig. 1c), which is sufficient to allow primitive streak-like differentiation. Moreover, it has also been shown that RhoA activation mediated by cell shape and cytoskeletal tension regulates adipocyte and osteogenic differentiation of human mesenchymal stem cells (McBeath et al., 2004), but a detailed investigation of Rho signaling in hESC has yet to be conducted. Additionally, PI3-kinase signaling has been implicated in definitive endoderm differentiation of hESC (McLean et al., 2007). Further investigation is required to elucidate the role of these signaling pathways on the colony-size-mediated effect described here.

By controlling the colony size, we are able to engineer the response of hESC in the presence of exogenous cytokines. Combining this technology with strategies to specify mesoderm and endoderm cells using exogenous factors (Chadwick et al., 2003; Nostro et al., 2008; Yang et al., 2008; Zhang et al., 2008) may reduced heterogeneity in cell responses and increase target cell yields.

Materials and methods

Cell lines and cell culture

All experiments described were performed using the hESC lines H9, I6, and HES2. hESC were maintained on irradiated mouse embryonic feeder cells (mEF) in serum-free medium consisting of knockout (KO) Dulbecco's modified Eagle's medium (DMEM; Invitrogen) and 20% KO serum replacement (Invitrogen) supplemented with 4 ng/ml recombinant human (rh) basic fibroblast growth factor (bFGF; PeproTech).

hESC differentiation

Prior to differentiation to remove mEF, hESC were passaged as clumps onto Matrigel (BD Biosciences)-coated substrates or micropatterned Matrigel substrates in defined serum-free medium consisting of X-Vivo10 medium (Cambrex) supplemented with 2 mM GlutaMAX (Invitrogen), 1×nonessential amino acids (NEAA; Invitrogen), 0.1 μM β-mercaptoethanol (BME; Sigma), 40 ng/ml bFGF (PeproTech), 0.1 ng/ml rh-TGFβ1 (R&D Systems), and 5 ng/ml rh-activin A (R&D Systems). Micropatterned Matrigel substrates and hESC colonies were generated as previously described (Peerani et al., 2007). Following overnight seeding of hESC, medium was replaced with X-Vivo10 medium supplemented with 2 mM GlutaMAX, 1× NEAA, 0.1 μM BME, and cytokines as indicated. Rh-BMP2 (R&D Systems) and rh-activin A (R&D Systems) were used at 50 ng/ml unless otherwise indicated.

Formation of forced aggregation embryoid bodies and hematopoietic assay

EB were formed through forced aggregation as previously described (Ng et al., 2005b; Ungrin et al., 2008). Briefly, following 48 h of treatment with BMP2 and activin A, micropatterned colonies of 200 or 1200 μm diameter were dissociated into single cells using TrypLE Express and then resuspended in serum-free hemogenic medium (XV-hem) supplemented with 10 μM Y-27632 Rho kinase (ROCK) inhibitor to enhance survival of hESC (Watanabe et al., 2007). XV-hem is composed of X-Vivo10 medium supplemented with 2 mM GlutaMAX, 1×NEAA, 0.1 μM BME, rh-stem cell factor (50 ng/ml; R&D Systems), rh-Flt-3 ligand (150 ng/ml; R&D Systems), and rh-vascular endothelial growth factor$_{165}$ (10 ng/ml; R&D Systems). The single-cell suspension was placed onto a microtextured surface containing pyramidal wells of 800 μm in width (Ungrin et al., 2008) and centrifuged at 400 g for 3 min to obtain pellets of 5000 cells per microwell. Prior to EB formation, microtextured surfaces were passivated using Pluronic F-127 for 1 h to facilitate EB removal. Following overnight culture, the forced-aggregate EB were removed from the microwells and rinsed with PBS to remove the ROCK inhibitor. EB were subsequently cultured in suspension in XV-hem medium for a further 8 days. For colony-forming assay, embryoid bodies were dissociated into single cells using TrypLE Express and resuspended in MethoCult GF+ semisolid medium (H4435; Stem Cell Technologies) at a density of 50,000 cells/ml. For cytospin preparation, the hematopoietic colonies were extracted from MethoCult cultures and Wright–Giemsa staining of cytospin preparations was carried out to assess nuclear and cytoplasmic morphology of the cells.

Endoderm differentiation assay

The endoderm differentiation protocol employed here was originally described by D'Amour et al. (2006). Following 48 h of treatment with BMP2 and activin A, micropatterned colonies were reseeded onto Matrigel-coated substrates in X-Vivo10 medium supplemented with 100 ng/ml activin A. Following 2 days of culture, the medium was replaced with RPMI medium 1640 (Gibco) supplemented with 2% fetal bovine serum (Gibco), 2 mM GlutaMAX, 50 u/ml penicillin/streptomycin (Invitrogen), and 50 ng/ml fibroblast growth factor 7 (or keratinocytes growth factor; R&D Systems). Following 3 more days of culture, the medium was replaced with DMEM (Invitrogen) supplemented with 1% B27 supplement (Gibco), 2 mM GlutaMAX, 50 u/ml penicillin/streptomycin, 50 ng/ml Noggin (R&D Systems), 250 nM cyclopamine (a kind gift from Dr. Derek van der Kooy, University of Toronto), and 2 μM retinoic acid.

Quantitative gene expression analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and purified using the RNeasy mini RNA isolation kit (Qiagen). cDNA was synthesized from purified RNA using the SuperScript III reverse transcriptase kit. Real-time quantification of transcripts using polymerase chain reaction was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and a 7900HT Fast real-time PCR system (Applied Biosystems). Both β-actin and GusB were used as endogenous controls and yielded very similar results, but the transcript levels presented here were calculated as $2^{\Delta(\Delta Ct)}$ normalized to β-actin. A list of primer sequences can be found in Supplementary Table 1.

Statistical analysis

Statistics were computed based on paired Student *t* tests. Error bars represent the standard error of the mean of at least three replicates using H9, I6, and HES2 hESC lines. Single and double asterisks indicate statistical significance of $P<0.05$ and $P<0.01$, respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2008.11.004.

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