Niche-mediated control of human embryonic stem cell self-renewal and differentiation

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Complexity in the spatial organization of human embryonic stem cell (hESC) cultures creates heterogeneous microenvironments (niches) that influence hESC fate. This study demonstrates that the rate and trajectory of hESC differentiation can be controlled by engineering hESC niche properties. Niche size and composition regulate the balance between differentiation-inducing and -inhibiting factors. Mechanistically, a niche size-dependent spatial gradient of Smad1 signaling is generated as a result of antagonistic interactions between hESCs and hESC-derived extra-embryonic endoderm (ExE). These interactions are mediated by the localized secretion of bone morphogenetic protein-2 (BMP2) by ExE and its antagonist, growth differentiation factor-3 (GDF3) by hESCs. Micropatterning of hESCs treated with small interfering (si) RNA against GDF3, BMP2 and Smad1, as well treatments with a Rho-associated kinase (ROCK) inhibitor demonstrate that independent control of Smad1 activation can rescue the colony size-dependent differentiation of hESCs. Our results illustrate, for the first time, a role for Smad1 in the integration of spatial information and in the niche-size-dependent control of hESC self-renewal and differentiation.

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Introduction

Human embryonic stem cells (hESCs) are pluripotent cells that are derived from the inner cell mass of blastocyst-stage embryos (Thomson *et al*, 1998). hESCs can be propagated extensively in culture while retaining the ability to differentiate into somatic cell types. Members of the transforming growth factor-beta (TGF- β) super-family, and the fibroblast growth factors (FGFs) have emerged as important regulators of hESC self-renewal (reviewed in Rao and Zandstra, 2005). The effect of TGF- β signaling is mediated by binding to cell-surface type I and type II receptors with threonine/serine kinase activity. Upon ligand binding, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates intracellular receptor-Smads (R-Smads) (reviewed in Shi and Massague, 2003). Upon phosphorylation, the R-Smads associate with Smad4 and localize to the nucleus and activate transcription. Activin, nodal and TGF- β signal through type I receptors activin receptor-like kinases (ALKs) ALK4, ALK5 and ALK7 and the R-Smads, Smad2 and 3. The bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) family signals through ALK1, ALK2, ALK3 and ALK6 and Smads 1, 5 and 8. Inhibitory-Smads (I-Smads), Smad6 and Smad7, negatively regulate TGF- β and BMP signaling by preventing R-Smads from binding to Smad4 or by forming stable interactions with the activated type I receptors. Maintenance of hESCs is associated with active Smad2/3 signaling and the suppression of BMP signaling mediated through Smads 1/5/8 (Besser, 2004; James *et al*, 2005; Xu *et al*, 2005). High levels of phosphorylated Smad1 (pSmad1) have been associated with hESC differentiation to trophectodermal and primitive endodermal lineages (Xu *et al*, 2002; Pera *et al*, 2004).

The FGF family, consisting of at least 22 ligands, mediates its effects through various isoforms of four distinct cell-surface FGF receptors (FGFRs1–4) (reviewed in Dailey *et al*, 2005; Eswarakumar *et al*, 2005; Mohammadi *et al*, 2005). FGF binding to FGFR is stabilized by cell-surface heparan-sulfate proteoglycans (HSPGs) that act as low-affinity receptors for FGFs. Ligand binding induces dimerization of FGFR and initiates receptor tyrosine kinase activity and signaling through the Ras-mitogen-associated protein kinase (MAPK), phosphatidylinositol-3 (PI-3) kinase and phospholipase C- γ (PLC- γ) pathways. Exogenous FGF-2 is routinely used for the culture of undifferentiated hESCs suggesting an important role for FGFs in the regulation of hESC fate (Rao and Zandstra, 2005).

Despite the addition of exogenous factors that manipulate the activation of the FGF and TGF- β pathways, the local cellular microenvironment and hence the signaling inputs varies significantly in hESC maintenance cultures. In fact, it is likely that hESCs are exposed to a wide range of signaling environments by virtue of the properties of hESC colonies (size, distribution and culture condition-specific associated differentiated cells), and individual hESC position in a particular

colony. As has been demonstrated for mouse ESC (Davey and Zandstra, 2006), hESC and their progeny interact to form supportive and nonsupportive microenvironments ('niches') that influence cell fate. Indeed hESC-derived fibroblasts have been used as feeder layers for the maintenance of undifferentiated hESCs (Stojkovic *et al*, 2005), demonstrating an interplay between hESCs and differentiated cells in culture (Bendall *et al*, 2007).

Given that most stem cell niches are associated with *in vivo* environments, ESCs (which are regularly used as models for early developmental events) may represent a powerful system to quantitatively investigate niche parameters and their effect on stem cell fate. Consistent with the properties of *in vivo* niches, localized effects in ESCs niches are likely mediated by interactions between exogenously controlled parameters and autocrine and paracrine secretion of endogenously produced factors. The relative magnitude and consequence of this endogenous signaling should, in turn, be a function of the local cellular microenvironment. In order to fully understand the molecular mechanisms that govern hESC fate control, we hypothesized it would be necessary to study the role of key regulators of hESC cell fate in the context of the local cellular microenvironment and the activation of pathways that are known to influence hESC fate.

To measure and control the effects of the microenvironment on hESC fate, we have developed a number of novel methods that allow us to quantitatively interrogate cell-specific localized signaling activation and to control spatial aspects of the hESC niche by patterning hESC colonies onto defined adhesive islands with controlled colony diameter and pitch (the distance between colonies). Our results demonstrate that two determinants of the hESC niche—colony size and cellular composition—dramatically impact hESC fate and signaling. Larger colonies with high local cell density microenvironments promote the maintenance of the undifferentiated phenotype in hESCs by suppressing Smad1 activation via increased activity of BMP antagonists such as GDF3. In contrast, differentiated cells, specifically extra-embryonic endoderm (ExE), antagonize self-renewal by the local secretion of BMP2. Using microcontact printing of adhesive ECM islands, we demonstrate for the first time, spatial control of the activation of Smad1 and consequently hESC fate. This understanding of the *in vitro* hESC niche identifies the importance of previously uncontrolled parameters in hESC biology and should yield new strategies to manipulate hESC fate.

Results

Our objective was to obtain a quantitative understanding of the role of the microenvironment on the modulation of endogenous hESC signaling and the regulation of hESC propagation. hESCs are typically cultured on feeder layers of mouse embryonic fibroblasts (MEFs), in the presence of complex serum-containing medium. In order to effectively interrogate the role of the microenvironment, we first established a better-defined system for hESC culture. Several feeder-free systems have been reported in the literature (reviewed in Rao and Zandstra, 2005); we adapted the conditions described in Li *et al* (2005) for our analysis. In our cultures, hESCs were propagated on MatrigelTM-coated plates in X-VIVO10TM medium supplemented with FGF-2

(40–80 ng/ml) and TGF- β 1 (0.1 ng/ml) (XFT). In XFT media, hESCs are karyotypically stable, maintain expression of pluripotency markers such as Oct-4, SSEA-4 and Tra-1-60 over greater than 30 passages, and robustly retained the ability to give rise to cells of all three germ layers *in vitro* in EB assays and *in vivo* in teratoma-formation assays (Supplementary Figure S1). This culture system has been validated in the CA1, H9 and I6 hESC cell lines.

Our hypothesis was that the local cellular microenvironment, including the composition and organization of hESC colonies and hESC derivatives, provides signals that influence hESC propagation. In order to test this hypothesis, we designed a series of experiments wherein exogenous cytokines were withdrawn from the culture medium and the differentiation of hESCs was followed over a 48-h period. This short time period was chosen in order to capture initial changes in colony composition that occur independently of the dramatic 'resetting' of the culture that occurs during passaging. We reasoned that if the local microenvironment provides signals supporting hESC propagation, a regional analysis of hESC culture under culture conditions with no exogenous growth factor input would reveal localized organization that could be correlated with cell fate. Given the propensity of hESC to die or differentiate when cultured as individual cells at low cell densities, we initially focused on this output.

To accurately measure the cellular microenvironment and the localized cell density for each cell, as well as the corresponding response of each cell to the withdrawal of exogenous cytokines, we initially screened conditions using the loss of the pluripotency marker Oct-4. We used image analysis and fluorescent microscopy to obtain the spatial location and the Oct-4 expression for each cell in culture (Figure 1A). The localized cell density for each cell was computed by counting the number of cells that surrounded it within a radial threshold of 300 μ m (Figure 1B). This threshold was determined by empirically plotting Oct-4 expression as a function of the localized cell density for radial thresholds ranging from 100 to 1000 μ m and choosing the largest threshold that maintained a correlation. This analysis is consistent with the supposition that autocrine and paracrine effects are likely restricted to a few cell diameters around any given cell (Francis and Palsson, 1997). Cells with equivalent niche properties (localized cell densities) were binned together (Figure 1C). Oct-4 histograms were generated for each bin (Figure 1D) and the percentage of Oct-4⁺ cells in each bin was plotted against the localized cell density (Figure 1E). Our results show that hESCs retain Oct-4 expression in regions of high-localized cell density upon withdrawal of exogenous cytokines, suggesting a role of hESC-supportive endogenous factor(s) that scale with the microenvironment. Importantly, the bulk cell density (i.e., the total number of cells per well) in all cultures was the same, the localized cell density is a function of the number of neighbors a cell has within a given radius and is a parameter that allows for the screening and identification of signaling effects due to cell-level organization.

Spatially dependent changes in Oct-4 expression could be associated with changes in local signaling activation. We next examined intracellular signaling pathways in hESCs in the context of the local cellular microenvironment. Specifically, we analyzed intracellular signaling through the Smad pathways mediated by members of the TGF- β superfamily that play an important role in hESC self-renewal and are known to

Figure 1 Development of a quantitative metric to assess the role of hESC microenvironment on hESC fate. Quantitative fluorescence microscopy was used to obtain images. (A) hESCs were stained for Hoechst (Ai) and Oct-4 (Aii). A mask was drawn around each nucleus (Aiii). The fluorescence intensity corresponding to the species of interest, in this case Oct-4, within this mask is obtained. A spatial map is generated using the centroid of the nucleus and the fluorescence intensity measurement indicated by the color bar (Aiv). Scale bar is 50 μm. (B) An algorithm is used to determine the number of neighbors within a 300 μm radius. This information is superimposed on the spatial location map to create heat maps (Bi). Blue indicates low number of cells and red indicates high density. The spatial location is superimposed on the Oct-4 fluorescence for each cell to obtain an Oct-4 map (Bii). Scale bar is 408 μm and the color threshold to determine positive populations is as indicated. (C) The cells are classified into bins based on the localized cell density, which is the number of neighbors within a 300 μm radius. The number of cells in each bin is shown here. (D) Oct-4 histograms are plotted for the cells in bin 1 and bin 7 which contain 0–100 cells (low) and 500–600 cells (high) as the localized cell density. (E) The percentage of Oct-4+ cells is plotted as a function of the localized cell density.

be modulated by the local secretion of activators and inhibitors (Rao and Zandstra, 2005; Levine and Brivanlou, 2006). Nuclear levels of pSmad1 in hESCs were found to be a function of local cell density (Figure 2A). A quantitative analysis of Smad1 activation demonstrated that a pSmad1 activation gradient was present very early in our hESC cultures such that the levels of nuclear pSmad1 are significantly lower in Oct-4-positive (Oct-4+) hESCs that are predominantly surrounded by Oct-4+ cells compared to Oct-4+ cells predominantly surrounded by Oct-4-negative (Oct-4-) cells (Figure 2B). To further understand the signaling interactions between the Oct-4+ and Oct-4- populations, cells were simultaneously interrogated for nuclear expression of Oct-4, hepatocyte nuclear factor 3β (HNF3β) and pSmad1. HNF3β was chosen as a marker on the basis of a preliminary screen with several markers of early differentiation. The Oct-4-negative cells expressed HNF3β suggesting endodermal differentiation in our system (Supplementary Figure S2). We suggest that this endoderm is extra-embryonic as these cells can be isolated (see Supplementary Materials and methods) and shown to express ExE markers including Gata6, Gata4, α-fetoprotein (AFP), Sox7, and the lack of HLA Class I

molecules (Supplementary Figure S3). Subpopulation analysis revealed that ExE cells had higher levels of nuclear pSmad1 than Oct-4+ cells (Figure 2Ci). Oct-4+ cells surrounded by a greater numbers of Oct-4+ cells expressed lower levels of pSmad1 (Figure 2Cii), whereas Oct-4+ cells surrounded by more HNF3β-positive (HNF3β+) cells had higher levels of pSmad1 (Figure 2Ciii). This observation is of particular importance as inspection of the spatial maps reveals certain areas of high cell density that have high levels of pSmad1 activation due to their high ExE content. These observations suggest that a pSmad1-promoting factor(s) is secreted by the HNF3β+ population. However, hESCs surrounded by a fixed number of HNF3β+ cells have nuclear pSmad1 levels that decrease as a function of increasing numbers of surrounding Oct-4+ cells (Figure 2Civ). This result strongly suggests the existence of endogenous factor(s) produced by Oct-4+ hESCs that antagonize the pSmad1-promoting factor(s) secreted by the HNF3β+ population. Furthermore, the relative impact of these endogenous factors is local cell density (niche-size) dependent.

We suggest that these pSmad1 signaling gradients are a general property of hESC cultures as these gradients have

Figure 2 The composition of the hESC microenvironment modulates Smad1 signaling. (A) Images of hESCs at low and high cell density within a single well showing inverse correlations between the number of Oct-4 + cells in the hESC microenvironment and pSmad1. Cells are stained for Hoechst, Oct-4, pSmad1. Scale bar is 50 μ m. (B) Quantification of the effect of local cell density can be visualized macroscopically by superimposing the single cell local cell density metric (Bii) and pSmad1 levels (Biii) onto spatial maps. Blue indicates low values and red indicates high values. Scale bar is 408 μ m. (C) PSmad1 levels in the HNF3 β + and Oct-4 + subpopulations (Ci). PSmad1 levels of the Oct-4 + subpopulation as a function of the localized Oct-4 + cell density (Cii). PSmad1 levels of the Oct-4 + subpopulation as a function of the localized HNF3 β density (Ciii). PSmad1 levels in the Oct-4 + subpopulation with fixed HNF3 β localized cell density and increasing Oct-4 + localized cell density. Data are normalized to the pSmad1 levels of the lowest localized cell density (Civ). (D) PSmad1 levels in hESCs after being pulsed for 90 min with unconditioned media (UCM), media conditioned by either H9 or I6 hESCs (hESC-CM), media conditioned by H9- or I6-derived extra-embryonic endoderm (ExE-CM), or 25 ng/ml of BMP2. (E) ELISA data for GDF3 and BMP2 present in hESC-CM and ExE-CM.

been found in CA1, H9 and I6 hESC cell lines maintained on MEF feeder layers (not shown) or the serum-free system presented here. While it was possible to detect phosphory-

lated Smad2 (pSmad2) levels, the dependence of pSmad2 levels on local cell density was more difficult to ascertain due to the low overall nuclear pSmad2 levels detected under our

experimental conditions. This result is not surprising given that our studies are performed in defined media in the absence of exogenous cytokines that promote signaling through the Smad2/3 pathway. While undifferentiated hESCs are capable of activating pSmad2/3 (Beattie *et al*, 2005; James *et al*, 2005), these observations are obtained with hESCs cultured using mEF-conditioned medium which has high levels of pSmad2-promoting cytokines.

To further demonstrate the signaling antagonism between the hESC and ExE populations on pSmad1 activation, serum-free media were separately conditioned by hESCs (hESC-CM) and isolated ExE (ExE-CM). Levels of pSmad1 in hESCs were measured after being pulsed with these conditioned media, with unconditioned media (UCM) and 25 ng/ml of BMP2 serving as controls (Figure 2D). As expected, the presence of hESC-derived or ExE-derived soluble factors decreased or increased pSmad1 activation, respectively. As BMP2 and GDF3 were identified by gene expression as possible pSmad1 agonists and antagonists in the system (see below), the conditioned media were tested for these proteins by ELISA (Figure 2E). GDF3 was only detected in hESC-CM and not in ExE-CM and UCM, whereas BMP2 was detected at levels approximately six times higher in ExE-CM than hESC-CM. These observations strongly suggest that pSmad1 levels in hESCs are dependent on the composition of hESCs and ExE in the local microenvironment (niche), which in turn determines the local balance between pSmad1 agonists (BMP2) and antagonists (GDF3).

Endogenous signaling through the FGFRs and the Smad signaling pathways has been associated with hESC self-renewal (Besser, 2004; Dvorak *et al*, 2005). To examine the role of endogenous FGF and TGF- β signaling in creating hESC culture heterogeneity, we studied the effect of TGF- β and FGF inhibitors on hESC differentiation. The purpose of the FGF/FGFR inhibitor studies was to reveal possible endogenous regulatory mechanisms through these pathways that could explain the effect of local cell density on Oct-4 expression. SB431542 (TI) is an inhibitor of ALK4, ALK5 and ALK7, but has no effect on ALK1, 2, 3 and 6 (Inman *et al*, 2002; Laping *et al*, 2002). PD173074 (FI) causes specific inhibition of the FGFR tyrosine kinase in a dose-dependent manner (Dimitroff *et al*, 1999; Bansal *et al*, 2003). Sustained exposure to these inhibitors leads to differentiation of hESCs (Vallier *et al*, 2005; Dvorak *et al*, 2006; and data not shown), confirming the reported significance of the FGF and TGF- β signaling pathways in hESC self-renewal. After a 36 h exposure to the inhibitors, population-level analysis revealed changes in Oct-4 expression relative to the case where no inhibitor was added (XV) (Figure 3A). Importantly however, when analysis was performed to examine the effect of the inhibitors on hESC as a function of the local microenvironments, significant differences were revealed (Figure 3B). A microenvironment-dependent resistance to differentiation in response to these inhibitors suggests the presence of endogenous factor(s) that act through a pathway distinct from the FGFRs and ALK4, ALK5 and ALK7. This result suggests the presence of other endogenous factor(s) that protect the cells from the effect of inhibitors in a local cellular microenvironment-dependent manner (since all the cells are subjected to the same external concentration of inhibitor). As SB431542 and PD173074 inhibit the kinase activity of ALK4/5/7 and the FGFRs, respectively, these inhibitors do not compete with

TGF- β or FGF ligands for binding to the extracellular domain of their respective receptors. Since high concentrations of FGF-2 are typically applied in feeder-free culture of hESCs (Li *et al*, 2005; Wang *et al*, 2005; Xu *et al*, 2005), we studied the effect of exogenous FGF-2 on spatial pSmad1 signaling gradients. Exogenous FGF-2 caused a decrease in nuclear pSmad1 levels in Oct-4+ cells (Figure 3C), relative to cells in culture where exogenous FGF-2 was not added. Further analysis showed that the decrease in pSmad1 levels upon addition of exogenous FGF-2 occurred independently of local cellular organization of the absolute number of Oct-4+ (Figure 3D) and Oct-4- cells (Figure 3E), as well as the local percentage of Oct-4+ cells (Figure 3F). We thus conclude that the endogenous pSmad1 gradient itself is independent of exogenous FGF but the overall levels of pSmad1 decrease in the presence of exogenous FGF-2. This is consistent with previous results where hESCs could be maintained in the undifferentiated state in feeder-free conditions using high concentrations of FGF-2 or a combination of noggin (a Smad1 signaling antagonist) and lower concentrations of FGF-2 (Wang *et al*, 2005; Xu *et al*, 2005). A possible role for FGF in the hESC niche is inducing phosphorylation of the linker region of Smad1 by MAPK, thereby preventing nuclear accumulation (Yamagata *et al*, 2005). We tested this possibility by pulsing cells for 90 min with XV media without cytokines as well as with XV supplemented with 80 ng/ml FGF (F), 25 ng/ml BMP2 (B), and 80 ng/ml FGF and 25 ng/ml of BMP2 (B + F). Cells were analyzed for Hoechst, Oct-4 and pSmad1 and total Smad1 (TSmad1) expression. The cytoplasmic and nuclear localization of Smad1 was obtained using image analysis (Supplementary Figure S4). While stimulation by FGF-2 alone decreased pSmad1 levels in the nucleus (Figure 3G), it did not change the cytoplasm to nuclear ratio (Figure 3H) of pSmad1, suggesting that there is no cytoplasmic accumulation of pSmad1. In contrast, TSmad1 does accumulate in the cytoplasm as its cytoplasm to nuclear ratio increases upon FGF-2 stimulation (Figure 3H). In combination, these observations suggest that FGF-2 prevents the phosphorylation of the Smad1, thereby preventing its translocation into the nucleus which in turn leads to the accumulation of the nonphosphorylated form of Smad1 in the cytoplasm. Interestingly, the high dose of FGF-2 used (80 ng/ml) is insufficient to prevent nuclear accumulation of pSmad1 in the presence of high BMP2 (25 ng/ml) as both the total cellular content and nuclear fraction of pSmad1 and TSmad1 increase upon BMP2 stimulation with or without FGF-2 (Figure 3G and H).

Our results thus far suggest the following: (a) the local cellular microenvironment influences hESC self-renewal through the modulation of endogenous factor(s); (b) Oct-4+ cells secrete endogenous factors including GDF3 to suppress Smad1 signaling in hESCs and promote the maintenance of undifferentiated hESCs; (c) HNF3 β + ExE cells secrete factors such as BMP2 to promote Smad1 signaling and negatively impact hESC self-renewal and (d) the endogenous factor(s) that promote maintenance of undifferentiated hESCs operate, at least in part, through a mechanism that is independent of FGFRs and ALK4, 5 and 7.

These results were obtained in experiments where early differentiation was induced in heterogeneous hESC cultures. Differentiation and Smad signaling were observed to be correlated with niche properties such as colony size and

Figure 3 Endogenous FGF and TGF- β signaling regulates hESC self-renewal and exogenous FGF-2 suppresses Smad1 signaling in Oct-4+ hESCs. HESC cells were cultured in XFT media. Cells were plated on Matrigel-coated plates in medium without any exogenous cytokines. After 11 h, the medium was replaced with fresh medium or medium containing FGF (FI) or TGF- β inhibitors (TI). After a further 37 h, the cells were fixed and analyzed. **(A)** The percentage of Oct-4+ cells under the different conditions after 48 h is shown. **(B)** The percentage of Oct-4+ cells in each condition is plotted as a function of the localized cell density, that is the local microenvironment. **(C)** The mean single-cell pSmad1 levels in the Oct-4+ subpopulation are plotted. Data are normalized to the mean single-cell pSmad1 value in the XV condition. Mean single-cell pSmad1 levels are plotted as a function of the number of Oct-4+ cells **(D)**, number of Oct-4- cells **(E)** and the fraction of Oct-4+ cells **(F)** within a 300 μ m radius. **(G)** Total cellular content (cytoplasmic and nuclear) of pSmad1 and total Smad1 (TSmad1) after 90-min stimulation by XV media alone (XV) or XV media supplemented with 80 ng/ml of FGF-2 (F), 25 ng/ml of BMP2 (B) or 80 ng/ml of FGF-2 and 25 ng/ml of BMP2 (B + F). **(H)** Cytoplasmic to nuclear ratio of pSmad1 and TSmad1 after stimulation. Error bars in (B) represents the standard error of the mean of five wells in a single representative trial that has been repeated seven times.

composition, suggesting an instructive role of the local microenvironment. However, an important limitation of these results is that they are correlative in nature. If the

local microenvironment indeed has an instructive role in the modulation of hESC self-renewal, it follows that controlling the spatial organization of cells can be used to modulate

signaling and thus directly impact cell fate. Therefore, we developed methods to culture hESCs in defined microenvironments by micropatterning colonies on extracellular

matrix substrate (Matrigel™) printed in distinct features (Figure 4A). HESCs were seeded as single cells onto the patterned substrates and cultured in X-VIVO10™ without

Figure 4 Micropatterning can be used to manipulate the hESC microenvironment and control hESC fate. To demonstrate that hESC microenvironment directly controls hESC fate, H9 hESCs were plated on patterned tissue-culture substrates with Matrigel using microcontact printing. Colony diameters (D) ranged from 200–800 μm and distance between colonies, pitch (P), was fixed at 500 or 1000 μm . (A) Quantitative fluorescent microscopy of patterned H9 cultures in XV media after withdrawal of all growth factors for 48 h and stained for Hoechst, Oct-4 and pSmad1. Scale bar is 200 μm . (B) Analysis of micropatterned colonies after 48 h. The percentage of Oct-4 cells as a function of colony size (Bi). pSmad1 in the Oct-4+ population as a function of colony size (Bii). Gene expression for Oct-4, Sox2, Nanog normalized to β -actin as a function of colony size (Biii). (C) To elucidate the molecular mechanism for the changes in pSmad1 levels, gene expression was quantified as a function of colony size for Smad1 agonist, BMP2, and antagonists, Follistatin, GDF3 and LeftyB and I-Smads, Smad6 and Smad7. Gene expression after normalization to β -actin and relative to the 200 μm data set (Ci). Ratios of the transcripts of Smad1 antagonists, Follistatin, GDF3 and LeftyB, to the transcripts of Smad1 agonist, BMP2, as a function of colony size (Cii). GDF3 gene expression as a function of colony size normalized to the percentage of Oct-4+ cells present (Ciii). For (Ci), statistical comparisons were made between gene expression for each cytokine at 400 and 800 μm colonies against the expression at 200 μm colonies. For (Cii), statistical comparisons were made between the ratio of the levels of transcripts of the antagonist to BMP2 between the 400 and 800 μm colonies against the ratio present in the 200 μm .

any exogenous growth factors. Cells were immunostained for Hoechst, Oct-4 and pSmad1 (Figure 4A and Supplementary Figure S5A). Larger colonies were also stained for Nanog, Tra-1-60 and Sox2 to further confirm expression of pluripotency-associated markers (Supplementary Figure S5B and S5C). The number of cells per colony, the number of cells per unit area and plating efficiency were calculated for each pattern type (Supplementary Figure S6). As expected, cell growth was not affected in these conditions and the micropatterned colonies remained as a monolayer at these early time points (Supplementary Figure S6A). Interestingly, as early as 6 h, the percentage of Oct-4+ cells in micropatterned colonies was the same regardless of colony size but changes in pSmad1 signaling were observed (Figure 4Bi and Bii). By day 2, the smaller colonies had differentiated significantly (Figure 4Bi). Importantly, the differences in the pluripotency-associated markers between colony sizes was not due to differences in the initial conditions as plating efficiency and cell density normalized to available attachment area were initially identical (Supplementary Figure S6).

To further confirm the loss of pluripotency-associated markers, real-time qPCR was used to quantify gene expression of Oct-4, Nanog and Sox2 on micropatterned colonies (Figure 4Biii). These latter experiments recapitulate the observations made in the nonpatterned cultures; namely that localized cell density correlates with self-renewal and the antagonism of the Smad1 pathway. Moreover, they suggest that this relationship is in fact causative such that when the microenvironment is independently controlled by fixing colony size, pSmad1 activation and hESC fate can be directly modulated.

To explore the molecular mechanisms by which colony size affects pSmad1 activation, real-time qPCR was used to quantify gene expression of the I-Smads, Smad6 and Smad7, as well as pSmad1 agonists and antagonists reported to be expressed in hESC cultures (Abeyta *et al*, 2004) (Figure 4Ci). While mRNA levels of BMP2, a known agonist of Smad1 activation, do not change with increasing colony size ($P=0.28\text{--}0.38$), the levels of GDF3 transcripts, a known ligand trap for BMP2 and therefore an antagonist of Smad1 activation, increased significantly (Figure 4Ci). No change in the expression of follistatin, another ligand trap of both BMPs and Activin, was observed ($P=0.60\text{--}0.80$, Figure 4Ci). LeftyB expression, which in other systems has been suggested to inhibit both Smad1 and Smad2 phosphorylation (Ulloa and Tabibzadeh, 2001), also increases with colony size (Figure 4Ci). Importantly, when the expression levels of the antagonists, GDF3 and LeftyB, are normalized to the level of transcripts of BMP2, an increasing trend is observed with colony size (Figure 4Cii). In contrast, Smad6 and Smad7 do not increase with colony size suggesting that their role in mediating pSmad1 is not colony size-dependent (Figure 4Ci). It must be noted that the correlation between GDF3 and colony size may reflect a size-dependent change in the cellular composition of the colony as the increase in GDF3 expression when normalized to the percentage of Oct-4+ cells in the colony falls within experimental error (Figure 4Ciii).

The above analysis suggests that the effect of colony size on hESC self-renewal is mediated by the ratio of the expression of pSmad1 agonists to antagonists. This mechanism was tested using two separate strategies. In the first strategy, micropatterned colonies were pulsed for 90 min with media

conditioned by small ($D=200\text{ }\mu\text{m}$, 200-CM) and large ($D=800\text{ }\mu\text{m}$, 800-CM) colonies and analyzed for the pSmad1 activation elicited by these conditioned media (Figure 5A). As expected, the same signaling antagonism observed earlier between hESCs and ExEs was recapitulated by the 800-CM and 200-CM media. The 200-CM increased pSmad1 levels in $D=800\text{ }\mu\text{m}$ colonies and the 800-CM decreased pSmad1 levels in $D=200\text{ }\mu\text{m}$ colonies (Figure 5A). Thus, media conditioned by small colonies had excess pSmad1 agonists and 800-CM media contained excess pSmad1 antagonists. In the second strategy, a number of tests were employed to manipulate the size-dependent balance between pSmad1 antagonists and agonists and consequently hESC fate. Specifically, 300 ng/ml of noggin and 100 nM of small interfering RNA (siRNA) directed against BMP2 (siBMP2), GDF3 (siGDF3) and Smad1 (siSmad1) were separately applied to micropatterned hESC colonies at the time of seeding (Figure 5B and C). As expected given the proposed mechanism, the addition of noggin, siBMP2, and siSmad1 to small colonies lowered pSmad1 levels and significantly increased the percentage of Oct-4+ cells (Figure 5B and C). It is not surprising that the increase in hESC self-renewal upon knockdown of BMP2 expression is marginal relative to noggin as it is likely that there are other members of the BMP family that promote signaling through the Smad1 pathway. In contrast, the addition of siGDF3 to larger colonies increased pSmad1 levels in proportion with colony size resulting in significant decreases in Oct-4 expression. These observations are consistent with earlier studies which suggested that overexpression of GDF3 supports undifferentiated hESC under conditions that promote differentiation (Levine and Brivanlou, 2006). Recently, a Rho-kinase associated inhibitor (ROCK) Y-27632 has been identified as a survival factor that improves hESC cloning efficiency (Watanabe *et al*, 2007). The addition of $10\text{ }\mu\text{M}$ Y-27632 after 24 h of seeding increased Oct-4 expression and decreased pSmad1 activation in small colonies but had no effect on larger colonies (Figure 5B and C). The efficacy of siRNA knockdown on protein expression was confirmed by TSmad1 immunostaining to be 40–60% (Figure 5D). Likewise, media conditioned by siGDF3- and siBMP2-transfected cells contained 65–80% less protein (Figure 5D). Together, these results demonstrate that the spatial control of hESC colony size by micropatterning regulates self-renewal by modulating properties of the hESC niche through a mechanism that involves altering the impact of endogenously secreted factors such as BMP2 and GDF3.

Discussion

In vivo, stem cells are generally found to reside in niches with defined tissue architecture. Arrangement of stem and support cells into niches organizes the timing and levels of signals stem cells receive, thus directing cell fate. However, stem cells may also be found in clusters in the absence of a clearly defined niche of support cells and still regulate cell fate in a spatially organized fashion (Alonso and Fuchs, 2003). This observation suggests that cells may be capable of forming niches in an autoregulatory manner (Davey and Zandstra, 2006). Such autoregulatory processes may serve to support and regulate stem cell populations during embryogenesis where a dynamic process of tissue development and

Figure 5 Niche-size-mediated control of self-renewal acts through the BMP/GDF3 pathway and by controlling pSmad1 activation. (A) The signaling antagonism between hESCs and ExE described earlier is recapitulated by the artificial signaling gradients established by micropatterning. Cells in $D=200\ \mu\text{m}$ and $D=800\ \mu\text{m}$ colonies were pulsed for 90 min with unconditioned media (XV), media conditioned by $D=200\ \mu\text{m}$ cells (200-CM), $D=800\ \mu\text{m}$ cells (800-CM), 25 ng/ml BMP2, hESC-CM and ExE-CM. As expected, 200-CM increased pSmad1 levels of larger colonies, whereas 800-CM decreased pSmad1 levels in smaller colonies. (B) Oct-4 expression in $D=200\ \mu\text{m}$ can be maintained by the addition of 100 nM of siRNA against BMP2 (siBMP2) or Smad1 (siSmad1), the addition of 300 ng/ml of Noggin or 10 μM Y-27632 Rho-kinase associated (ROCK) inhibitor. Larger colonies $D>400\ \mu\text{m}$ differentiate in 100 nM of siRNA against GDF3 (siGDF3). However, Y-27632 and 100 nM siSmad1 have little effect on larger colonies. (C) pSmad1 levels decrease in the presence of siBMP2, noggin, siSmad1 and Y-27632 in small colonies, whereas pSmad1 levels increase in larger colonies in the presence of siGDF3. Interestingly, no effect of pSmad1 is observed when siSmad1 and Y-27632 are added to larger colonies. (D) Protein loss of Tsmad1 by siSmad1 was detected by quantified fluorescence microscopy while ELISA assays were used to confirm loss of BMP2 protein by siBMP2 in 200 μm colonies and loss of GDF3 protein by siGDF3 in larger colonies.

rearrangement occurs. The coordinated series of temporal and spatial events that occur in the mammalian embryo, including the existence of Smad signaling gradients during axis formation and gastrulation support the importance of these parameters in cell fate control *in vivo* and *in vitro* (Goumans and Mummery, 2000). A stem cell niche is defined by production of local signal(s) and spatial organization of cells receptive to those signals to generate location-dependent control over contrasting cell-fate decisions such as self-renewal and differentiation. It is readily observed that ESCs tend to maintain tight contacts with their neighbors, growing in clusters to the general exclusion of morphologically differentiated progeny. Our spatial analysis demonstrates the modulation of local cell density can be used to generate directed patterns of self-renewal and differentiation. Such patterning of cell fate may also be a powerful tool for examining the interaction between ESCs and their differentiated progeny. This approach would allow for the creation of precise gradients of signaling in colonies through the engineered control of colony size and geometry and provide new means to control hESC fate.

Several laboratories have reported poor hESC cloning efficiencies and difficulties in passaging hESCs as single cells. Our data suggest that there is a critical colony size required to antagonize pSmad1 activity and prevent differentiation. The data also suggests that ROCK inhibitor Y-27632, which recently has been observed to enable single hESC cell cloning, may have a similar mechanistic foundation (Watanabe *et al*, 2007). The balance between differentiation and self-renewal will also be dependent on the local composition of the hESC niche including BMP2-secreting ExE. Our results suggest a mechanism by which the expression of pSmad1 antagonists overcomes the expression of pSmad1 agonists in microenvironments established by larger colony sizes and increased localized cell density. This phenomenon in turn leads to decreased levels of pSmad1 and hESC maintenance in these microenvironments. A schematic of our proposed mechanism is shown in Figure 6.

While the increase in undifferentiated hESC in small colonies induced by BMP2 siRNA is significant, it was insufficient to completely overcome endogenous differentiation-inducing signals. The addition of high concentrations of

Figure 6 Two niche determinants—composition and size—regulate hESC self-renewal by local modulation of pSmad1 agonists and antagonists. (A) pSmad1 levels in hESCs are a balance of agonist(s) secreted by differentiated progeny including HNF3 β + extra-embryonic endoderm and antagonists secreted by hESCs (Oct-4 +). The levels of pSmad1 of a single hESC will be dependent on the localized cell density of the Oct-4 + and HNF3 β + neighbors around it. (B) By manipulating colony size through micropatterning, the levels of Smad1 antagonists, GDF3 and LeftyB, increase while BMP2 remains constant resulting in pSmad1 levels that decrease with colony size. This decrease in pSmad1 levels can be correlated with the increased pluripotency seen with increasing colony size.

noggin or Smad1 siRNA at seeding elicited larger increases in undifferentiated hESC support. Together, these results suggest that BMP agonists in addition to BMP2 are influencing cell fate in smaller colonies. Small colonies may also lack other endogenous factors required to prevent differentiation independently of pSmad1 suppression. This perspective is supported by the data showing that noncompetitive inhibitors to the receptor for these molecules, in particular inhibitors of TGF- β , increased the rate of differentiation of cells at lower local densities. Nonetheless, the differentiation induced by GDF3 siRNA in large colonies demonstrates that colony size-induced suppression of pSmad1 is necessary for the prevention of differentiation. While it has been generally shown at the population level that high pSmad1 activation is associated with differentiation, we show here specifically that pSmad1 levels for individual hESCs is highly variant and dependent on the local composition and number of undifferentiated and differentiated cells in the immediate microenvironment, that is the 'niche'. Furthermore, we demonstrate that micropatterning hESC colonies controls properties of this niche to regulate the extent of pSmad1 activation and differentiation.

In the context of the hESC niche properties described here, we propose that the addition of exogenous FGF-2 and noggin, which have been identified as supplements for the serum-free maintenance of hESCs, are acting as a means to exogenously perturb the hESC niche by biasing the signaling interactions between undifferentiated and differentiated cells towards self-renewal. While we have demonstrated that the pSmad1 signaling gradient is established independently of exogenous FGF-2, FGF-2 may still have an instructive role (albeit indirect) in the niche as it has been shown that FGF-2 stimulation of hESCs leads to the suppression of BMP4 and activation of Gremlin-1 (Greber *et al*, 2007). Furthermore, it has recently been shown that FGF-2 interacts cooperatively with IGF-2 in the hESC niche to maintain self-renewal (Bendall *et al*, 2007).

We have demonstrated that endogenous signaling and pluripotent gene expression change with colony size. However, it must be noted that further study of cell-specific hESC gene expression in different colony sizes is required as the analysis presented here reports changes in gene expression for a population of cells cultured in different-sized colonies. For instance, the increase in expression of GDF3

and LeftyB in larger colonies may be representative of the increased number of Oct-4 + cells in these colonies. When the relative number of transcripts is normalized to the percentage of Oct-4 + cells in a colony, the increase in gene expression falls within experimental error (Figure 4Ciii). This observation suggests that the decreased pSmad1 levels associated with large colonies may be due to the paracrine accumulation of GDF3 alone rather than an increase in GDF3 gene expression. While it has been demonstrated by biochemical methods that GDF3 acts as a ligand trap for BMPs in hESCs (Levine and Brivanlou, 2006), it has also been shown in other mammalian systems to act as a Nodal-like agonist (Chen *et al*, 2006). We demonstrate here that the addition of GDF3 siRNA results in pSmad1 activation and subsequent differentiation, confirming the relevance of GDF3 on BMP-like signaling either by direct or indirect inhibition.

Underlying mechanisms that may be responsible for the apparent colony size-specific changes in gene expression include paracrine signaling feedback (or feed forward) loops that occur before any changes in hESC fate commitment, as we have recently observed for STAT3 signaling in mESC (Davey *et al*, 2007). This mechanism is consistent with data demonstrating that the addition of exogenous BMP2 positively regulates BMP2 gene expression in hESCs during ExE differentiation (Pera *et al*, 2004). A similar mechanism may be in place to influence the trajectory of differentiation in $D = 200 \mu\text{m}$ colonies. Likewise, we can speculate that the initial low pSmad1 environment established by larger colonies results from the positive regulation of BMP antagonists GDF3 and LeftyB, or the negative regulation of BMP2 or both. While the above discussion stresses the effect of paracrine secretion of soluble factors that scale extrinsically with colony size, colony size will change the ratio of perimeter to internal cells as well as the levels and distribution of mechanical stress within a colony (Nelson *et al*, 2005). A quantitative study of the effects of these variables would yield considerable insight into the properties of the hESC niche. The results presented in this work demonstrate a role for niche size on hESC self-renewal control and provide a quantitative framework to test other niche-related factors in a systematic manner.

Materials and methods

Cell lines and cell culture

H9, CA1 and I6 cell lines were used in the experiments described. H9 and I6 cells were obtained from the Israel Institute of Technology. The CA1 hES cells are fully characterized and approved by the Stem Cell Oversight Committee at the Canadian Institute of Health Research. (Correspondence concerning the isolation and characterization of the CA1 hESC cells should be directed to Andras Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5; nagy@mshri.on.ca.) HESCs were routinely cultured on feeder layers of irradiated mEFs in knockout (KO)-Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 20% KO-serum replacement (SR) (Invitrogen) and supplemented with 4 ng/ml FGF-2 (PeproTech). Cells were dissociated into small clumps using 0.1% collagenase IV (Invitrogen) and passaged 1:3–1:4 every 4–5 days. For feeder-free culture of hESCs in XFT medium, cells were cultured on tissue-culture plates coated with growth factor reduced MatrigelTM (GFR-MG, BD Biosciences) in completely defined X-VIVO10TM medium (Cambrex) with 2 mM L-Glutamine (Invitrogen), 1 × nonessential amino acids (NEAA) (Invitrogen), 0.1 μM β-mercaptoethanol (Sigma) and supplemented with 40–80 ng/ml FGF-2 and 0.1 ng/ml TGF-β1 (R&D Systems).

HESC differentiation assays

Cells cultured under feeder-free conditions (XFT) were dissociated into small clumps and seeded in GFR-MG-coated plates in X-VIVO10TM medium supplemented with 2 mM L-Glutamine (Invitrogen), 1 × NEAA (Invitrogen), 0.1 μM β-mercaptoethanol (Sigma) (XV medium). After 11 h, cells were washed with XV medium and incubated for a further 37 h with fresh XV medium with no supplementation (XV) or with 80 ng/ml FGF-2 or 10 mM TGF-β inhibitor SB431542 (Sigma) or 20 nM FGF inhibitor PD173074 (kind gift from Dr Shereen Ezzat, University of Toronto, Toronto, Ontario, Canada).

Microcontact printing of GFR-MG on tissue-cultures substrates

Poly(dimethylsiloxane) (PDMS) stamps with the diameter, D , of circular features from 200 to 800 μm (D , diameter) and the distance (or pitch P) between features from 500 to 1000 μm (P , pitch) were fabricated using standard soft lithography techniques (Younan Xia, 1998). The microcontact printing process employed the protocol detailed by (Tan *et al* (2004)). Briefly, PDMS stamps were immersed in a solution of GFR-MG diluted in 1:30 in pH = 5.0 phosphate-buffered saline (PBS) for 1 h at 4°C. Stamps were rinsed three times with sterile ddH₂O, dried gently with N₂ gas, and carefully placed on the tissue-culture-treated microscope slide (Nalge Nunc). The slide and the stamp were then placed in a humidity chamber (RH = 55–70%) for 10 min. The stamps were then removed from the surface of the slide and a silicone gasket (Grace Biolabs) was sealed around the patterned area to form a leak-proof well. Substrates were then passivated with a 5% wt PluronicTM F-127 (Sigma-Aldrich) solution in ddH₂O for 1 h.

Seeding of hESCs on patterned MatrigelTM substrates

HESCs maintained on mEFs were dissociated in 0.25% Trypsin with 1 mM EDTA (Trypsin-EDTA, Invitrogen) for 3 min. Trypsin was inactivated by adding media containing 15% fetal bovine serum (FBS) (Hyclone). To remove FBS, cells were centrifuged and resuspended in PBS. The cells were then resuspended in XFT medium. Cells were seeded overnight at the density of 1×10^6 cells per well and then washed three times and resuspended with XV medium for another 48 h. For the appropriate experiments, XV medium was supplemented with either 300 ng/ml of noggin (PeproTech) or 10 μM Y-27632 ROCK inhibitor (Calbiochem).

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Direct ELISA for GDF3 and BMP2

Supernatant was collected, centrifuged and filtered as described in Supplementary methods. A 100 μl of solution was placed on a Maxisorp Nunc-immunoplates (Nunc-Nalge) overnight at 4°C. Plates were washed three times with PBS and blocked overnight at 4°C with 5wt% BSA in PBS (blocking solution). Wells were incubated overnight at 4°C with mouse anti-BMP2 (R&D Systems) or goat anti-GDF3 (Santa Cruz) in blocking solution. Wells were washed three times with PBS. Wells were incubated for 1 h with goat anti-mouse FITC (Sigma) or sheep anti-goat FITC (Sigma) and washed three times with PBS. Fluorescence was detected using the SpectraMAX GeminiXS system (Molecular Devices).

Knockdown of BMP2, GDF3, Smad1 on micropatterned substrates by siRNA

The DharmaFECTTM 1 (Dharmacon) delivery system was used to introduce predesigned siRNA against GDF3 (100 nM, id#138551: Ambion), BMP2 (100 nM, id#:147099, Ambion), Smad1 (100 nM, id#:4086, SMARTpool) according to the manufacturer's protocol using OptiMEM (Invitrogen) as basal media. Cells were dissociated as single cells using the above protocol and seeded in XFT media plus the transfection media. Cells were allowed to seed overnight and washed the following day. Cells were fixed 48 h later.

Image analysis and generation of chloropleth maps

Fluorescent images were obtained and quantitatively analyzed using the Target Activation or Cell Health Profiling (CHP) assay algorithms available with the Cellomics Arrayscan V^{TI} platform (Cellomics). These algorithm provide nuclear intensity of DNA content through Hoechst 33342 staining, pSmad1/5/8, Oct-4 and HNF3β for individual cells as well as the spatial (x,y) coordinates of the centroid of nucleus. CHP also allows for the dilation of the nuclear mask to quantify cytoplasmic localization of fluorescence intensity. To generate plots of fluorescence intensity versus localized cell density, scripts were written in Python (<http://www.python.org>) to calculate the number of cells in a 300 μm distance using the spatial coordinates and fluorescent intensity data provided by the Target Activation algorithm. To generate chloropleth maps, each cell was represented by a filled circle placed at the location of the centroid of the nucleus with a color intensity corresponding to average nuclear intensity of the marker indicated using the Python Imaging Library software platform toolbox.

Statistical analysis

Statistics were computed based on the paired or unpaired Student's t -test as appropriate. Error bars on plots represent the standard error of the mean of three or more replicates ($n > 3$) using at least two different cell lines unless indicated in the figure legend. Single (*) and double (**) asterisks indicate statistical significance of $P < 0.05$ and $P < 0.01$, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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