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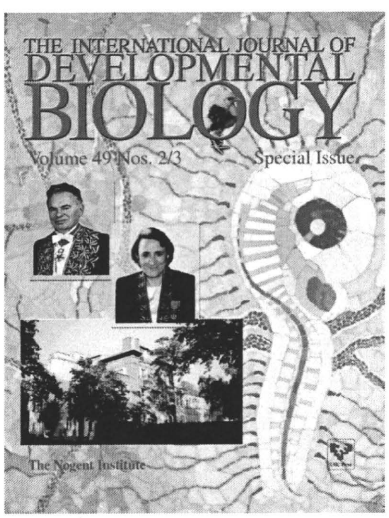


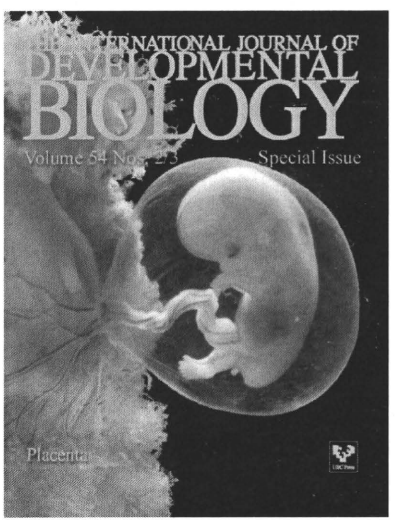


Supplementary Table 1 The components of hESF series media.

Supplementary Table 2 Primers for Q-PCR.

Supplementary Figure Legends

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REGULAR ARTICLE

# Inhibition of ERK1/2 prevents neural and mesendodermal differentiation and promotes human embryonic stem cell self-renewal

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**Abstract** Extracellular signal-regulated kinases (ERKs) have many important functions during embryogenesis. However, their role in embryonic stem (ES) cells is controversial. Previous studies reported that, in contrast to mouse ES cells, human ES cells differentiate if ERK1/2 is inhibited. We reexamined the role of ERK1/2 in human ES cells using a chemically defined culture system and found that when ERK1/2 is blocked with specific chemical inhibitors, neural and mesendodermal differentiation is prevented, but cells become sensitive to BMP-induced differentiation. Inhibition of ERK1/2 significantly reduced the clonogenicity of human ES cells by preventing cell adhesion and survival. When this negative effect was avoided, we were able to maintain human ES cell self-renewal for more than 3 months in the presence of ERK1/2 inhibitors in a chemically defined culture system containing FGF2 and activin A but no BMP4. Our results suggest that the functional outcome of FGF/ERK1/2 signaling in human ES cells is influenced by the relative levels of activin A/TGFβ and BMP activity. In contrast to mouse ES cells, a low level of BMP4 is sufficient to initiate extraembryonic differentiation when ERK1/2 is inhibited. While similar to mouse ES cells, activation of ERK1/2 in human ES cells is required for proper neural and mesendodermal differentiation.


## Introduction

ERK belongs to the MAPK (mitogen-activated protein kinase) family (Johnson and Lapadat, 2002). ERK1/2 (ERK1 and 2) is activated through a chain of phosphorylation events by RAS/RAF/MEK1/2 following the binding of receptor tyrosine kinases (RTKs) by their specific extracellular ligands (such as FGFs and EGFs) (Dreesen and Brivanlou, 2007). ERK1/2 signaling plays important roles in early embryogenesis. In preimplantation mouse embryos, the FGF–ERK1/2 pathway drives primitive endoderm development and suppresses NANOG expression (Chazaud et al., 2006). Prior to mouse gastrulation, FGF-ERK1/2 signaling becomes highly active in extraembryonic ectoderm and promotes the growth of trophoblast stem cells *in vitro* (Corson et al., 2003; Tanaka et al., 1998). During vertebrate gastrulation, ERK1/2

*Abbreviations:* ERK, extracellular signal-regulated kinase; ES, embryonic stem; FGF, fibroblast growth factor; BMP, bone morphogenetic protein; TGFβ, transforming growth factor β; MAPK, mitogen-activated protein kinase.

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signaling is required for neural ectoderm and mesendoderm differentiation and primitive streak formation (Ciruna and Rossant, 2001; Morrison et al., 2008; Stavridis et al., 2007; Yao et al., 2003). The evidence cited above strongly suggests that FGF–ERK1/2 signaling has diverse functions and acts in a cell-context-dependent manner.

In mice, ERK1/2 inhibitor enhances the growth of undifferentiated ES cells (Burdon et al., 1999). It was recently demonstrated that the pluripotency of mouse ES cells is best preserved in the presence of inhibitors of FGF receptor tyrosin kinase, MEK1/2 (activators of ERK1/2) and GSK3 (the "3I" condition) in a chemically defined environment (Ying et al., 2008). These authors argued that mouse ES cells naturally exist in a metastable ground state of self-renewal and that the maintenance of this state requires inhibition of their natural tendency to differentiate, which can be prevented by inhibition of specific signaling pathways that promote their differentiation. By applying MEK1/2 and GSK3 inhibitors and LIF, germline-competent ES cells were derived and propagated from refractory mouse strains, such as the Type I diabetes NOD strain, as well as from rat embryos (Nichols et al., 2009; Li et al., 2008). In contrast to the results from rodent ES cells, in human ES cells the inhibition of FGF receptor tyrosin kinase or MEK1/2, and therefore ERK1/2, has been reported to lead to extraembryonic differentiation (Xu et al., 2002; Pera et al., 2004; Li et al., 2007).

In human ES cell culture, FGF2 has been shown to activate both ERK1/2 and the PI3K–AKT pathway, which promotes cell proliferation (Dreesen and Brivanlou, 2007; Eiselleova et al., 2009). Mouse ES cells overexpressing a constitutively active AKT mutant showed enhanced capacity for self-renewal and became resistant to differentiation (Watanabe et al., 2006). As ERK1/2 is one of the multiple intracellular effectors downstream of FGF signaling and cross talks with other key signaling pathways, it is essential to dissect its function under strictly defined conditions. However, most studies of FGF2 and ERK1/2 in human ES cells have been carried out in undefined systems, in the presence of feeder cells, or in a conditioned medium containing Knockout serum replacement (KSR). As the function of FGF2–ERK1/2 signaling is cell-context dependent, it is difficult to draw definitive conclusions by comparison to the results obtained from mouse ES cells grown under fully defined conditions.

We have recently developed a minimal chemically defined system, in which the effect of exogenous growth factors and small molecules can be analysed without the confounding influence of undefined components (Furue et al., 2008). Using this system, we have now reexamined the role of ERK1/2 signaling in human ES cells and found that under defined conditions, ERK1/2 signaling permits neural and mesendodermal differentiation of human ES cells, but that it can also act to inhibit BMP signaling. It is these latter effects that have led to the previous conflicting conclusions.

## Results

### Inhibiting ERK1/2 prevents mesendodermal induction in human ES cells

High concentrations of activin A (100 ng/ml) are commonly used to induce mesendodermal lineages from human ES cells (D'Amour et al., 2005). However, we found that it alone was not sufficient to mediate differentiation. In HUES1 and SHEF5 cells, the addition of 10 ng/ml FGF2 is necessary to achieve robust mesendodermal gene expression and epithelial-to-mesenchymal transformation (EMT), upon which the cells lost their compact colony morphology and started to spread out (Fig. 1A). Expression of the mesendodermal genes *T* (*BRACHYURY*), *GSC* (*GOOSCOID*), *FOXA2*, and *SOX17* was significantly upregulated by combined treatment of 100 ng/ml activin A and FGF2 in cells (Figs. 1B, 2A, and Supplementary Fig. 2). OCT4 protein expression was maintained in cells which were only treated with 100 ng/ml of activin A. After addition of FGF2, its expression was reduced, while strong FOXA2 staining was evident in the nucleus (Fig. 1C). We also detected stronger phosphorylation of ERK1/2 but not AKT, associated with higher dosages of activin A (Fig. 1D).

We employed two widely used chemical inhibitors of ERK1/2 and PI3K, U0126 and LY294002, respectively (Bain et al., 2007), to investigate the role of ERK1/2 and AKT signaling. For these studies, the cells were seeded on type 1 collagen gel and cultured in our previously described defined-medium system hESF (Furue et al., 2008), which includes nine components, is supplemented with low concentrations of activin A (10 ng/ml), and, hence, is named hESF9A (Supplementary Table 1). When HUES1 human ES cells were grown in hESF9A, U0126 (10 μM) abolished the phosphorylated (activated) form of ERK1/2, while LY294002 (10 μM) markedly reduced the phophorylated (activated) form of AKT (Supplementary Fig. 1). Phosphorylation of either AKT or GSK3β was not affected by U0126 (Supplementary Fig. 1).

To determine whether the activation of ERK1/2 is responsible for the enhanced differentiation, we treated the cells with U0126 or LY294002 for 5 days. At 1 μM, the ERK1/2 inhibitor U0126 markedly reduced cell spreading, while at 5–20 μM, it restored the ES morphology (Fig. 2A, panels b–e), with increased expression of *OCT4* and *NANOG* and inhibition of the upregulation of *BRACHYURY*, *GSC*, *FOXA2*, and *SOX17* (Fig. 2B). At 50 μM, U0126 caused significant cell death (Fig. 2A, panel f). By contrast, the PI3 kinase inhibitor LY294002 did not prevent cell spreading or recover the expression of the pluripotency genes *OCT4* and *NANOG* at any concentration tested (Figs. 2C and D). Moreover, it enhanced the expression of *GSC*, *FOXA2*, and *SOX17* at higher concentrations (Fig. 2D). However, at 20 μM, LY294002 showed strong cell toxicity (Fig. 2C, panel f). Taken together, these results showed that the ERK1/2 branch of FGF2 signaling promotes mesendodermal differentiation and, consequently, inhibition of ERK1/2 signaling prevents human ES cells exiting from the undifferentiated state though this route.

### Inhibiting ERK1/2 prevents neural differentiation

We next tested the role of ERK in neural differentiation. To examine whether this is the case in human ES cells, we triggered neural differentiation by first passaging cells in larger clumps (more than 200 cells), with subsequent withdrawal of FGF2 and activin A from the culture medium from the second day. Under these conditions, the human ES cells adhered poorly to the substrate and formed floating cell aggregates (Fig. 3A, panel a) in which early neural marker genes (*SIX3* and *PAX6*) were upregulated (Fig. 3B). (Similar

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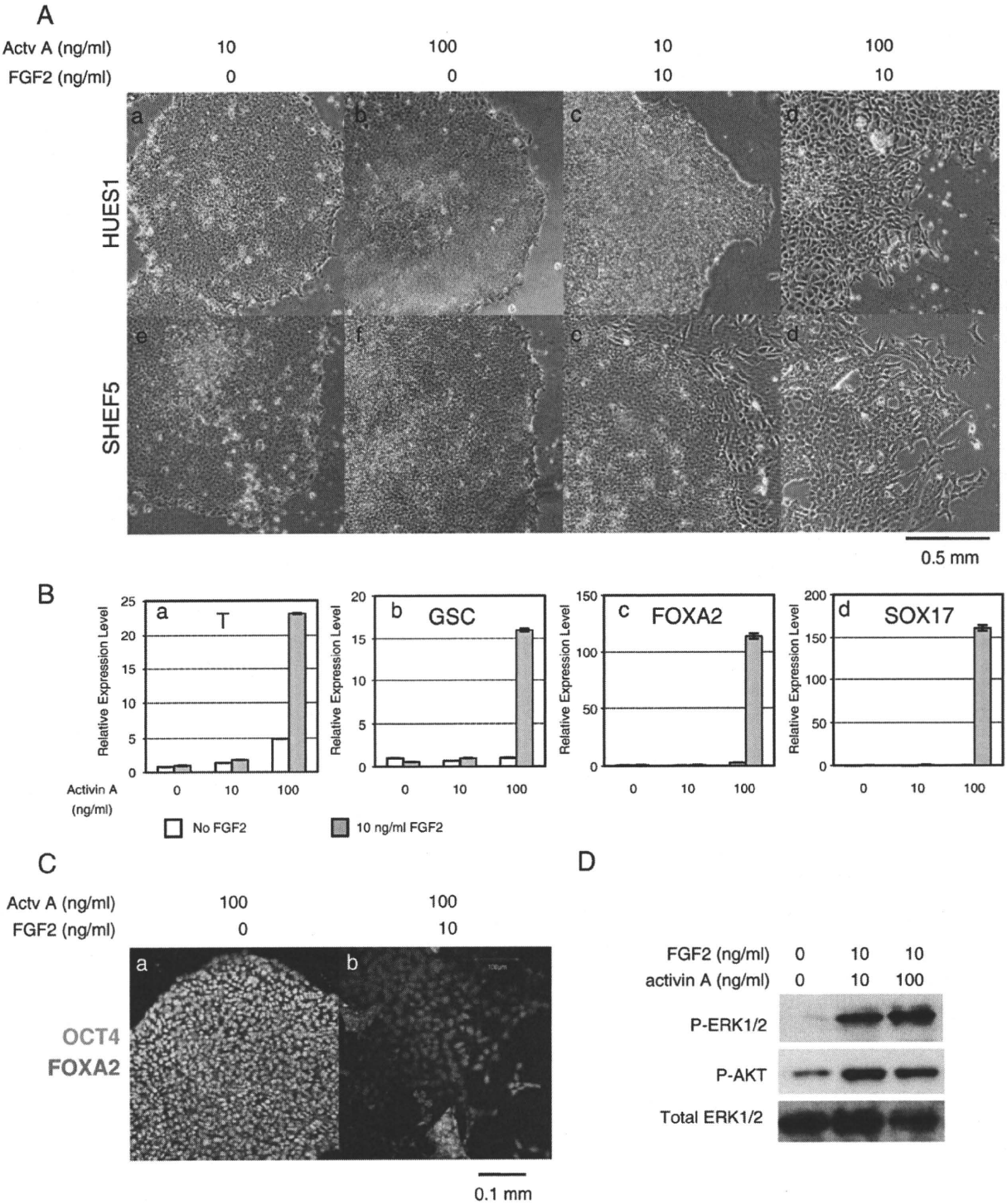


**Figure 1** Addition of FGF2 led to robust mesendodermal differentiation induced by a high dosage of activin A. (A) Morphology of HUES1 and SHEF5 ES cells cultured in 10 and 100 ng/ml of activin A with or without FGF2 after 5 days. Note that 100 ng/ml activin A plus 10 ng/ml of FGF2 strongly induced epithelial-to-mesenchymal transition. (B) Q-PCR analysis revealed robust upregulation of mesendodermal marker genes under these conditions. (C) Immunostaining of OCT4 (green) and FOXA2 (red) in normal HUES1 cells treated with 100 ng/ml of activin A with or without FGF2 for 5 days. (D) A higher dosage of activin A caused stronger ERK1/2 but not AKT phosphorylation. Karyotypically normal HUES1 cells were first cultured in hESF8 (without FGF2 and activin A) for 48 h, and then treated with FGF2 and activin A at the indicated concentration for 30 min before Western blot analysis.

results were obtained for SHEF5 cells; Supplementary Figs. 2C and D.) Treating the floating cell aggregates with U0126 for 5 days improved their ability to spread out on the substrate in a concentration-dependent manner (Fig. 3C, panels b–d). The best concentration in this experiment was 10 μM U0126, in which the cell aggregates flattened out and

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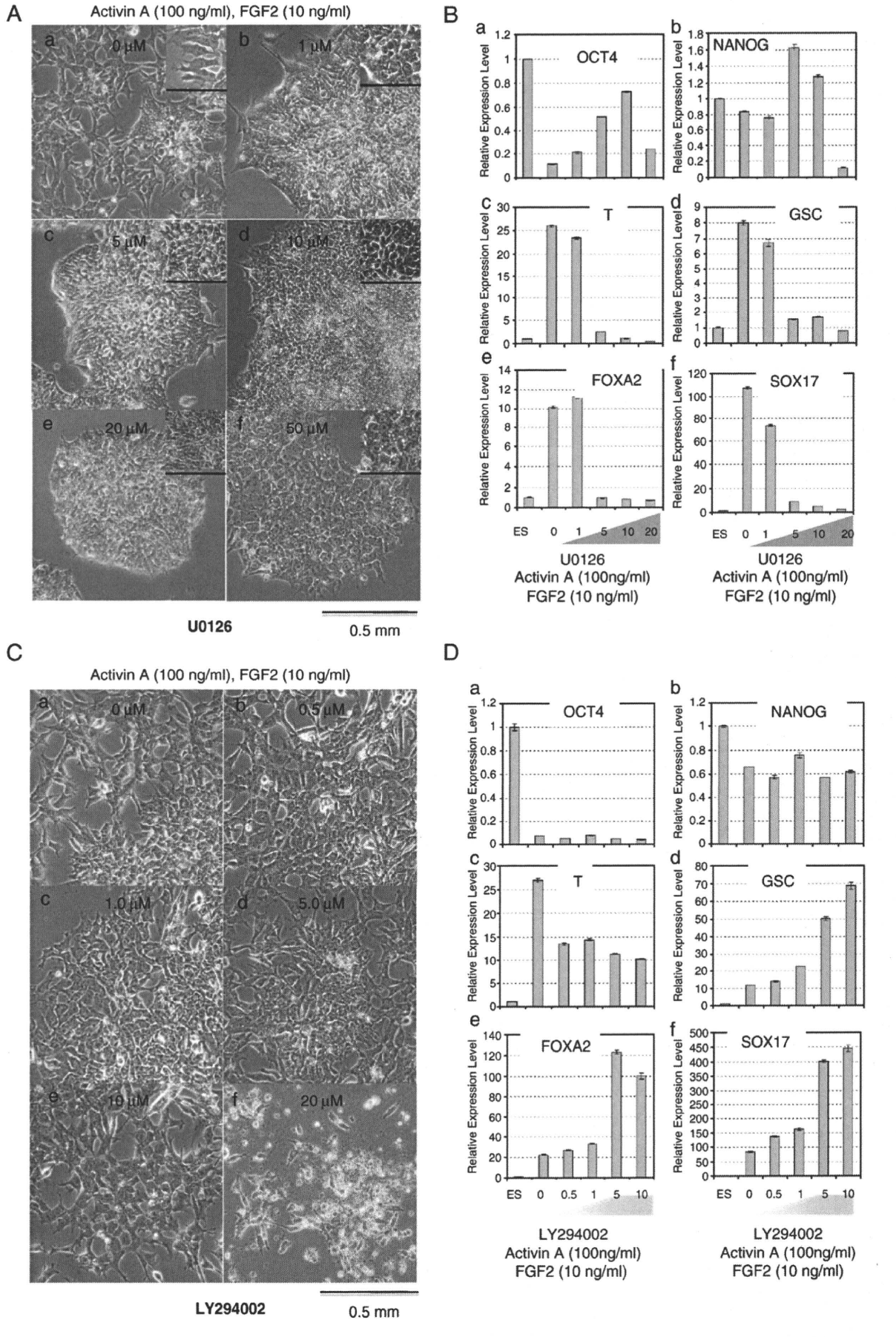


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formed compact colonies typical of undifferentiated ES cells (Fig. 3A, panel d). Moreover, the upregulation of neural marker genes was inhibited (Fig. 3C). However, at 20 and 50 μM, U0126 appeared to be toxic, since we observed many vesicles emerging inside the cell and a dramatic increase in cell death (Fig. 3A, panels e and f). We then passaged U0126-treated and nontreated cells and cultured them in hESF9A for 7 days. Immunostaining was carried out to further examine the expression of OCT4, SIX3, and PAX6. We found that without prior U0126 treatment, cells formed "rosette-like" clusters and the expression of OCT4 protein was markedly reduced (Fig. 3C, panel a), while in the same cultures many rosette cells formed showing SIX3 and PAX6 nuclear staining (Fig. 3C, panels b and c). By contrast, after U0126 treatment, HUES1 cells did not form rosettes and maintained uniform nuclear OCT4 staining (Fig. 3C, panel d), while SIX3 and PAX6 proteins were not detected (Fig. 3C, panels e and f). U0126 also prevented neural differentiation from SHEF5 cells (Supplementary Figs. 2C and D).

## ERK1/2 activation is required to counterbalance BMP-induced differentiation in monolayer culture

Human ES cells express several BMP ligands (Sperger et al., 2003). Moreover, the Knockout serum replacement in the conventional human ES cell culture medium includes BMP-like activities (Xu et al., 2005). Thus, we next tested whether ERK1/2 activation can counteract BMP-induced differentiation in human ES cells using the hESF-based monolayer culture system. After exposure to BMP4 for 5 days, human ES cell colonies lost their compact morphology, appearing flattened (Fig. 4A, panel a), and also lost the expression of the OCT4 protein (Fig. 4B, panel a). By contrast, when cells were cultured in the presence of 50 ng/ml of FGF2 together with BMP4, they maintained an undifferentiated morphology as well as nuclear OCT4 expression (Figs. 4A and B, panel c). Thus, FGF2 signaling appears to counteract the BMP pathway. However, when U0126 was added to BMP4 in the absence of FGF2, these flat cells appeared even earlier and more extensively than with BMP4 alone (Fig. 4A, panel b). We could not detect any OCT4 protein in these cells (Fig. 4B, panel b). U0126 treatment also almost abolished the ability of FGF2 to rescue the cells from BMP4-induced differentiation, as many flat cells were observed under the BMP4+FGF2 U0126 condition and OCT4 staining was absent in most of them (Figs. 4A and B, panel d). In agreement with the morphological changes, the RNA levels of the early extraembryonic lineage marker AFP increased markedly in BMP4-treated cells, but were downregulated when high concentrations of FGF2 were added (Supplementary Fig. 3). Cells cultured in the presence of BMP4, FGF, and U0126 showed similar upregulation of AFP as those treated with BMP4 alone (Supplementary Fig. 3). This suggests that one major role of FGF2–ERK1/2 signaling is to suppress the differentiation induced by BMP, which may explain the reports that ERK1/2 inhibition promotes the differentiation of human ES cells cultured in media containing KSR (Xu et al., 2002; Li et al., 2007).

## ERK1/2 function is required for the attachment of human ES cells at low cell density

In the presence of U0126, we observed a reduction in the attachment of the human ES cells to the collagen substrate after passaging, resulting in a high proportion of cells floating in the media. To test the effect of U0126 on cell attachment more easily, we used a culture-adapted HUES1 subline that carried an extra chromosome 12p, because these cells survive better after trypsin dissociation into single cells. In condition A, HUES1 cells cultured in hESF9A without U0126 were dissociated into single cells and plated at 2000 cells/cm$^2$ density in hESF9A without U0126. In condition B, HUES1 cells were first cultured with U0126 for 5 days; the drug was then removed 2 h before harvesting and the cells were plated at the same density in hESF9A without U0126. In condition C, the cells were cultured without U0126 but plated in hESF9A containing U0126. The day after seeding, we found similar numbers of cells attached to the substrate in conditions A and B, whereas almost no cells attached in condition C (Figs. 5A and B). Thus, ERK1/2 activity appears to be required for cell adhesion and subsequent survival under single-cell or low-density conditions. After culturing the cells for 5 days after seeding in hESF9A, we added U0126 back into the condition B group and maintained the cultures for a further 5 days. A high-content screen was carried out to analyse the growth and differentiation status of the cells (Figs. 5C and D). On average, cultures maintained under conditions A and B had similar numbers of colonies and numbers of cells per field imaged, indicating that once cells are attached and have reached a certain density, U0126 does not affect their growth significantly. However, there were markedly more OCT4- and SSEA4-positive cells per field in condition B (when cells were cultured at later stages in U0126) than in condition A, suggesting that inhibition of ERK1/2 by U0126 reduced spontaneous differentiation (Fig. 5D, panels a and b). Under condition C, when cells were seeded in U0126 but cultured without it, there were very few colonies and only a small proportion of cells expressed OCT4 and SSEA4 (Fig. 5C, panel c, and D condition C). We also tested the effect of U0126 on karyotypically normal SHEF6 cells. Similar to HUES1, U0126 significantly reduced the number of single cells attached to the substrate and, consequently, almost no colony formed in this group (Supplementary Fig. 4). Taken together, these results indicate that ERK1/2 inhibition has a negative impact on cell attachment to the substrate.

**Figure 2** (A) Morphology of HUES1 ES cells cultured in activin A (100 ng/ml) plus FGF2 (10 ng/ml) treated with increasing concentrations of U0126. Scale bar: 0.5 mm. (B) Q-PCR analysis of marker-gene expression. Their relative expression levels were compared to the levels in ES cells grown under normal serum-free culture conditions. (C) Morphology of hESCs cultured in activin A (100 ng/ml) plus FGF2 (10 ng/ml) treated with increasing concentrations of LY294002. Scale bar: 0.5 mm. (D) Q-PCR analysis of marker-gene expression. Their relative expression levels were compared to the levels in human ES cells grown in hESF9A.

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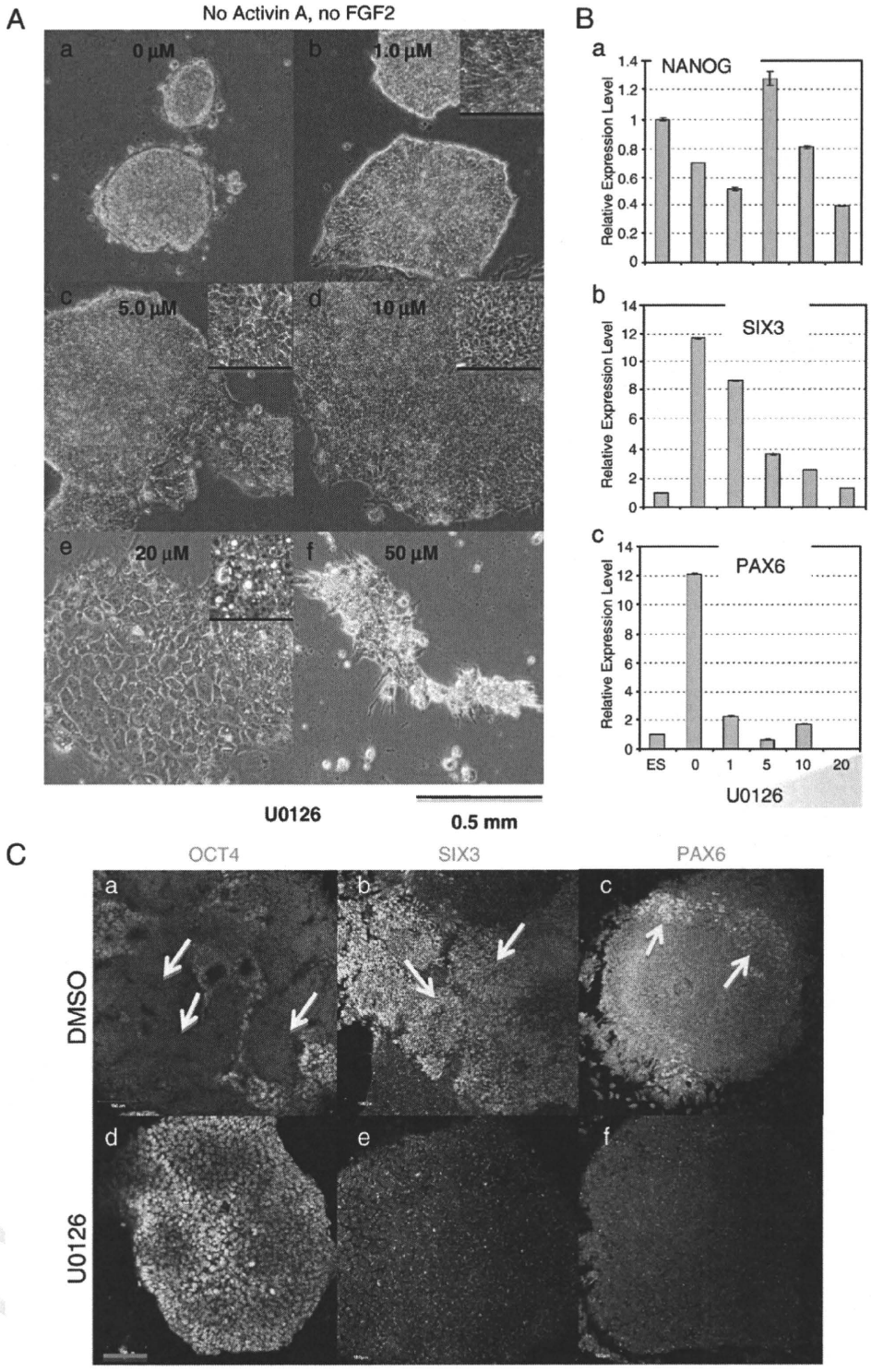


**Figure 3** (A) Morphology of human ES cells cultured without activin A or FGF2 treated with increasing concentrations of U0126. Scale bar: 0.5 mm. Inset scale bar: 0.1 mm. (B) Q-PCR analysis of *NANOG*, *SIX3*, and *PAX6* expression. Their relative expression levels were compared to the levels in ES cells grown under normal serum-free culture conditions. (C) Immunostaining of OCT4, SIX3, and PAX6 (in green) in cells first treated without (a–c) or with U0126 (10 μM) (d–f) in hESF8 for 5 days, passaged, and then cultured in hESF9A. Note the absence of significant OCT4 staining in the absence of U0126, especially from the neural rosettes (panel a, arrows), and the retention of OCT4 expression the presence of U0126. By contrast, note the expression of SIX3 and PAX6, especially their nuclear localisation in the rosettes (arrows) in the absence of U0126 (panels b, c), but the absence of these transcription factors when the cells were cultured with U0126 (panels e, f). DNA: blue. Scale bar: 100 μm.

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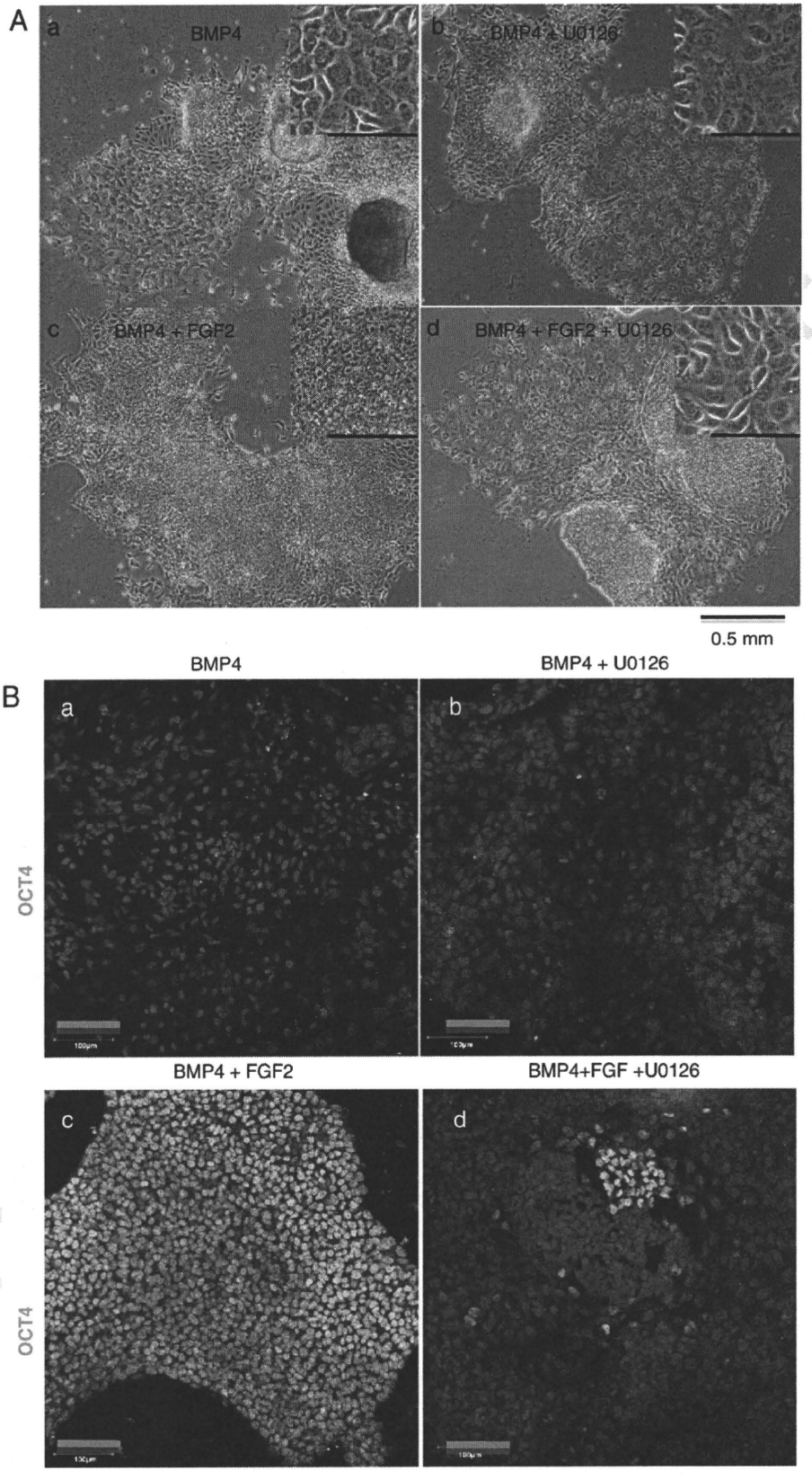


**Figure 4** (A) Morphology of HUES1 cells cultured in different BMP4 (1 ng/ml), FGF2 (40 ng/ml), and U0126 (10 μM) combinations as indicated on the top of each graph. Scale bar: 0.5 mm. (B) Immunostaining of OCT4 (in green) of above experiment. DNA: blue. Scale bar: 100 μm.

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## Long-term culture of human ES cells in a chemically defined medium containing U0126

Since U0126 can inhibit ES cell differentiation under chemically defined conditions, we assessed whether it can be used to support the self-renewal of several human ES cells during long-term culture in the hESF9A medium. To reduce the adverse effect of U0126 on cell adhesion, it was omitted from the medium when the cells were seeded as well as the following day; subsequently, cultures were fed with a medium containing U0126 until the next passage. After 3 passages (approx 3 weeks), three independent human ES cell lines, SHEF4, SHEF6, and the adapted HUES1 subline, grew to produce very large undifferentiated colonies in the hESF9A medium with 5 μM U0126 (Fig. 6A, panels a, c, and e), whereas without U0126 increased spontaneous differentiation was evident for the SHEF4 and SHEF6 cell lines (Fig. 6A, panels b and d). The adapted HUES1 subline also showed better colony morphology when cultured with U0126 (Fig. 6A, panel f). After culturing in U0126 for more than 3 months, SHEF4 and SHEF6 cells retained a normal karyotype, while no additional karyotype changes were detected in the adapted HUES1 line (Supplementary Fig. 6A). The SHEF4 and SHEF6 human ES cell lines expressed significantly higher levels of OCT4 and SSEA4 when grown in the presence of U0126 compared to nontreated cells (Fig. 6B). Adapted HUES1 cells displayed a less increased expression of OCT4 and SSEA4 when grown in the presence of U0126. This might reflect the innately different properties of individual human ES cell lines (Fig. 6B). To confirm the specificity of U0126, we used PD0325901, another ERK1/2 inhibitor used by

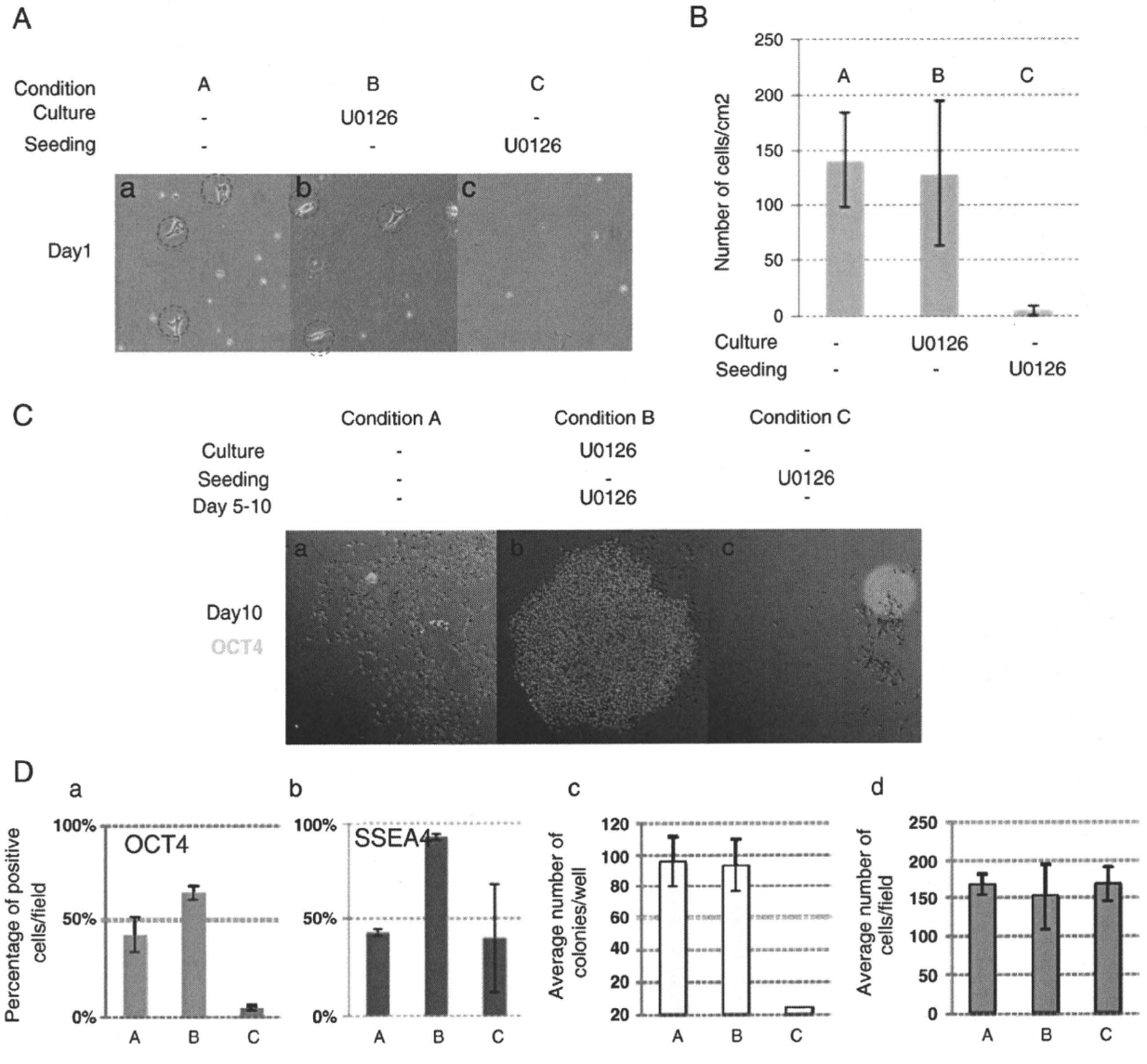


**Figure 5** Inhibition of ERK1/2 prevented human ES cell attachment to the substrate. (A) Phase-contrast images of HUES1 cells attached to the substrate under different conditions (listed above each graph). (B) Bar-graph quantisation of HUES1 cell attachment after passaging with or without U0126. Seeding density was 2000 cells/cm$^2$. (C, a–c) Immunostaining of OCT4 expression under different conditions (listed above each graph). (D) Bar-graph presentation of the percentage of (a) OCT4- or (b) SSEA4-positive cells per field, (c) number of colonies per well, and (d) number of cells per field. The bars represent the mean of all measured colonies by the In Cell Analyser Developer's Toolbox and the error bar is the standard error of the mean (SEM).

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Ying and colleagues on mouse ES cells (Ying et al., 2008). We found that 0.3 μM PD0325901 was sufficient to block the phosphorylation of ERK1/2 in SHEF4 cells (Supplementary Fig. 6A). It reduced differentiation of the SHEF4 and SHEF6 cell lines during a period of 4 weeks (Supplementary Fig. 6B). Human ES cells cultured in PD0325901 or U0126 for 1–3 months retained the ability to undergo neural, mesendodermal, and extraembryonic differentiation when appropriate inducing conditions were applied. As shown in Supplementary Figs. 5B and 6D, differentiated cells strongly expressed TUJ1 (neural), BRACHYURY, FOXA2, SOX17 (mesendoderm), and CDX2 (extraembryonic) and downregulated OCT4. Our results show that the inhibition of ERK1/2 signaling helps to prevent differentiation of human ES cells in chemically defined environments and does not affect the pluripotency of these cells.

## Discussion

In this study, we analysed the function of ERK1/2 in human ES cells under chemically defined culture conditions. Contrary to some previous reports that inhibition of ERK1/2 causes human ES cell differentiation (Li et al., 2007), our data demonstrate that inhibition of ERK1/2 signaling can be beneficial for self-renewal. Blocking ERK1/2 prevented mesendoderm and neural induction and, consequently, the

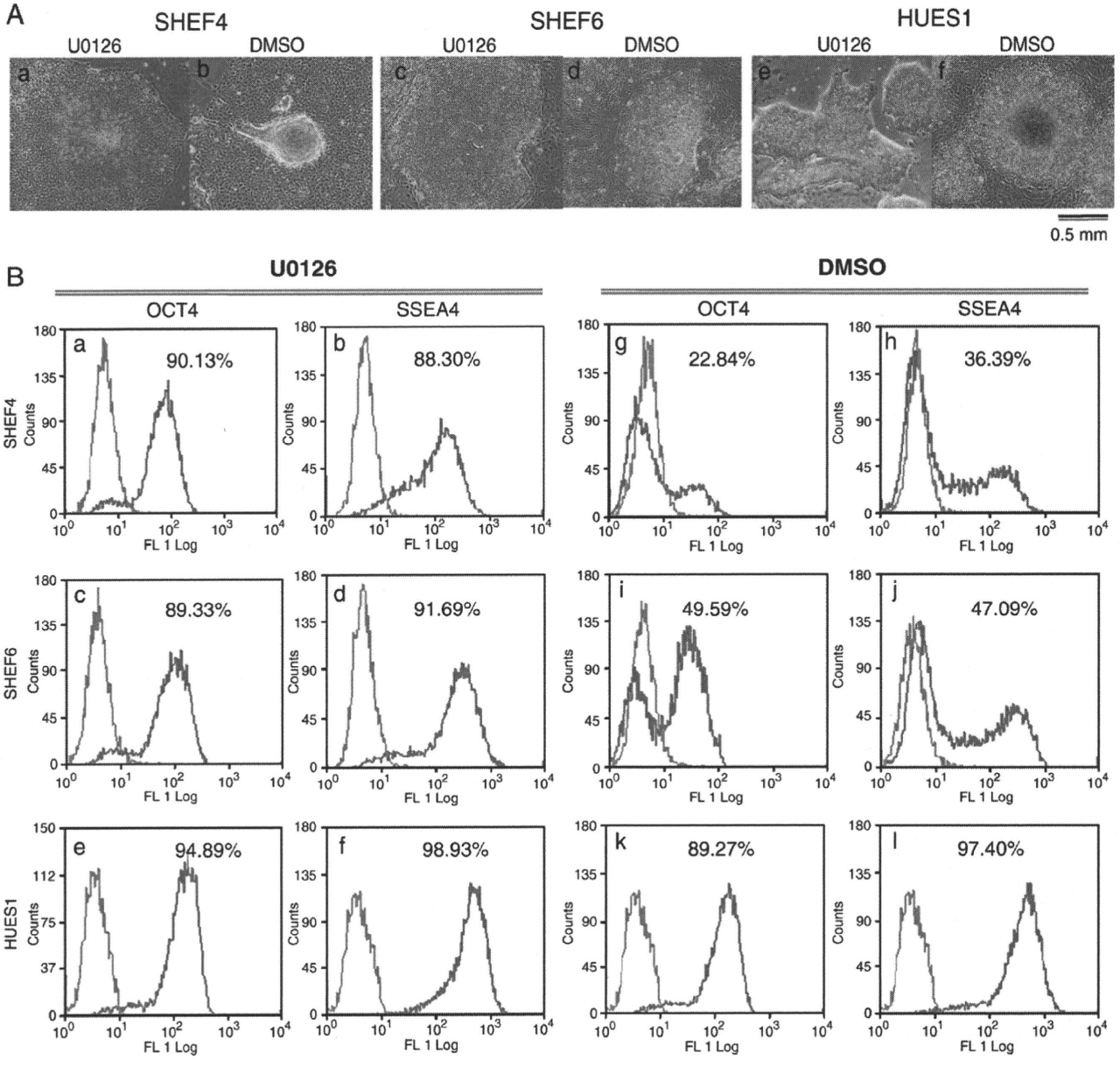


**Figure 6** (A) Morphology of human ES cells cultured for 3 passages with or without U0126 in hESF9A. (B) FACS analysis of OCT4 and SSEA4 expression in human ES cells cultured with or without U0126 for 3 passages (3 weeks). The gray histogram is for SHEF4, SHEF6, and HUES1 cells stained with P3X (negative control). The red histograms represent the experimental population.

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ES cell phenotype was retained. However, ERK1/2 activation was needed to suppress BMP signaling under normal ES cell-culture conditions. Human ES cells retained OCT4 expression in the presence of a high dosage of FGF2 and 1 ng/ml of BMP4. When ERK1/2 inhibitor U0126 was added, cells rapidly differentiated toward an extraembryonic fate (Fig. 4). In this respect, human ES cells appear to be fundamentally different from mouse ES cells, as both FGF4 -/- and ERK2 -/- mouse ES cells were resistant to BMP4-induced differentiation (Kunath et al., 2007). This difference could explain why inhibition of ERK1/2 causes human ES cell differentiation in a KSR-containing medium that includes BMP-like activity (Xu et al., 2002, 2005). As the activation of ERK1/2 can suppress BMP signaling, blocking ERK in effect enhances BMP pathway activities and, consequently, cells differentiate toward extraembryonic fates. In a defined culture system supplemented with FGF2 and activin A but no exogenous BMP ligand, ERK1/2 is not required to suppress BMP signaling, and since inhibition of ERK1/2 stops both neural and mesendoderm differentiation, the cells will remain in the undifferentiated state.

In human ES cell cultures, FGF2 appeared to constitute some of the functions of LIF in mouse ES cells. In mice, parallel to the STAT3–JAK pathway that upregulates KLF4 and promotes pluripotency, LIF also activates both PI3K–AKT and MAPK1/2–ERK1/2 pathways (Niwa et al., 2009). AKT increases the expression level of the transcription factor TBX3, which is located upstream of the pluripotency factors NANOG and SOX2 (Niwa et al., 2009). On the other hand, the MAPK1/2–ERK1/2 pathway downregulates TBX3 and NANOG to encourage lineage commitment (Niwa et al., 2009). Both LIF and endogenously expressed FGF4 can elevate ERK activity, which gives ES cells a tendency to initiate spontaneous neural differentiation under serum- and feeder-free culture conditions (Stavridis et al., 2007; Ying et al., 2003a). In this scenario, addition of BMP4 suppressed neural differentiation by inducing the expression of Id proteins through SMAD1 (Ying et al., 2003b). Blocking ERK1/2 can prevent mouse ES cell neural differentiation and "trap" them in the "ground state" of self-renewal (Stavridis et al., 2007; Ying et al., 2008). In a separate study, Pluripotin, a small molecule that inhibits both ERK1/2 and Ras-GAP, was also able to sustain mouse ES cell self-renewal in the absence of BMP4 under chemically defined conditions, possibly through the same mechanism (Chen et al., 2006). Although LIF can stimulate STAT3 phosphorylation and, therefore, its activation, in human and primate ES cells this has no detectable effect on the pluripotency network (Daheron et al., 2004; Sumi et al., 2004). FGF2 strongly activates both AKT and ERK1/2. The AKT branch is critical for ES cell self-renewal. Watanabe and colleagues showed that overexpressing a constitutive active mutant form of AKT was able to maintain the self-renewal of both mouse and primate ES cells (Watanabe et al., 2006). In our experiments, inhibition of AKT through blocking PI3K accelerated mesendodermal differentiation of human ES cells under inducing conditions (Figs. 2C and D). Under chemically defined conditions, we found that ERK1/2 activation is required for proper neural and mesendodermal lineage specification. These two important functions seem to be conserved between human and mouse ES cells. ERK1/2 inhibition significantly reduces spontaneous differentiation as long as there is no BMP activity present and can thus serve as a useful strategy to maintain human ES cell self-renewal under defined conditions.

The interactions among ERK1/2, BMP, and TGFβ signaling pathways are complex and may depend on the relative levels of BMP and TGFβ signaling. It has been shown that ERK1/2 can phosphorylate the linker region of SMAD1 (a key transducer of BMP signals), and thus lead to its cytoplasmic retention and degradation (Fuentealba et al., 2007; Sapkota et al., 2007). Similarly, it can either directly phosphorylate SMAD2/3 or indirectly through other kinases such as CDK4/6, to regulate SMAD2/3 stability and subsequently the strength of TGFβ signaling (Schmierer and Hill, 2007). In a separate study from our laboratory by Avery et al., in a KSR-based medium inhibition of an activin A receptor-like kinase (ALK) strengthened BMP signaling, as indicated by stronger phosphorylation of the C-terminus of SMAD1/5/8 (its active form) and the upregulation of BMP-responsive genes such as MSX1. Knocking down SMAD4 reduced SMAD1/5/8 activation, abolished the upregulation of differentiation genes, and partially rescued the expression of the pluripotency genes OCT4 and NANOG (Avery et al., 2010). This implies that activin A/TGFβ signaling can sequester SMAD4 (the co-SMAD essential for both BMP and TGFβ pathways) from the BMP pathway, thus in effect attenuating the strength of the BMP activity. FGF2–ERK1/2 enhances activin A signaling and, in turn, antagonises BMP signaling and the associated extra-embryonic lineage differentiation. BMP4 is also reported to induce mesoderm differentiation from EBs (Takei et al., 2009). In this circumstance, the cell–cell interaction and the spatial organisation of the EBs may also have an impact on cell signaling and fate decisions.

MAPK–ERK1/2 signaling cascades also play critical roles in cell adhesion. In our experiments, U0126 markedly reduced cell attachment during passaging and prevented EMT during induced mesendodermal differentiation. Two recent publications showed that inhibition of FGF signaling affected human ES cell adhesion, which resulted in the upregulation of caspase 3 and anoikis, while a high concentration of FGF2 increased human ES cell clonogenicity and reduced the number of cells floating up in the culture (Eiselleova et al., 2009; Wang et al., 2009). ROCK inhibitor Y27632 could not rescue the reduction in cell adhesion caused by ERK1/2 inhibition (Supplementary Fig. 4). ERK1/2 inhibition is particularly detrimental to human ES cells when they are at low density. This is likely because cells are more dependent on the survival signal provided by cell–substrate adhesion. We noted that, as human ES cells grew to higher density, U0126 or PD0325901 treatment no longer impairs cell survival as much. Thus, close cell–cell contact must send out additional prosurvival signals. One of the candidate pathways is Notch signaling. RNAi knocking down of NOTCH1 and 2, or their canonical effector CBF-1, or blocking Notch signaling with the γ-secretase inhibitor L-685,458 all markedly reduced the growth of human ES cells (Fox et al., 2008). It has been shown that the engagement Notch receptor led to the activation of AKT, the transcription factor STAT3, and mammalian target of rapamycin to promote the survival of stem cells (Androutsellis-Theotokis et al., 2006). E-cadherin-mediated cell adhesion has also been shown to activate AKT though PI3K to stimulate epithelial cell growth (Pece et al., 1999). Therefore, cell–

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cell contact-dependent AKT activation may be able to compensate for the detrimental effect caused by ERK1/2 inhibition in human ES cells once they reach a certain density.

Based on our results, we propose that ERK1/2 plays multiple important roles in human ES cells (Fig. 7): it is required for neural and mesendoderm induction and essential for cell attachment to the substrate; however, it can suppress BMP signaling and prevent extraembryonic differentiation. Our model could explain the discrepancies regarding the roles of FGF2 and ERK1/2 in human ES cells and mouse ES cell self-renewal. Further dissecting the "balancing action" among FGF, BMP, and activin A/TGFβ pathways under chemically defined conditions will offer insights into how these developmental signals regulate mammalian ES cell fate decisions.

## Materials and methods

### Cell lines and maintenance

HUES1 (normal and adapted), SHEF4, SHEF5 and SHEF6 human ES cells were used in this study. SHEF4, SHEF5, and SHEF6 cells were maintained on feeders in an ES medium containing KO-DMEM (Invitrogen) supplemented with nonessential amino acids, 0.1 μM 2-mercaptoethanol, 1 mM glutamine, 20% serum replacement (Invitrogen), and 4 ng/ml FGF2 (Peprotech) (Amit et al., 2000). HUES1 cells were maintained on feeders in an HUES1 medium containing KO-DMEM supplemented with nonessential amino acids, 0.1 mM 2-mercaptoethanol, glutamine, 8% serum replacement, 8% plasminate (Bayer), and 4 ng/ml FGF2 (Cowan et al., 2004). In both cases, the cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. The cells were passaged with 1 mg/ml collagenase IV (Invitrogen) (for SHEF lines) and 0.05% trpsin/0.04% EDTA (Sigma) (for HUES1), respectively. The cells were seeded onto a 25 $cm^2$ flask (NUNC) that had been previously coated with 0.1% porcine gelatin (Sigma) and mitomycin C (Sigma)-inactivated MEF as feeder cells.

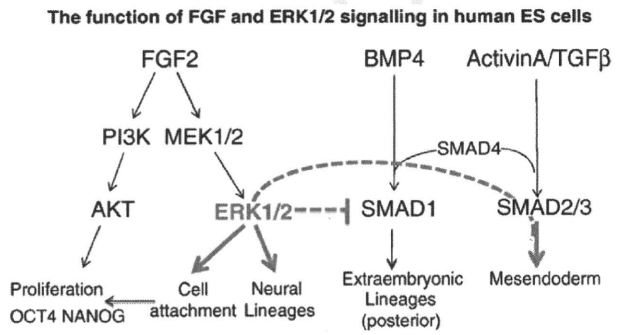


**Figure 7** Model depicting multiple roles of ERK1/2 in human ES cells. FGF2 activated both AKT and ERK1/2. ERK1/2 promotes neural and mesendodermal (together with a high dosage of activin A/TGFβ) differentiation from ES cells while it suppresses BMP-induced extraembryonic lineage differentiation. It is also needed for human ES cell attachment to the substrate, which is essential for ES cell survival and proliferation.

### Human ES cell culture in serum-free, feeder-free, and chemically defined medium

Prior to growth-factor-treatment experiments, human ES cells were grown for more than 3 passages in the absence of feeders in a serum-free and chemically defined medium on type I collagen gel (Nita Gelatin, Japan) (Furue et al., 2008). The hESF basal medium was purchased from the Cell Science & Technology Institute Inc., Sendai, Japan. The components of hESF8, 9, and 9A are listed in Supplementary Table 1. Because cells are very sensitive to the passaging strategy used, including the size of colonies that are passaged, the addition of activin A permits the maintenance of more robust and reproducible cultures of undifferentiated cells. Thus, hESF9A containing 10 ng/ml of FGF2 and activin A each was used for regular serum-free culture. Before the start of each series of experiments, when necessary, manual passaging was used to ensure a uniform undifferentiated starting cell population for all treatment groups.

### Growth-factor treatment and differentiation protocols

Serum-free, feeder-free cultured human ES cells were seeded in 12-well plates at a density of $4\times10^4$/well. One day after seeding, the cells were washed with an hESF8 medium, and then fed with hESF8 and with activin A (R&D systems), BMP4, and FGF2 (Peprotech) added at the desired concentrations. The medium was changed every 2 days with freshly added growth factors. Cells were collected after 5 days and analysed. For neural differentiation, we used a protocol modified from Zhang et al. (2001). Briefly, SHEF4 and SHEF6 cells were first cultured as floating EBs in hESF8 with 4 ng/ml of FGF2 for 1 week. These EBs were then plated in 6-well plates coated with poly-L-ornithine hydrobromide (PLOH) and 2 μg/$cm^2$ Laminin (LN) (Sigma) and fed with hESF8 and 20 ng/ml of FGF for 1 week. Afterward, colonies with neural rosette morphology were manually picked and cultured in suspension in the same medium for another 7 days. These rosettes were replated on a PLOH/LN surface for 7–10 days. Rosettes with extensively long neurites were picked and dissociated to obtain a relatively pure population of neuronal cells. To initiate mesendodermal or extraembryonic differentiation, cells were treated with 100 ng/ml activin A plus 10 ng/ml FGF2 or 10 ng/ml of BMP4 on 2 μg/$cm^2$ FN-coated surface for 7 days.

### Antibodies and chemical inhibitors

The following antibodies were used in this study: SMAD4, OCT4, ERK1/2, and BRACHYURY (Santa Cruz); CDX2, FOXA2, NANOG, and SOX17 (R&D systems); SIX3 (a rabbit polyclonal antibody raised against polypeptide RLQ HQA IGP SGM RSL AEP GC which corresponds to the C terminus of mouse SIX3; the same sequence was present in human SIX3); PAX6 and FORSE1 (Developmental Studies Hybridoma Bank, IA, USA); phospho-ERK1/2, phospho-AKT, phospho-GSK3β, total GSK3 β (Cell Signaling); SOX2 and actin (Abcam, Cambridge UK). The monoclonal antibody to SSEA4 was produced from a culture of hybridoma 813-70 (Kannagi et al., 1983). The chemical inhibitors U0126, LY294002, and PD0325901 were

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purchased from Promega, Merck, and AXON Medchem (Groningen, the Netherlands), respectively.

## Western blot

For Western blot analysis, HUES1 cells were first cultured in hESF9A for 48 h and then washed briefly with PBS and cultured in hESF8 (without FGF and activin A) for 48 h. Afterward, 10 ng/ml of FGF2 and chemical inhibitors (U0126 10 μM, LY294002 10 μM, PD0325901 0.3 μM, or DMSO only) were added, while 30 min later cells were detached and lysed on ice in a sample-loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, PhosSTOP (Roche), and 0.002% bromophenol blue). The amount of $2\times10^5$ cells-equivalent lysate was loaded per lane.

## Quantitative PCR

Total RNA was extracted with TRIZOL (Invitrogen). The amount of 1 μg RNA of each sample was used for reverse transcription (20 μl) using Superscript III (Invitrogen). Q-PCRs were performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in an iCycler (Bio-Rad). Each gene was done in triplicate (Fig. 1) or duplicate (Figs. 2 and 3). The sample input was normalised against the $C_t$ (critical threshold) value of the housekeeping gene GAPDH. Relative quantification of each gene was performed using the iCycler Excel program. The error bar is the standard deviation of the $C_t$ value. Primer sequences are listed in Supplementary Table 2.

## Immunostaining, FACS, and high-content screen

For in situ immunostaining and high-content screen, cells were fixed in 6-well plates with 4% PFA in PBS. OCT4 and SSEA4 staining was scanned in an In Cell Analyser 1000 (Amersham Biosciences) and analysed using the Developer's Toolbox. OCT4, SIX3, and PAX6 staining was imaged with an Olympus FV1000 confocal microscope. For FACS analysis, cells were incubated with 0.05% trypsin in 0.2% EDTA in 37 °C, 10% $CO_2$ incubator until most dissociated into single cells. Trypsin inhibitor (Sigma T6522) (1 mg/ml in DMEM) was immediately added to inactivate the trypsin. Cells were subsequently rinsed off the flask or 6-well plate with an hESF8 medium, centrifuged down, and washed 3 times in PBS containing 4 mg/ml polyvinylpyrrolidone (Sigma) to prevent them from sticking together. They were fixed in 4% PFA in PBS 0.1% Tween 20 (PBST) for 20 min at room temperature. For intracellular staining of Oct4, 0.2% Triton X-100 was included in the fixative. Afterward, cells were washed 3 times in PBST, blocked with 5% FBS–PBST for 1 h, and incubated with the desired primary and secondary antibodies. FACS was done using a DAKO Cytomation $CyAn_{ADP}$ machine.

## Y27632 treatment and clonogenicity assay

SHEF6 cells were dissociated into single cells as described above and seeded at 10,000 cells/$cm^2$ in FN (2 μg/$cm^2$) coated 6-well plates in hESF9A. Y27632 (10 μM) or U0126 (10 μM) was added 2 h prior to passaging and overnight during seeding as described in Watanabe et al. (2007). The medium was replaced the next day. Cells were grown in hESF9A for another 10 days, then fixed briefly, and stained with alkaline phosphatase (AP) Red Substrate Solution (Sigma). AP-positive colonies were photographed and counted.

## Uncited reference

Harb et al., 2008 Q1

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.scr.2010.06.002.

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LETTER

# Advantages and difficulties in culturing human pluripotent stem cells in growth factor-defined serum-free medium

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Dear Editor,

Serum- and feeder-free culture conditions have received a great deal of attention for culturing human embryonic stem (hES) cells or induced pluripotent stem (iPS) cells although hES/iPS cells are still most commonly maintained on inactivated mouse embryonic fibroblast feeders (MEF) in medium supplemented with FBS (Thomson et al. 1998; Reubinoff et al. 2000), or proprietary replacements, such as knockout serum replacement (KSR) together with fibroblast growth factor-2 (FGF-2) (Amit et al. 2000; Draper et al. 2004). Use of culture media containing undefined or undisclosed components has limited the development of applications for pluripotent cells because of our lack of knowledge of their responses to specific cues that control self-renewal, differentiation, and lineage selection. Therefore, a defined serum-free medium consisting of minimum essential components for culturing hES/iPS cells could contribute to advances in the field.

Previously, we have developed a defined growth factor-supplemented serum-free medium, hESF9, for the culture of human ES cells on a type I collagen substrate without feeders (Furue et al. 2008). This medium consists of hESF basal medium supplemented with heparin and only four protein components: insulin, transferrin, albumin conjugated with oleic acid, and FGF-2 (10 ng/ml). Under these culture conditions, FGF-2 promotes proliferation of hES cells in a concentration-dependent manner. Heparin, which is known to enhance the activity of FGF, also promotes proliferation of hES cells in a concentration-dependent manner in the absence of FGF-2 suggesting that endogenous FGF-2 is produced by hES cells. In conventional cultures with KSR-based medium, the proliferative effects of FGF-2 or heparin are not detectable although it is well known that FGF-2 supports hES cell growth. Thus, a defined serum-free medium consisting of minimum essential components could aid in elucidating hES/iPS cell responses to specific cues that control self-renewal, differentiation, and lineage selection.

The modern era of serum-free growth began in the mid-1970s when Izumi Hayashi and Gordon Sato (1976) defined conditions for growth of a rat pituitary cell line in a hormonally defined serum-free medium that did not alter cell phenotypes or cell growth. N2 supplements for neural

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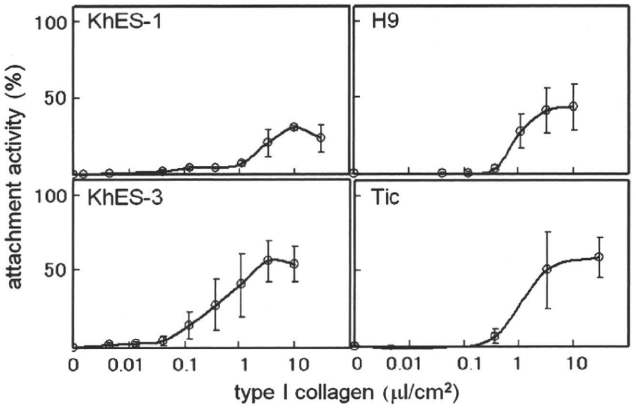


**Figure 1.** Attachment and proliferation of hES and iPS cells on type I collagen. The attachment of hES/iPS cells to ECM components was measured by the procedures followed by Fassler and Meyer (1995). Briefly, a 96-well microplate (Corning Costar, Corning, NY) was coated with each adhesion molecule at 37°C for 3 h. hES/iPS cells were seeded at confluent density ($3\times10^6$ cells $cm^{-2}$) on type I collagen (Nitta Gelatin, Inc.) coated plates in hESF9 (Cell Science & Technology Institute, Inc.). After 3 d, the attached cells were fixed and stained for 30 min with 0.4% crystal violet (Sigma) in methanol. After the plate was washed and dried, a solution (1% acetic acid and 30% ethanol in water) was added to the wells to dissolve the crystal violet. The absorbance of 595 nm, which indicated the concentration of the dissolved crystal violet, was measured with a microplate reader (model 550; Bio-Rad, Hercules, CA). Each graph shows the percentage of the attached cells on type I collagen in hESF9 relative to the attached cells on fibronectin (Sigma) as 100% as all the cell lines attached to fibronectin in hESF9. *Bar*=SE ($n$=3).

cells were developed by Bottenstein and Sato (1979). Later, we described a variety of serum-free media that can be used to propagate and analyze differentiated cells (Barnes and Sato 1980; Sato 1987; Furue et al. 1994; Okamoto et al. 1996; Sato et al. 2002). Serum contains variable amounts of hormones, soluble growth and differentiation factors, attachment factors, and undefined components. Also MEFs secrete a variety of nutrients or growth factors most of which have not been identified. The precise formulation of KSR is not in the public domain, and although "serum-free," it is likely to contain a variety of animal products (PCT/US98/00467; WO98/30679). The formulations of several other products for serum-free culture from several companies are also proprietary. These undefined or disclosed components of the culture conditions hamper analysis of the mechanisms that control cell behavior.

However, there are several difficulties in culturing hES/iPS cells in defined conditions without MEFs. First, propagation of pluripotent hES/iPS cells has been difficult to achieve even in conventional culture media due to their propensity to differentiate (Skottman and Hovatta 2006; Adewumi et al. 2007) as most researchers working with hES/iPS cells know. When cells grown to confluence are split, many differentiated cells appear in the next passage. The variability of batches of MEFs or KSR affects the tendency of cells to differentiate. The dissociation method is the most problematic. Even in conventional cell culture, imprecise handling during dissociation decreases cell survival as single hES/iPS cells cannot survive. Centrifugation at high speed decreases cell survival or promotes cell aggregation. Unless the cells are handled with care for several passages on MEFs in KSR-based medium, the cells do not survive or differentiate when transferred to feeder-free cultures. We had used EDTA to dissociate hES cell colonies in the feeder-free culture method using hESF9 medium. As the attachment activity of undifferentiated hES cells is different from that of differentiated cells, the undifferentiated hES cells were able to be transferred to the next passage, which led to the successful continuous culture of undifferentiated cells. However, this method requires previous practical experience with human primary

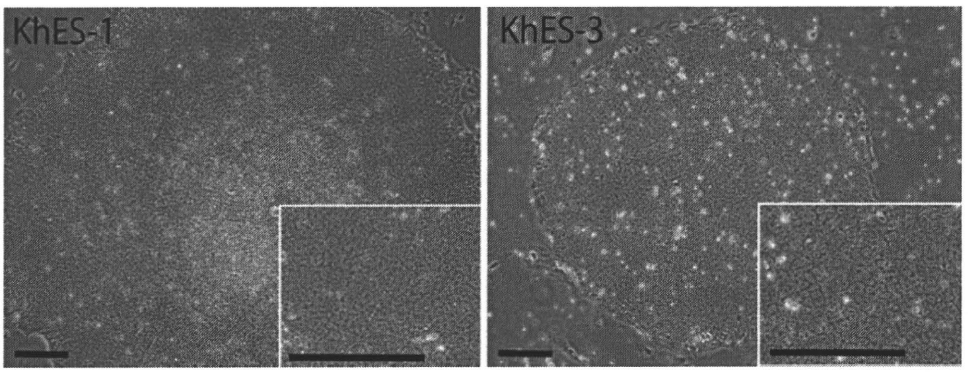


**Figure 2.** hES cells cultured on fibronectin in hESF9 medium. Phase-contrast microphotographs of KhES-1 at passage 16 and KhES-3 at passage 6 cultured on fibronectin in hESF9 medium. After 2 d, the KhES-1 cells and KhES-3 cells (from Kyoto University) cultured on MEF in KSR-based medium were passaged by the routine procedure onto the new MEF; the medium was changed to hESF9 medium, and then the cells were cultured on MEF for more than 4 d. Previously, we dissociated the hES cells with EDTA, but some of cell lines could not survive after the dissociation with EDTA. Then, we have utilized 1 U/ml dispase (Roche Applied Science) for approximately 1 min at 37°C to dissociate the hES/iPS colonies into the small clumps. The cells were washed twice at 20 g and then seeded on fibronectin-coated flask (Corning Costar) in hESF9 medium.

cell cultures. As hESF9 medium consists of minimum essential components, improper handling greatly affects cell viability and culture outcome.

Second, there are variations in the characteristics of hES/iPS cell lines as most of the researchers working on hES/iPS cells have come to realize. hES cell lines, Shef1, Shef5, and HUES1 cultured in the University of Sheffield were able to attach to type I collagen and grow well in hESF9 medium (Furue et al. 2008). However, we found that attachment activity of the KhES-1 line (Nakatsuji 2005), which was established in Kyoto University, to type I collagen seemed low, suggesting that there is a difference between the cell lines in their attachment ability. Owing to regulatory issues for hES cell importation into Japan, we were unable to directly compare KhES-1 cells with Shef1 or HUES1. We have examined the attachment and growth activity of other hES lines (KhES-3 from Kyoto University, Kyoto, Japan; H9 from National Stem Cell Bank, WiCell, Madison, WI) and the MRC-5-derived iPS cell line Tic (JCRB 1331, JCRB Cell Bank, Osaka, Japan), which was established in the National Center for Child Health and Development, on type I collagen (Nitta Gelatin, Osaka, Japan) in hESF9 medium (Cell Science & Technology Institute, Sendai, Japan). The results show that there is a difference between hES/iPS cell lines in attachment activity to type I collagen. This result suggests that there is a difference among cell lines in integrin signaling (Fig. 1).

Based on these findings, we have modified the culture protocol and tried to culture KhES-1 and KhES-3 cell lines on fibronectin (Sigma, St. Louis, MO) in hESF9 medium without feeders (Fig. 2). Matrigel, a basement membrane preparation from the Engelbreth–Holm–Swarm mouse tumor, is often used for feeder-free culture for hES/iPS cells with MEF-conditioned medium (Draper et al. 2004). However, it contains a complex and ill-defined mixture of fibronectin, laminin, type IV collagen, entactin, and heparan sulfate proteoglycans, and various growth factors such as FGF-2, EGF, PDGF, and NGF (Yang et al. 2003). Ludwig et al. (2006) have reported that in place of matrigel, a combination of collagen IV, fibronectin, laminin, and vitronectin supported robust, long-term proliferation of human ES cells in their chemically defined medium TeSR1. We have previously reported by using defined serum-free culture conditions for mouse embryonic stem (mES) cell that integrins regulate mES cell self-renewal. mES cells remained undifferentiated when cultured on type I and type IV collagen or poly-D-lysine whereas they differentiated when cultured on laminin or fibronectin where LIF-induced self-renewal signaling was decreased (Hayashi et al. 2007). Now, we are investigating the role of integrins in the pluripotency of hES/iPS cells.

For robust cultures, we have further modified the culture protocol. We have used 1 U/ml dispase (Roche Applied Science, Indianapolis, IN) to dissociate the cell colonies and washed the dispase with the medium supplemented with recombinant human albumin (1 mg/ml, Millipore, Bedford, MA). If differentiated cells appear in the culture, addition of low concentration of activin (2~10 ng/ml, R&D Systems, Minneapolis, MN) or middle concentration of noggin (10~20 ng/ml, R&D Systems) seems also to inhibit the differentiated cell growth as previously reported (Beattie et al. 2005; James et al. 2005; Vallier et al. 2005; Wang et al. 2005). However, addition of these growth factors confounds the analysis of the actions of other exogenous factors. We are using hESF9 medium to develop a drug screening test.

It would be convenient if the cell culture novice could propagate and passage any type of cell without difficulty. Unfortunately, this is currently not the case for undifferentiated hES/iPS cell lines. Although serum has proved to be a universal medium supplement that allowed the isolation and characterization of a few normal diploid cell lines and numerous abnormal transformed cell lines over the years, the use of serum or other undefined medium components impedes our ability to understand cell responses to controlled environmental stimuli. There are advantages and disadvantages to culturing hES/iPS cells under defined serum-free culture conditions, and the suitability of any particular medium depends on the purpose of the experiment.

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# BMP4 induction of trophoblast from mouse embryonic stem cells in defined culture conditions on laminin

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**Abstract** Because mouse embryonic stem cells (mESCs) do not contribute to the formation of extraembryonic placenta when they are injected into blastocysts, it is believed that mESCs do not differentiate into trophoblast whereas human embryonic stem cells (hESCs) can express trophoblast markers when exposed to bone morphogenetic protein 4 (BMP4) in vitro. To test whether mESCs have the potential to differentiate into trophoblast, we assessed the effect of BMP4 on mESCs in a defined monolayer culture condition. The expression of trophoblast-specific transcription factors such as *Cdx2*, *Dlx3*, *Esx1*, *Gata3*, *Hand1*, *Mash2*, and *Plx1* was specifically upregulated in the BMP4-treated differentiated cells, and these cells expressed trophoblast markers. These results suggest that BMP4 treatment in defined culture conditions enabled mESCs to differentiate into trophoblast. This differentiation was inhibited by serum or leukemia inhibitory factor, which are generally used for mESC culture. In addition, we studied the mechanism underlying BMP4-directed mESC differentiation into trophoblast. Our results showed that BMP4 activates the Smad pathway in mESCs inducing *Cdx2* expression, which plays a crucial role in trophoblast differentiation, through the binding of Smad protein to the *Cdx2* genomic enhancer sequence. Our findings imply that there is a common molecular mechanism underlying hESC and mESC differentiation into trophoblast.

**Keywords** BMP4 · Smad · *Cdx2* · Trophoblast · Mouse embryonic stem cells

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## Introduction

Mouse embryonic stem cells (mESCs) are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst, which differentiate into all the three germ layers in vitro and in vivo (Evans and Kaufman 1981; Martin 1981). However, mESCs are thought to be incapable of differentiating into trophoblast because they do not contribute to placenta in chimeric mouse (Beddington and Robertson 1989). To obtain placental trophoblast from mESCs, genetic manipulations of transcription factors or signaling molecules have been reported, such as the decreased expression of *Oct3/4* (Niwa et al. 2000) or *Sox2* (Masui et al. 2007) or overexpression of *Cdx2* (Niwa et al. 2005; Tolkunova et al. 2006), *Eomes* (Niwa et al. 2005), *Ras* (Lu