

Cre/CAG-CAT^{loxP/-}-EGFP transgenic mouse blastocysts. When these cells were cultured using the protocol established for inducing NC cells, they expressed EGFP. In the future, studies using a defined serum-free culture system such as ours could be a useful tool for clarifying the mechanisms of NC induction and further differentiation into other lineages.

In conclusion, we have developed a protocol using a defined monolayer culture condition for mES cell differentiation into NC cells. We previously reported that removing LIF from our simple serum-free culture medium consisting of basal medium, LIF, and six other factors resulted in the apoptosis of mES cells, while removing LIF from the conventional culture medium containing serum induced spontaneous differentiation of mES cells. Ying *et al.* (Ying *et al.*, 2003) demonstrated that LIF and BMP-4 maintained an undifferentiated state of mES cells in a medium supplemented with N2 (Bottenstein and Sato, 1979) consisting of five factors (insulin, transferrin, selenium, putrescine, and progesterone), and B27 (Brewer *et al.*, 1993) consisting of 21 factors. However, they recently reported another culture condition using N2 and inhibitors without B27 (Ying *et al.*, 2008). These findings are consistent with our findings, suggesting that fewer stimulators are beneficial to regulate cell differentiation of mES cells. Our culture condition is useful for elucidating the effects of various exogenous factors on NC cell differentiation. Further, this method could be applied to clinical research, and we are currently adapting this method for human ES and iPS cells.

Materials and Methods

Cell Culture

The mouse ES cell line D3 was routinely maintained in ESF7 medium (Cell Science & Technology Institute, Tokyo, Japan) in 75 cm² plastic flasks (Corning, New York) coated with type I collagen (Nita Gelatin, Osaka, Japan) in a humidified atmosphere of 5% CO₂ at 37°C. ESF7 comprises ESF basal medium (Cell Science & Technology Institute) supplemented with seven defined factors: insulin, transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA), and 10 ng/ml LIF, as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2007; Hayashi *et al.*, 2010).

For differentiation, the cells were inoculated at a density of 5 × 10³ cells/cm² on laminin-coated plates in ESF5 medium. The ESF5 medium is ESF basal medium supplemented with 10 µg/ml insulin, 5 µg/ml transferrin, 10 µM 2-mercaptoethanol, 10 µM 2-ethanolamine, and 20 nM sodium selenite (Furue *et al.*, 2005). When the ESF5 medium was supplemented with FGF-2, 100 ng/ml heparan sulphate (Sigma) was added to the culture medium to enhance FGF-2 activity.

Tdt-mediated dUTP-biotin nick-end labeling (TUNEL) assay and immunostaining

The cells were subjected to a TUNEL assay using an ApopTag fluorescence direct *in situ* apoptosis detection kit (Chemicon). After the TUNEL assay, the cells were immunostained with antibodies, as described previously (Hayashi *et al.*, 2010). Briefly, cells were fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.1% Triton X-100 or ice-cold acetone. The cells were then reacted with the following primary antibodies: anti-nestin (1:100; ARP), anti-P0 (1:200; Aves Labs), anti-p75 (1:200; Chemicon), anti-AP-2α (1:100; Abcam), anti-type II collagen (1:500; Abcam), anti-type I collagen (1:500; Abcam), anti-Hoxa2 (1:100; Santa Cruz Biotechnology), A2B5 (1:100; Chemicon), anti-O4 (1:300; R&D Systems), anti-peripherin (1:100; Chemicon), anti-Krox-20 (1:200; Covance), and anti-SMA (1:400; Sigma). Primary antibody binding was

visualized with AlexaFluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA), AlexaFlu-594-conjugated donkey anti-mouse IgG (Invitrogen). Fluorescence images were acquired using a Nikon fluorescence microscope with a CCD camera (Hamamatsu Photonics) and analyzed with Aquacosmos software (Hamamatsu Photonics).

Real-time RT-PCR

RT-PCR was performed as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2007). Briefly, total RNA was extracted from the cultured cells using a total RNA extraction kit (Agilent) and reverse-transcribed using a Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix according to the supplier's directions (Qiagen, Hilden, Germany) with an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA). The primer sequences are listed in Supplementary Table 1. The relative expression of mRNA was calculated and compared with the expression in each control.

Undifferentiated cell line derived from Protein-0 (P0) promoter-Cre/Floxed-EGFP transgenic mice

All experiments in this study were approved by the ethics committees of Keio University and the University of Tokyo, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the U.S. Institutes of Health. P0-Cre/Floxed-EGFP double-transgenic mice (ICR background mice) were established as described previously (Nagoshi *et al.*, 2008). Blastocysts were collected from 3.5 days after coitus and cultured in DMEM supplemented with 20% FBS and 1000 unit/ml LIF or ESF7 including 2000 unit/ml LIF supplemented with 5% FBS on mitomycin-treated primary CF-1 mouse embryonic fibroblasts (MEF feeders; Millipore, Phillipsburg, NJ) on a 0.1% gelatin-coated 35-mm dish in a humidified atmosphere of 5% CO₂ at 37°C. Each typical ES-like clone was picked up individually, digested by a 27-gauge needle and passaged. Cell clones expressing both the Cre and EGFP genes were selected and then cultured continuously.

Acknowledgements

This study was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M. K. F. and M. A., and by an International Cooperative Research Project grant of the Japan Science and Technology Agency to M. A.

References

- BASCH, M.L. and BRONNER-FRASER, M. (2006). Neural crest inducing signals. *Adv Exp Med Biol* 589: 24-31.
- BHATTACHARYYA, A., FRANK, E., RATNER, N. and BRACKENBURY, R. (1991). P0 is an early marker of the Schwann cell lineage in chickens. *Neuron* 7: 831-844.
- BOTTENSTEIN, J.E. and SATO, G.H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc Natl Acad Sci USA* 76: 514-517.
- BREWER, G.J., TORRICELLI, J.R., EVEGE, E.K. and PRICE, P.J. (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 35: 567-576.
- ECKERT, D., BUHL, S., WEBER, S., JAGER, R. and SCHORLE, H. (2005). The AP-2 family of transcription factors. *Genome Biol* 6: 246.
- FURUE, M., OKAMOTO, T., HAYASHI, Y., OKOCHI, H., FUJIMOTO, M., MYOISHI, Y., ABE, T., OHNUMA, K., SATO, G.H., ASASHIMA, M. *et al.* (2005). Leukemia inhibitory factor as an anti-apoptotic mitogen for pluripotent mouse embryonic stem cells in a serum-free medium without feeder cells. *In vitro Cell Dev Biol Anim* 41: 19-28.
- FURUE, M. and SAITO, S. (1998). Hepatocyte growth factor regulates activin bA mRNA in submandibular gland. *In vitro Cell. Dev. Biol.* 34: 520-523.
- FURUE, M.K., NA, J., JACKSON, J.P., OKAMOTO, T., JONES, M., BAKER, D., HATA, R., MOORE, H.D., SATO, J.D. and ANDREWS, P.W. (2008). Heparin promotes the growth of human embryonic stem cells in a defined serum-free

- medium. *Proc Natl Acad Sci USA* 105: 13409-13414.
- GILBERT, S. and COLLEGE, S. (2000). Cell-cell communication in development. In *Dev Biol*, (ed. SINAUER, A.). Sinauer Association, Inc, Sunderland, MA, USA, pp.143-181.
- HAYASHI, I. and SATO, G.H. (1976). Replacement of serum by hormones permits growth of cells in a defined medium. *Nature* 259: 132-134.
- HAYASHI, Y., FURUE, M.K., OKAMOTO, T., OHNUMA, K., MYOISHI, Y., FUKUHARA, Y., ABE, T., SATO, J.D., HATA, R. and ASASHIMA, M. (2007). Integrins regulate mouse embryonic stem cell self-renewal. *Stem Cells* 25: 3005-3015.
- HAYASHI, Y., FURUE, M.K., TANAKA, S., HIROSE, M., WAKISAKA, N., DANNO, H., OHNUMA, K., OEDA, S., AIHARA, Y., SHIOTA, K. et al. (2010). BMP4 induction of trophoblast from mouse embryonic stem cells in defined culture conditions on laminin. *In vitro Cell Dev Biol Anim* 46: 416-430.
- KAWAGUCHI, J., MEE, P.J. and SMITH, A.G. (2005). Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. *Bone* 36: 758-769.
- KAWAMOTO, S., NIWA, H., TASHIRO, F., SANO, S., KONDOH, G., TAKEDA, J., TABAYASHI, K. and MIYAZAKI, J. (2000). A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett* 470: 263-268.
- KAWASAKI, H., MIZUSEKI, K., NISHIKAWA, S., KANEKO, S., KUWANA, Y., NAKANISHI, S., NISHIKAWA, S.I. and SASAI, Y. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* 28: 31-40.
- LAMB, T.M., KNECHT, A.K., SMITH, W.C., STACHEL, S.E., ECONOMIDES, A.N., STAHL, N., YANCOPOLOUS, G.D. and HARLAND, R.M. (1993). Neural induction by the secreted polypeptide noggin. *Science* 262: 713-718.
- LE DOUARIN, N.M. and DUPIN, E. (2003). Multipotentiality of the neural crest. *Curr Opin Genet Dev* 13: 529-536.
- LEE, G., CHAMBERS, S.M., TOMISHIMA, M.J. and STUDER, L. (2010). Derivation of neural crest cells from human pluripotent stem cells. *Nat Protoc* 5: 688-701.
- LEMKE, G. and CHAO, M. (1988). Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. *Development* 102: 499-504.
- LEMKE, G., LAMAR, E. and PATTERSON, J. (1988). Isolation and analysis of the gene encoding peripheral myelin protein zero. *Neuron* 1: 73-83.
- LEND AHL, U., ZIMMERMAN, L.B. and MCKAY, R.D. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* 60: 585-595.
- MCKAY, R. (1997). Stem cells in the central nervous system. *Science* 276: 66-71.
- MIZUSEKI, K., SAKAMOTO, T., WATANABE, K., MUGURUMA, K., IKEYA, M., NISHIYAMA, A., ARAKAWA, A., SUEMORI, H., NAKATSUJI, N., KAWASAKI, H. et al. (2003). Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. *Proc Natl Acad Sci USA* 100: 5828-5833.
- MOLNE, M., STUDER, L., TABAR, V., TING, Y.T., EIDEN, M.V. and MCKAY, R.D. (2000). Early cortical precursors do not undergo LIF-mediated astrocytic differentiation. *J Neurosci Res* 59: 301-311.
- MORALES, A.V., BARBAS, J.A. and NIETO, M.A. (2005). How to become neural crest: from segregation to delamination. *Semin Cell Dev Biol* 16: 655-662.
- MORIKAWA, S., MABUCHI, Y., NIIBE, K., SUZUKI, S., NAGOSHI, N., SUNABORI, T., SHIMMURA, S., NAGAI, Y., NAKAGAWA, T., OKANO, H. et al. (2009). Development of mesenchymal stem cells partially originate from the neural crest. *Biochem Biophys Res Commun* 379: 1114-1119.
- MORIKAWA, Y., ZEHIR, A., MASKA, E., DENG, C., SCHNEIDER, M.D., MISHINA, Y. and CSERJESI, P. (2009). BMP signaling regulates sympathetic nervous system development through Smad4-dependent and -independent pathways. *Development* 136: 3575-3584.
- MUJTABA, T., MAYER-PROSCHEL, M. and RAO, M.S. (1998). A common neural progenitor for the CNS and PNS. *Dev Biol* 200: 1-15.
- NAGOSHI, N., SHIBATA, S., KUBOTA, Y., NAKAMURA, M., NAGAI, Y., SATOH, E., MORIKAWA, S., OKADA, Y., MABUCHI, Y., KATOH, H. et al. (2008). Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad. *Cell Stem Cell* 2: 392-403.
- PANCHISION, D.M. and MCKAY, R.D. (2002). The control of neural stem cells by morphogenic signals. *Curr Opin Genet Dev* 12: 478-487.
- PICCOLO, S., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). Dorsoroventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* 86: 589-598.
- RAJAN, P., PANCHISION, D.M., NEWELL, L.F. and MCKAY, R.D. (2003). BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. *J Cell Biol* 161: 911-921.
- SAKAKIBARA, S., IMAI, T., HAMAGUCHI, K., OKABE, M., ARUGA, J., NAKAJIMA, K., YASUTOMI, D., NAGATA, T., KURIHARA, Y., UESUGI, S. et al. (1996). Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. *Dev Biol* 176: 230-242.
- SASAI, Y., LU, B., STEINBEISSER, H. and DE ROBERTIS, E.M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature* 376: 333-336.
- SATO, J.D., BARNES, D., HAYASHI, I., HAYASHI, J., HOSHI, H., KAWAMOTO, T., MATSUDA, R., MCKEEHAN, W.L., MATSUZAKI, K., OKAMOTO, T. et al. (2002). Specific cells and their requirements. In *Basic Cell Culture: A Practical Approach, 2nd Edn.*, (ed. J.M. DAVIS). Oxford University Press, Oxford, England., pp.227-274.
- SELA-DONENFELD, D. and KALCHEIM, C. (1999). Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. *Development* 126: 4749-4762.
- YAMAUCHI, Y., ABE, K., MANTANI, A., HITOSHI, Y., SUZUKI, M., OSUZU, F., KURATANI, S. and YAMAMURA, K. (1999). A novel transgenic technique that allows specific marking of the neural crest cell lineage in mice. *Dev Biol* 212: 191-203.
- YING, Q.L., NICHOLS, J., CHAMBERS, I. and SMITH, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115: 281-292.
- YING, Q.L., WRAY, J., NICHOLS, J., BATLLE-MORERA, L., DOBLE, B., WOODGETT, J., COHEN, P. and SMITH, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453: 519-523.
- ZHOU, Y. and SNEAD, M.L. (2008). Derivation of cranial neural crest-like cells from human embryonic stem cells. *Biochem Biophys Res Commun* 376: 542-547.
- ZIMMERMAN, L.B., DE JESUS-ESCOBAR, J.M. and HARLAND, R.M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86: 599-606.

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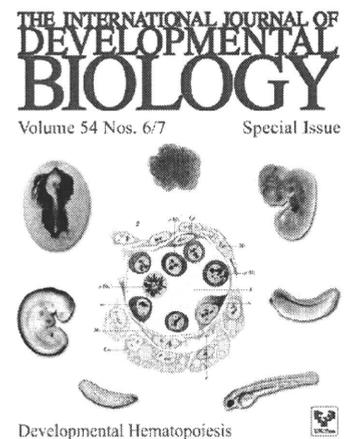
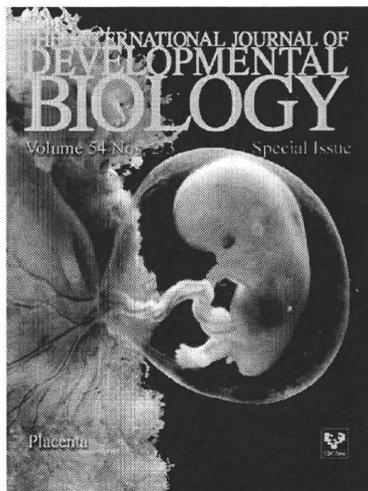
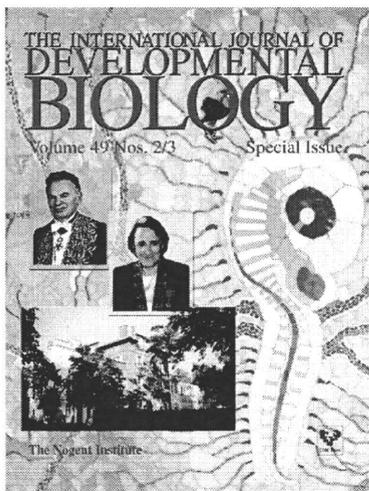
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Supplementary Table 1 The components of hESF series media.

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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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Received 11 May 2010; received in revised form 23 June 2010; accepted 28 June 2010

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/TGF β 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.

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Introduction

ERK belongs to the MAPK (mitogen-activated protein kinase) family (Johnson and Lapadat, 2002). ERK1/2 (ERK1 and 2) is

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

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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

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doi:10.1016/j.scr.2010.06.002

Please cite this article as: Na, J., et al., Inhibition of ERK1/2 prevents neural and mesendodermal differentiation and promotes human embryonic stem cell self-renewal, *Stem Cell Res.* (2010), doi:10.1016/j.scr.2010.06.002

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 μ M) abolished the phosphorylated (activated) form of ERK1/2, while LY294002 (10 μ M) markedly reduced the phosphorylated (activated) form of AKT (Supplementary Fig. 1). Phosphorylation of either AKT or GSK3 β 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 μ M, the ERK1/2 inhibitor U0126 markedly reduced cell spreading, while at 5–20 μ M, 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 μ M, 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 μ M, 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 through 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

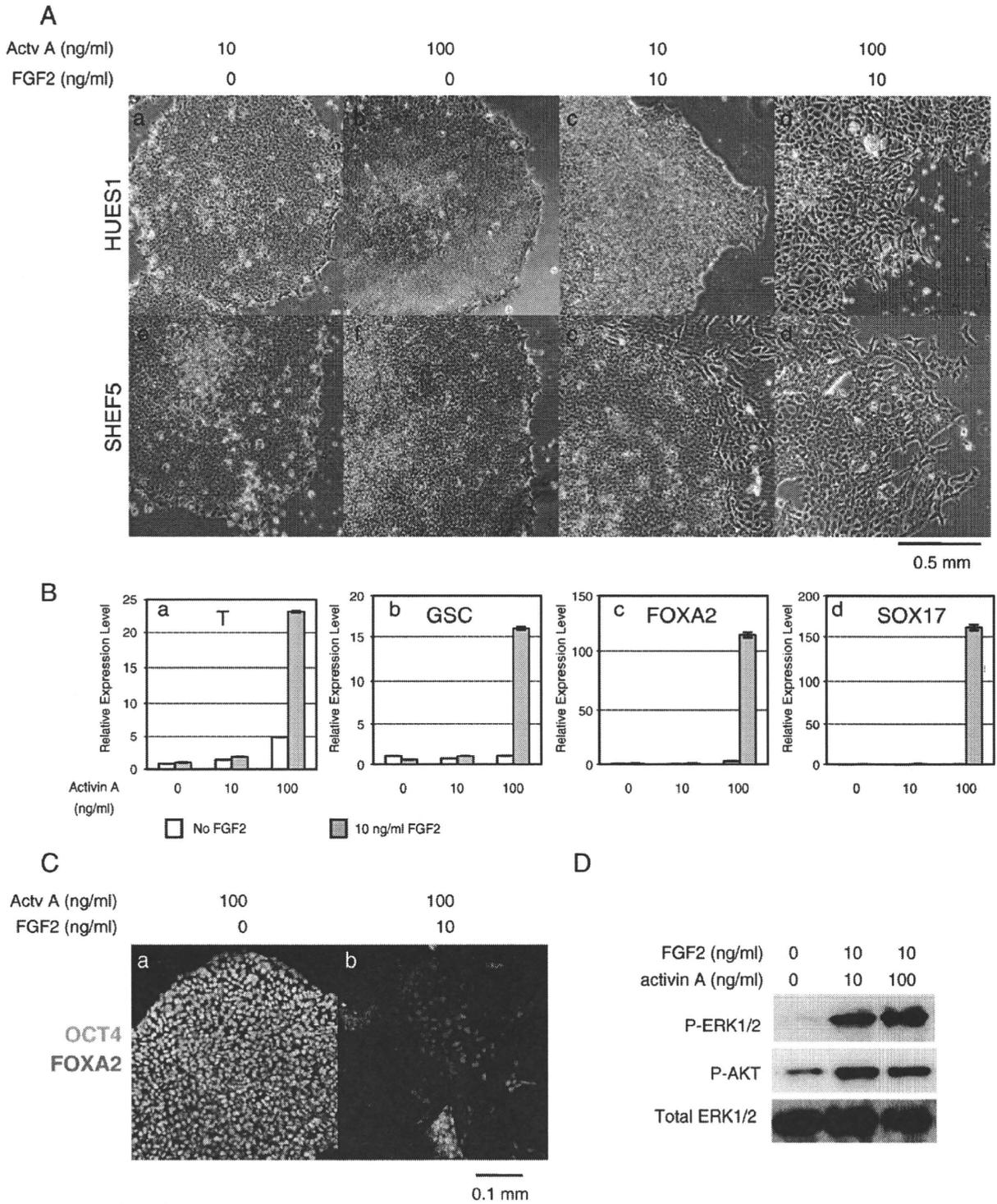
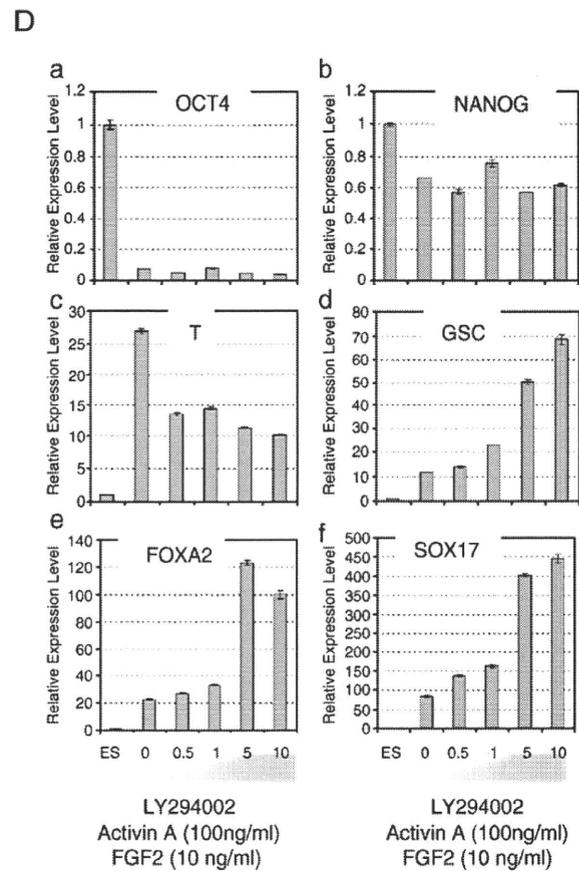
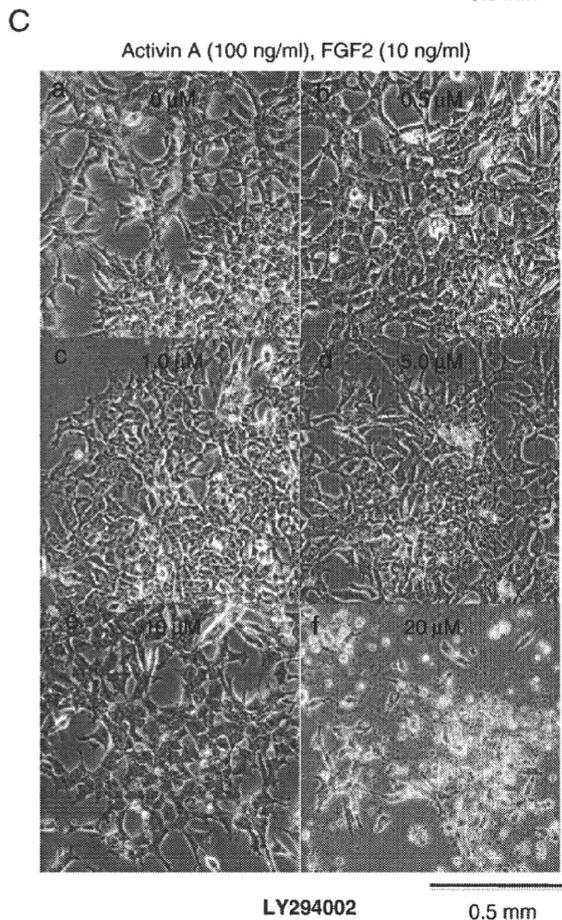
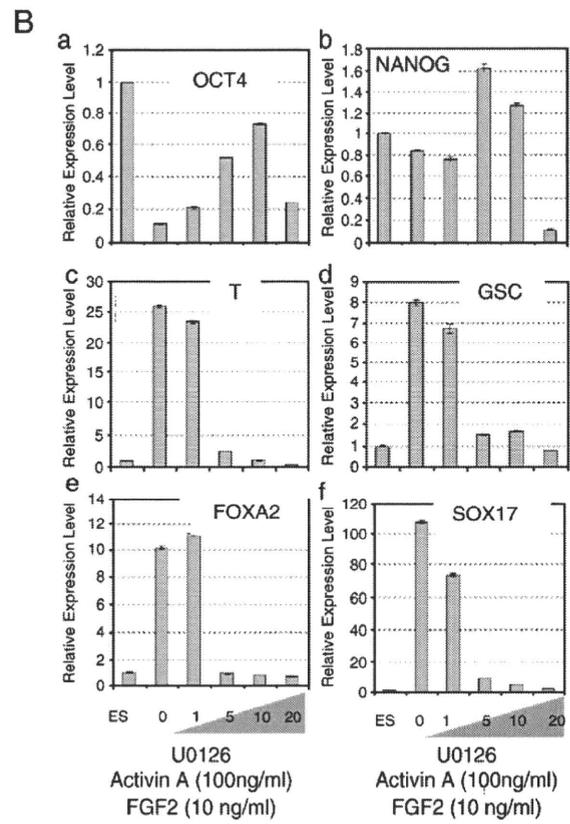
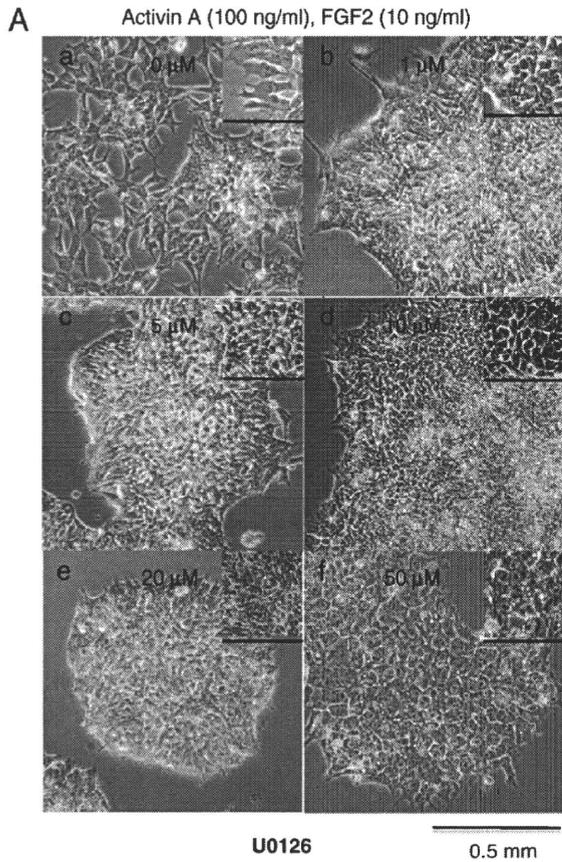


Figure 1 Addition of FGF2 led to robust mesendodermal differentiation induced by a high dosage of activin A. (A) Morphology of HUES1 and SHEF5 ES cells cultured in 10 and 100 ng/ml of activin A with or without FGF2 after 5 days. Note that 100 ng/ml activin A plus 10 ng/ml of FGF2 strongly induced epithelial-to-mesenchymal 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.

161 results were obtained for SHEF5 cells; Supplementary
 162 Figs. 2C and D.) Treating the floating cell aggregates with
 163 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 μ M U0126, in which the cell aggregates flattened out and



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167 formed compact colonies typical of undifferentiated ES cells
 168 (Fig. 3A, panel d). Moreover, the upregulation of neural
 169 marker genes was inhibited (Fig. 3C). However, at 20 and
 170 50 μ M, U0126 appeared to be toxic, since we observed many
 171 vesicles emerging inside the cell and a dramatic increase in
 172 cell death (Fig. 3A, panels e and f). We then passaged U0126-
 173 treated and nontreated cells and cultured them in hESF9A for
 174 7 days. Immunostaining was carried out to further examine
 175 the expression of OCT4, SIX3, and PAX6. We found that
 176 without prior U0126 treatment, cells formed "rosette-like"
 177 clusters and the expression of OCT4 protein was markedly
 178 reduced (Fig. 3C, panel a), while in the same cultures many
 179 rosette cells formed showing SIX3 and PAX6 nuclear staining
 180 (Fig. 3C, panels b and c). By contrast, after U0126 treatment,
 181 HUES1 cells did not form rosettes and maintained uniform
 182 nuclear OCT4 staining (Fig. 3C, panel d), while SIX3 and PAX6
 183 proteins were not detected (Fig. 3C, panels e and f). U0126
 184 also prevented neural differentiation from SHEF5 cells
 185 (Supplementary Figs. 2C and D).

186 ERK1/2 activation is required to counterbalance 187 BMP-induced differentiation in monolayer culture

188 Human ES cells express several BMP ligands (Sperger et al.,
 189 2003). Moreover, the Knockout serum replacement in the
 190 conventional human ES cell culture medium includes BMP-
 191 like activities (Xu et al., 2005). Thus, we next tested whether
 192 ERK1/2 activation can counteract BMP-induced differentia-
 193 tion in human ES cells using the hESF-based monolayer
 194 culture system. After exposure to BMP4 for 5 days, human ES
 195 cell colonies lost their compact morphology, appearing
 196 flattened (Fig. 4A, panel a), and also lost the expression of
 197 the OCT4 protein (Fig. 4B, panel a). By contrast, when cells
 198 were cultured in the presence of 50 ng/ml of FGF2 together
 199 with BMP4, they maintained an undifferentiated morphology
 200 as well as nuclear OCT4 expression (Figs. 4A and B, panel c).
 201 Thus, FGF2 signaling appears to counteract the BMP
 202 pathway. However, when U0126 was added to BMP4 in the
 203 absence of FGF2, these flat cells appeared even earlier and
 204 more extensively than with BMP4 alone (Fig. 4A, panel b). We
 205 could not detect any OCT4 protein in these cells
 206 (Fig. 4B, panel b). U0126 treatment also almost abolished
 207 the ability of FGF2 to rescue the cells from BMP4-induced
 208 differentiation, as many flat cells were observed under the
 209 BMP4+FGF2 U0126 condition and OCT4 staining was absent
 210 in most of them (Figs. 4A and B, panel d). In agreement with
 211 the morphological changes, the RNA levels of the early
 212 extraembryonic lineage marker AFP increased markedly in
 213 BMP4-treated cells, but were downregulated when high
 214 concentrations of FGF2 were added (Supplementary Fig. 3).
 215 Cells cultured in the presence of BMP4, FGF, and U0126
 216 showed similar upregulation of AFP as those treated with
 217 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/cm² 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 condi-
 tions. 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 differen-
 tiation 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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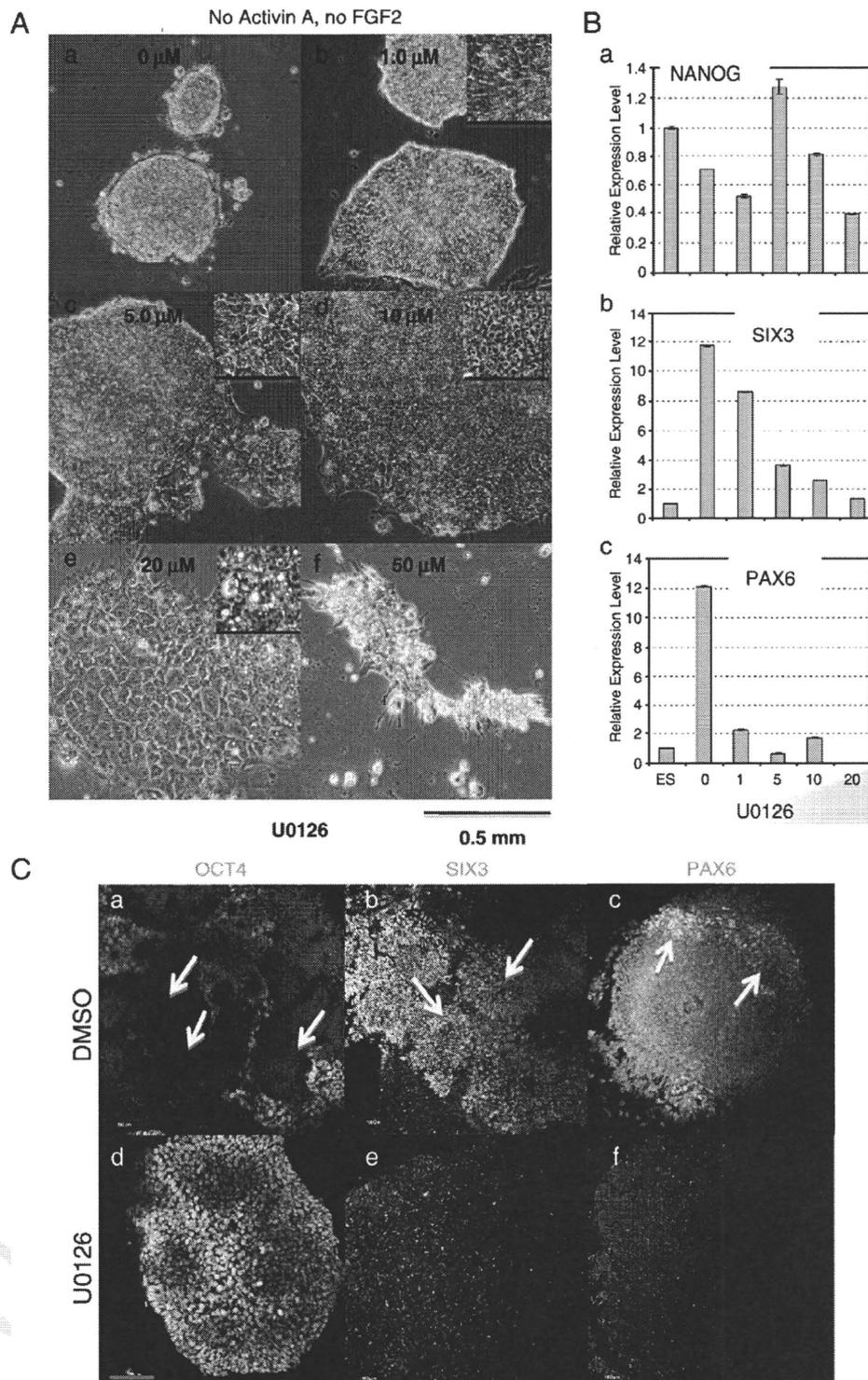


Figure 3 (A) Morphology of human ES cells cultured without activin A or FGF2 treated with increasing concentrations of U0126. Scale bar: 0.5 mm. Inset scale bar: 0.1 mm. (B) Q-PCR analysis of *NANOG*, *SIX3*, and *PAX6* expression. Their relative expression levels were compared to the levels in ES cells 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.

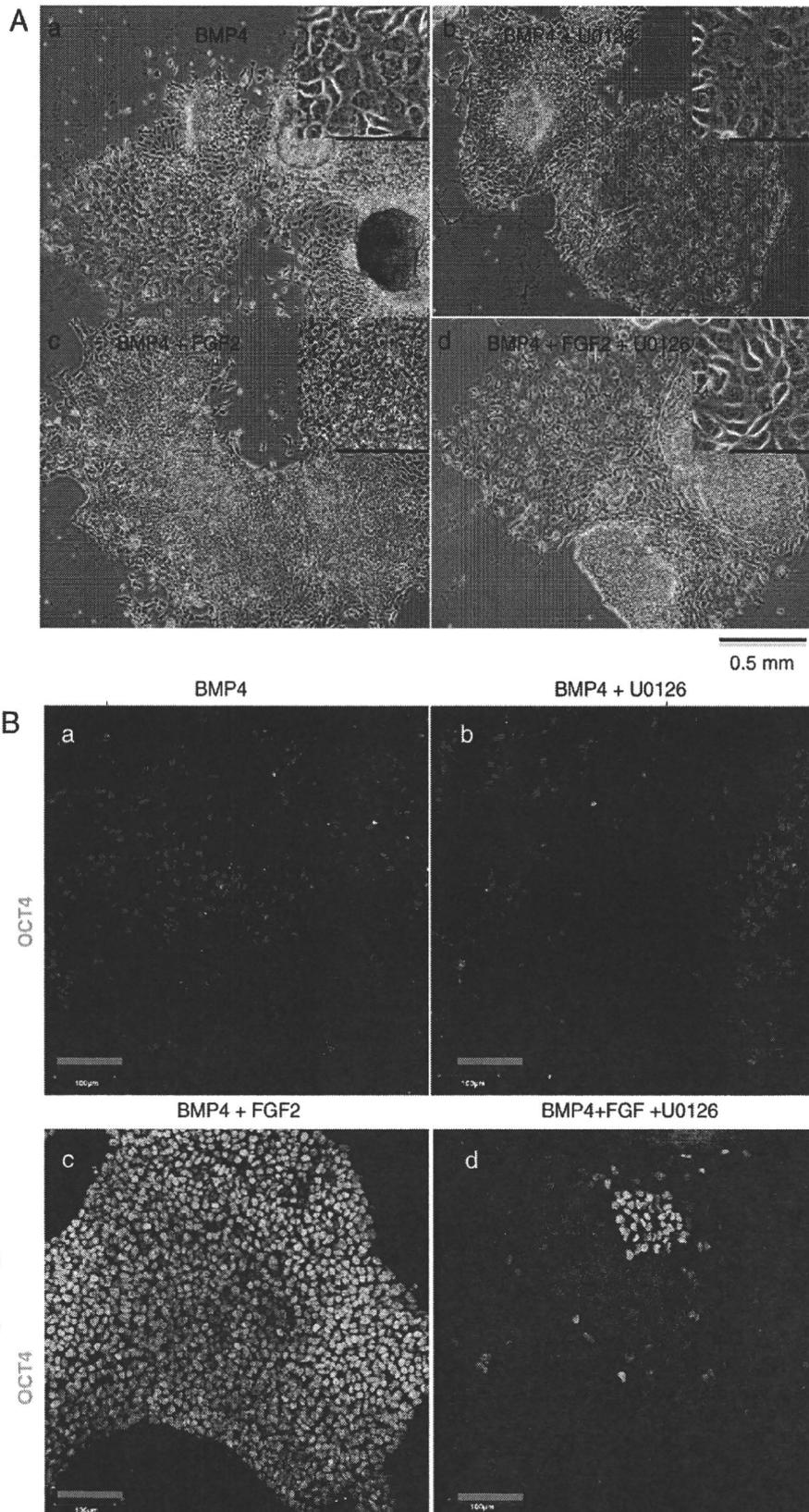


Figure 4 (A) Morphology of HUES1 cells cultured in different BMP4 (1 ng/ml), FGF2 (40 ng/ml), and U0126 (10 μM) combinations as indicated on the top of each graph. Scale bar: 0.5 mm. (B) Immunostaining of OCT4 (in green) of above experiment. DNA: blue. Scale bar: 100 μm.

269 **Long-term culture of human ES cells in a chemically**
 270 **defined medium containing U0126**

271 Since U0126 can inhibit ES cell differentiation under chemi-
 272 cally defined conditions, we assessed whether it can be used to
 273 support the self-renewal of several human ES cells during long-
 274 term culture in the hESF9A medium. To reduce the adverse
 275 effect of U0126 on cell adhesion, it was omitted from the
 276 medium when the cells were seeded as well as the following
 277 day; subsequently, cultures were fed with a medium contain-
 278 ing U0126 until the next passage. After 3 passages (approx
 279 3 weeks), three independent human ES cell lines, SHEF4,
 280 SHEF6, and the adapted HUES1 subline, grew to produce very
 281 large undifferentiated colonies in the hESF9A medium with
 282 5 μ M U0126 (Fig. 6A, panels a, c, and e), whereas without

283 U0126 increased spontaneous differentiation was evident for
 284 the SHEF4 and SHEF6 cell lines (Fig. 6A, panels b and d). The
 285 adapted HUES1 subline also showed better colony morphology
 286 when cultured with U0126 (Fig. 6A, panel f). After culturing in
 287 U0126 for more than 3 months, SHEF4 and SHEF6 cells retained
 288 a normal karyotype, while no additional karyotype changes
 289 were detected in the adapted HUES1 line (Supplementary
 290 Fig. 6A). The SHEF4 and SHEF6 human ES cell lines expressed
 291 significantly higher levels of OCT4 and SSEA4 when grown in
 292 the presence of U0126 compared to nontreated cells (Fig. 6B).
 293 Adapted HUES1 cells displayed a less increased expression of
 294 OCT4 and SSEA4 when grown in the presence of U0126. This
 295 might reflect the innately different properties of individual
 296 human ES cell lines (Fig. 6B). To confirm the specificity of
 297 U0126, we used PD0325901, another ERK1/2 inhibitor used by

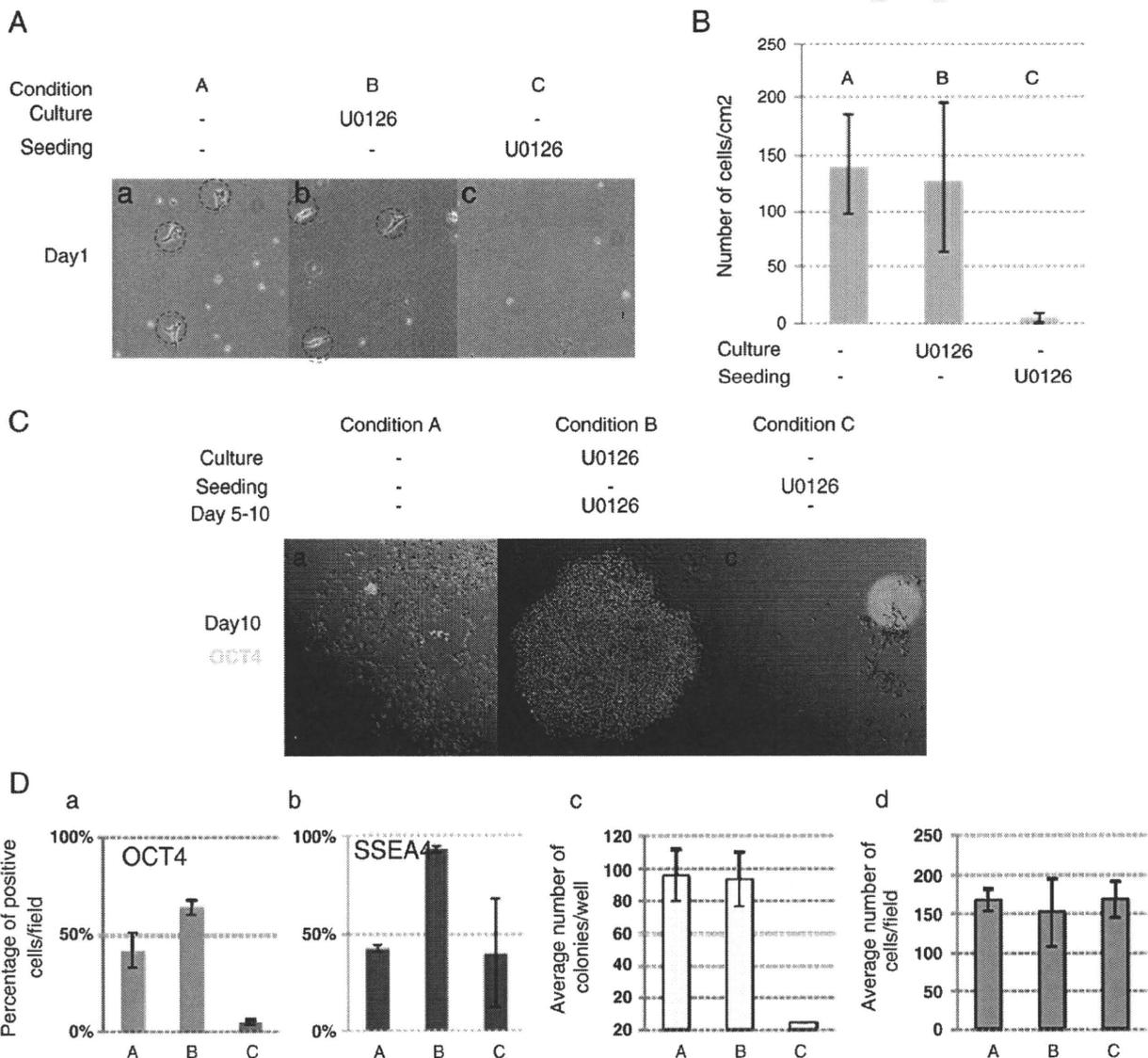


Figure 5 Inhibition of ERK1/2 prevented human ES cell attachment to the substrate. (A) Phase-contrast images of HUES1 cells attached to the substrate under different conditions (listed above each graph). (B) Bar-graph quantisation of HUES1 cell attachment after passaging with or without U0126. Seeding density was 2000 cells/cm². (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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298 Ying and colleagues on mouse ES cells (Ying et al., 2008). We
 299 found that 0.3 μ M PD0325901 was sufficient to block the
 300 phosphorylation of ERK1/2 in SHEF4 cells (Supplementary
 301 Fig. 6A). It reduced differentiation of the SHEF4 and SHEF6 cell
 302 lines during a period of 4 weeks (Supplementary Fig. 6B).
 303 Human ES cells cultured in PD0325901 or U0126 for 1–3 months
 304 retained the ability to undergo neural, mesendodermal, and
 305 extraembryonic differentiation when appropriate inducing
 306 conditions were applied. As shown in Supplementary Figs. 5B
 307 and 6D, differentiated cells strongly expressed TUJ1 (neural),
 308 BRACHYURY, FOXA2, SOX17 (mesendoderm), and CDX2 (ex-
 309 traembryonic) and downregulated OCT4. Our results show that
 310 the inhibition of ERK1/2 signaling helps to prevent differen-

311 tiation of human ES cells in chemically defined environments
 312 and does not affect the pluripotency of these cells.

Discussion

313

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

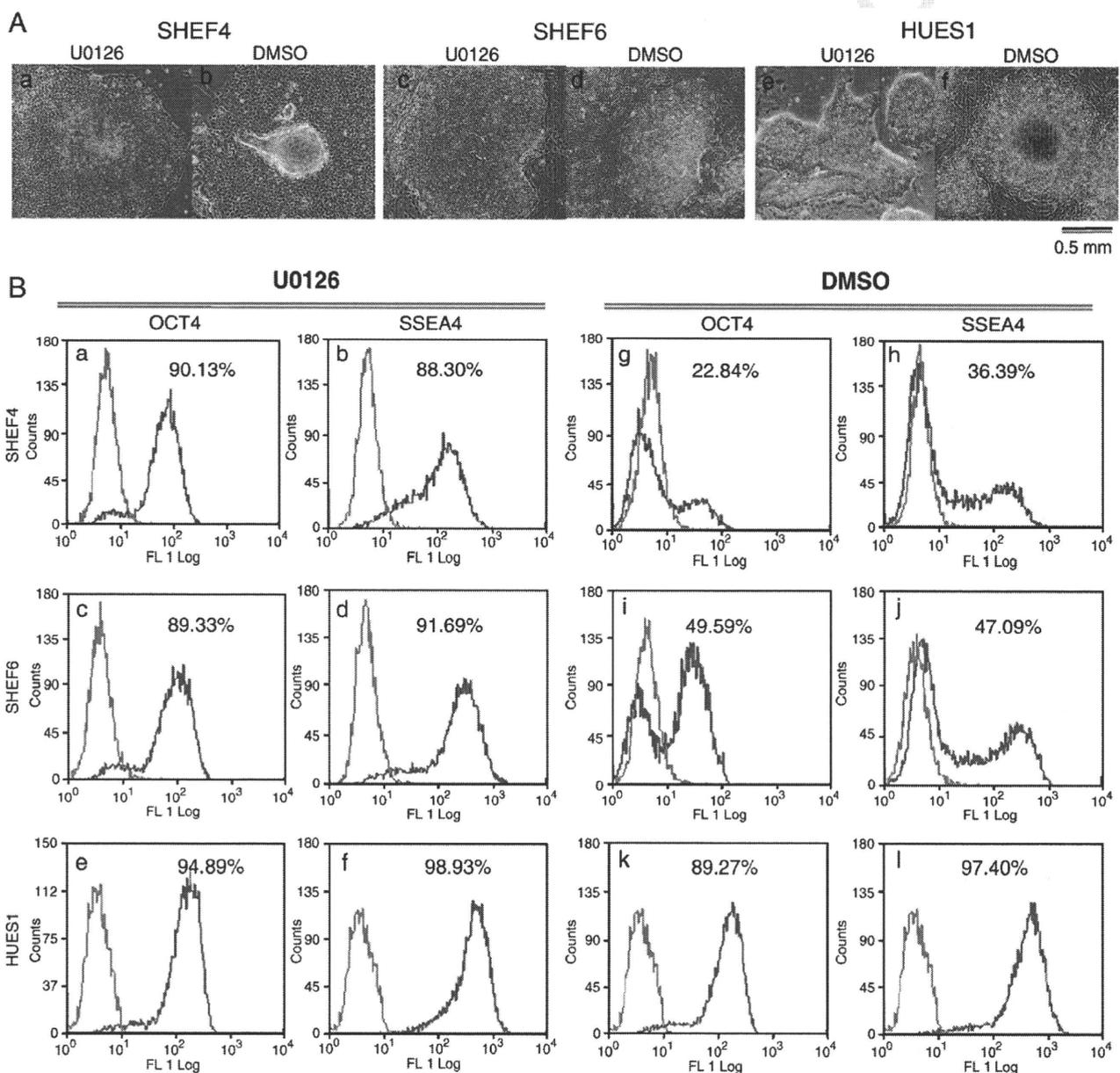


Figure 6 (A) Morphology of human ES cells cultured for 3 passages with or without U0126 in hESF9A. (B) FACS analysis of OCT4 and SSEA4 expression in human ES cells cultured with or without U0126 for 3 passages (3 weeks). The gray histogram is for SHEF4, SHEF6, and HUES1 cells stained with P3X (negative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

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

342 In human ES cell cultures, FGF2 appeared to constitute
343 some of the functions of LIF in mouse ES cells. In mice,
344 parallel to the STAT3–JAK pathway that upregulates KLF4
345 and promotes pluripotency, LIF also activates both PI3K–AKT
346 and MAPK1/2–ERK1/2 pathways (Niwa et al., 2009). AKT
347 increases the expression level of the transcription factor
348 TBX3, which is located upstream of the pluripotency factors
349 NANOG and SOX2 (Niwa et al., 2009). On the other hand, the
350 MAPK1/2–ERK1/2 pathway downregulates TBX3 and NANOG
351 to encourage lineage commitment (Niwa et al., 2009). Both
352 LIF and endogenously expressed FGF4 can elevate ERK
353 activity, which gives ES cells a tendency to initiate
354 spontaneous neural differentiation under serum- and feeder-
355 free culture conditions (Stavridis et al., 2007; Ying et al.,
356 2003a). In this scenario, addition of BMP4 suppressed neural
357 differentiation by inducing the expression of *Id* proteins
358 through SMAD1 (Ying et al., 2003b). Blocking ERK1/2 can
359 prevent mouse ES cell neural differentiation and “trap” them
360 in the “ground state” of self-renewal (Stavridis et al., 2007;
361 Ying et al., 2008). In a separate study, Pluripotin, a small
362 molecule that inhibits both ERK1/2 and Ras-GAP, was also
363 able to sustain mouse ES cell self-renewal in the absence of
364 BMP4 under chemically defined conditions, possibly through
365 the same mechanism (Chen et al., 2006). Although LIF can
366 stimulate STAT3 phosphorylation and, therefore, its activa-
367 tion, in human and primate ES cells this has no detectable
368 effect on the pluripotency network (Daheron et al., 2004;
369 Sumi et al., 2004). FGF2 strongly activates both AKT and
370 ERK1/2. The AKT branch is critical for ES cell self-renewal.
371 Watanabe and colleagues showed that overexpressing a
372 constitutive active mutant form of AKT was able to maintain
373 the self-renewal of both mouse and primate ES cells
374 (Watanabe et al., 2006). In our experiments, inhibition of
375 AKT through blocking PI3K accelerated mesendodermal
376 differentiation of human ES cells under inducing conditions
377 (Figs. 2C and D). Under chemically defined conditions, we
378 found that ERK1/2 activation is required for proper neural
379 and mesendodermal lineage specification. These two impor-
380 tant functions seem to be conserved between human and
381 mouse ES cells. ERK1/2 inhibition significantly reduces
382 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 TGF β signaling
pathways are complex and may depend on the relative levels
of BMP and TGF β 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 TGF β 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/TGF β signaling can sequester SMAD4 (the co-SMAD
essential for both BMP and TGF β 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 publica-
tions 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 through PI3K to stimulate
epithelial cell growth (Pece et al., 1999). Therefore, cell–

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

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

461 Materials and methods

462 Cell lines and maintenance

463 HUES1 (normal and adapted), SHEF4, SHEF5 and SHEF6
464 human ES cells were used in this study. SHEF4, SHEF5, and
465 SHEF6 cells were maintained on feeders in an ES medium
466 containing KO-DMEM (Invitrogen) supplemented with nones-
467 sential amino acids, 0.1 μ M 2-mercaptoethanol, 1 mM gluta-
468 mine, 20% serum replacement (Invitrogen), and 4 ng/ml
469 FGF2 (Peprotech) (Amit et al., 2000). HUES1 cells were
470 maintained on feeders in an HUES1 medium containing KO-
471 DMEM supplemented with nonessential amino acids, 0.1 mM
472 2-mercaptoethanol, glutamine, 8% serum replacement, 8%
473 plasminate (Bayer), and 4 ng/ml FGF2 (Cowan et al., 2004).
474 In both cases, the cells were cultured at 37 °C in a humidified
475 atmosphere of 5% CO₂ in air. The cells were passaged with
476 1 mg/ml collagenase IV (Invitrogen) (for SHEF lines) and
477 0.05% trypsin/0.04% EDTA (Sigma) (for HUES1), respectively.
478 The cells were seeded onto a 25 cm² flask (NUNC) that had
479 been previously coated with 0.1% porcine gelatin (Sigma) and
480 mitomycin C (Sigma)-inactivated MEF as feeder cells.

Human ES cell culture in serum-free, feeder-free, and chemically defined medium 481 482

483 Prior to growth-factor-treatment experiments, human ES 483
484 cells were grown for more than 3 passages in the absence of 484
485 feeders in a serum-free and chemically defined medium on 485
486 type I collagen gel (Nita Gelatin, Japan) (Furue et al., 2008). 486
487 The hESF basal medium was purchased from the Cell Science 487
488 & Technology Institute Inc., Sendai, Japan. The components 488
489 of hESF8, 9, and 9A are listed in Supplementary Table 1. 489
490 Because cells are very sensitive to the passaging strategy 490
491 used, including the size of colonies that are passaged, the 491
492 addition of activin A permits the maintenance of more robust 492
493 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. 497 498

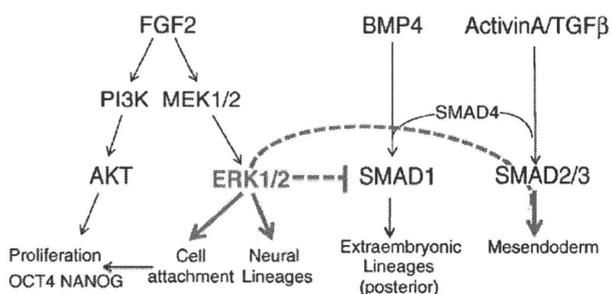
Growth-factor treatment and differentiation protocols 499 500

501 Serum-free, feeder-free cultured human ES cells were 501
502 seeded in 12-well plates at a density of 4×10^4 /well. One 502
503 day after seeding, the cells were washed with an hESF8 503
504 medium, and then fed with hESF8 and with activin A (R&D 504
505 systems), BMP4, and FGF2 (Peprotech) added at the desired 505
506 concentrations. The medium was changed every 2 days with 506
507 freshly added growth factors. Cells were collected after 507
508 5 days and analysed. For neural differentiation, we used a 508
509 protocol modified from Zhang et al. (2001). Briefly, SHEF4 509
510 and SHEF6 cells were first cultured as floating EBs in hESF8 510
511 with 4 ng/ml of FGF2 for 1 week. These EBs were then plated 511
512 in 6-well plates coated with poly-L-ornithine hydrobromide 512
513 (PLOH) and 2 μ g/cm² Laminin (LN) (Sigma) and fed with 513
514 hESF8 and 20 ng/ml of FGF for 1 week. Afterward, colonies 514
515 with neural rosette morphology were manually picked and 515
516 cultured in suspension in the same medium for another 516
517 7 days. These rosettes were replated on a PLOH/LN surface 517
518 for 7–10 days. Rosettes with extensively long neurites were 518
519 picked and dissociated to obtain a relatively pure population 519
520 of neuronal cells. To initiate mesendodermal or extraem- 520
521 bryonic differentiation, cells were treated with 100 ng/ml 521
522 activin A plus 10 ng/ml FGF2 or 10 ng/ml of BMP4 on 2 μ g/ 522
523 cm² FN-coated surface for 7 days. 523

Antibodies and chemical inhibitors 524

525 The following antibodies were used in this study: SMAD4, 525
526 OCT4, ERK1/2, and BRACHYURY (Santa Cruz); CDX2, FOXA2, 526
527 NANOG, and SOX17 (R&D systems); SIX3 (a rabbit polyclonal 527
528 antibody raised against polypeptide RLQ HQA IGP SGM RSL 528
529 AEP GC which corresponds to the C terminus of mouse SIX3; 529
530 the same sequence was present in human SIX3); PAX6 and 530
531 FORSE1 (Developmental Studies Hybridoma Bank, IA, USA); 531
532 phospho-ERK1/2, phospho-AKT, phospho-GSK3 β , total GSK3 β 532
533 (Cell Signaling); SOX2 and actin (Abcam, Cambridge UK). 533
534 The monoclonal antibody to SSEA4 was produced from a 534
535 culture of hybridoma 813-70 (Kannagi et al., 1983). The 535
536 chemical inhibitors U0126, LY294002, and PD0325901 were 536

The function of FGF and ERK1/2 signalling in human ES cells



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

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

539 Western blot

540 For Western blot analysis, HUES1 cells were first cultured in
541 hESF9A for 48 h and then washed briefly with PBS and
542 cultured in hESF8 (without FGF and activin A) for 48 h.
543 Afterward, 10 ng/ml of FGF2 and chemical inhibitors (U0126
544 10 μ M, LY294002 10 μ M, PD0325901 0.3 μ M, or DMSO only)
545 were added, while 30 min later cells were detached and
546 lysed on ice in a sample-loading buffer (0.125 M Tris-HCl, pH
547 6.8, 4% SDS, 20% glycerol, PhosSTOP (Roche), and 0.002%
548 bromophenol blue). The amount of 2×10^5 cells-equivalent
549 lysate was loaded per lane.

550 Quantitative PCR

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

562 Immunostaining, FACS, and high-content screen

563 For in situ immunostaining and high-content screen, cells were
564 fixed in 6-well plates with 4% PFA in PBS. OCT4 and SSEA4
565 staining was scanned in an In Cell Analyser 1000 (Amersham
566 Biosciences) and analysed using the Developer's Toolbox.
567 OCT4, SIX3, and PAX6 staining was imaged with an Olympus
568 FV1000 confocal microscope. For FACS analysis, cells were
569 incubated with 0.05% trypsin in 0.2% EDTA in 37 °C, 10% CO₂
570 incubator until most dissociated into single cells. Trypsin
571 inhibitor (Sigma T6522) (1 mg/ml in DMEM) was immediately
572 added to inactivate the trypsin. Cells were subsequently rinsed
573 off the flask or 6-well plate with an hESF8 medium, centrifuged
574 down, and washed 3 times in PBS containing 4 mg/ml
575 polyvinylpyrrolidone (Sigma) to prevent them from sticking
576 together. They were fixed in 4% PFA in PBS 0.1% Tween 20
577 (PBST) for 20 min at room temperature. For intracellular
578 staining of Oct4, 0.2% Triton X-100 was included in the fixative.
579 Afterward, cells were washed 3 times in PBST, blocked with 5%
580 FBS-PBST for 1 h, and incubated with the desired primary and
581 secondary antibodies. FACS was done using a DAKO Cytomation
582 CyAn_{ADP} machine.

583 Y27632 treatment and clonogenicity assay

584 SHEF6 cells were dissociated into single cells as described
585 above and seeded at 10,000 cells/cm² in FN (2 μ g/cm²)
586 coated 6-well plates in hESF9A. Y27632 (10 μ M) or U0126
587 (10 μ M) was added 2 h prior to passaging and overnight
588 during seeding as described in Watanabe et al. (2007). The

medium was replaced the next day. Cells were grown in 589
hESF9A for another 10 days, then fixed briefly, and stained 590
with alkaline phosphatase (AP) Red Substrate Solution 591
(Sigma). AP-positive colonies were photographed and 592
counted. 593

Uncited reference

Harb et al., 2008

Acknowledgments

This work was supported by a Medical Research Council 597
Collaborative Career Development Fellowship in Stem Cell 598
Research to J.N., and grants from the MRC and ESTOOLS, an 599
integrated project of the 6th Framework Programme of the 600
European Commission. We thank Dr. M. Jones, Mr. D. Harley, 601
and Dr. P. Gokhale for assistance with FACS and high-content 602
screen analysis, Prof. Harry Moore for providing SHEF cell 603
lines, and Prof. Marysia Placzek for SIX3 antibody. Confocal 604
images were acquired using the Light Microscopy Facility 605
funded by the Wellcome Trust (Grant GR077544AIA). 606

Appendix A. Supplementary data

Supplementary data associated with this article can be 608
found, in the online version, at 10.1016/j.scr.2010.06.002. 609

References

- Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P.,
Waknitz, M.A., Itskovitz-Eldor, J., Thomson, J.A., 2000. Clonally
derived human embryonic stem cell lines maintain pluripotency
and proliferative potential for prolonged periods of culture. *Dev.*
Biol. 227, 271–278. 615
- Androutsellis-Theotokis, A., Leker, R.R., Soldner, F., Hoepfner, D.
J., Ravin, R., Poser, S.W., Rueger, M.A., Bae, S.K., Kittappa, R.,
McKay, R.D., 2006. Notch signalling regulates stem cell numbers
in vitro and in vivo. *Nature* 442, 823–826. 619
- Avery, S., Zafarana, G., Gokhale, P.J., Andrews, P.W., 2010. The
Role of SMAD4 in human embryonic stem cell self-renewal and
stem cell fate. *Stem Cells.* 622
- Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan,
H., Klevernic, I., Arthur, J.S., Alessi, D.R., Cohen, P., 2007. The
selectivity of protein kinase inhibitors: a further update. 623
Biochem. J. 408, 297–315. 626
- Burdon, T., Tracey, C., Chambers, I., Nichols, J., Smith, A., 1999.
Suppression of SHP-2 and ERK signalling promotes self-renewal of
mouse embryonic stem cells. *Dev. Biol.* 210, 30–43. 629
- Chazaud, C., Yamanaka, Y., Pawson, T., Rossant, J., 2006. Early lineage
segregation between epiblast and primitive endoderm in mouse
blastocysts through the Grb2-MAPK pathway. *Dev. Cell* 10, 615–624. 632
- Chen, S., Do, J.T., Zhang, Q., Yao, S., Yan, F., Peters, E.C., Scholer, H.
R., Schultz, P.G., Ding, S., 2006. Self-renewal of embryonic stem
cells by a small molecule. *Proc. Natl. Acad. Sci. USA* 103, 635
17266–17271. 636
- Ciruna, B., Rossant, J., 2001. FGF signaling regulates mesoderm cell
fate specification and morphogenetic movement at the primitive
streak. *Dev. Cell* 1, 37–49. 639
- Corson, L.B., Yamanaka, Y., Lai, K.M., Rossant, J., 2003. Spatial and
temporal patterns of ERK signaling during mouse embryogenesis. 640
Development 130, 4527–4537. 642

- 643 Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer,
644 J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers,
645 D., Melton, D.A., 2004. Derivation of embryonic stem-cell lines
646 from human blastocysts. *N Engl J. Med.* 350, 1353–1356.
- 647 Daheron, L., Opitz, S.L., Zaehres, H., Lensch, M.W., Andrews, P.W.,
648 Itskovitz-Eldor, J., Daley, G.Q., 2004. LIF/STAT3 signaling fails to
649 maintain self-renewal of human embryonic stem cells. *Stem Cells*
650 22, 770–778.
- 651 D'Amour, K.A., Agulnick, A.D., Eliazar, S., Kelly, O.G., Kroon, E.,
652 Baetge, E.E., 2005. Efficient differentiation of human embryonic
653 stem cells to definitive endoderm. *Nat. Biotechnol.* 23, 1534–1541.
- 654 Dreesen, O., Brivanlou, A.H., 2007. Signaling pathways in cancer and
655 embryonic stem cells. *Stem Cell Rev.* 3, 7–17.
- 656 Eiselleova, L., Matulka, K., Kriz, V., Kunova, M., Schmidtova, Z., Neradil,
657 J., Tichy, B., Dvorakova, D., Pospisilova, S., Hampl, A., Dvorak, P.,
658 2009. A complex role for FGF-2 in self-renewal, survival, and
659 adhesion of human embryonic stem cells. *Stem Cells* 27, 1847–1857.
- 660 Fox, V., Gokhale, P.J., Walsh, J.R., Matin, M., Jones, M., Andrews,
661 P.W., 2008. Cell-cell signaling through NOTCH regulates human
662 embryonic stem cell proliferation. *Stem Cells* 26, 715–723.
- 663 Fuentealba, L.C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H.,
664 Pera, E.M., De Robertis, E.M., 2007. Integrating patterning
665 signals: Wnt/GSK3 regulates the duration of the BMP/Smad1
666 signal. *Cell* 131, 980–993.
- 667 Furue, M.K., Na, J., Jackson, J.P., Okamoto, T., Jones, M., Baker, D.,
668 Hata, R., Moore, H.D., Sato, J.D., Andrews, P.W., 2008. Heparin
669 promotes the growth of human embryonic stem cells in a defined
670 serum-free medium. *Proc. Natl. Acad. Sci. USA* 105, 13409–13414.
- 671 Harb, N., Archer, T.K., Sato, N., 2008. The Rho-Rock-Myosin
672 signaling axis determines cell-cell integrity of self-renewing
673 pluripotent stem cells. *PLoS ONE* 3, e3001.
- 674 Johnson, G.L., Lapadat, R., 2002. Mitogen-activated protein kinase
675 pathways mediated by ERK, JNK, and p38 protein kinases.
676 *Science* 298, 1911–1912.
- 677 Kannagi, R., Cochran, N.A., Ishigami, F., Hakomori, S., Andrews, P.
678 W., Knowles, B.B., Solter, D., 1983. Stage-specific embryonic
679 antigens (SSEA-3 and -4) are epitopes of a unique globo-series
680 ganglioside isolated from human teratocarcinoma cells. *EMBO J.*
681 2, 2355–2361.
- 682 Kunath, T., Saba-El-Leil, M.K., Almousailleakh, M., Wray, J.,
683 Meloche, S., Smith, A., 2007. FGF stimulation of the Erk1/2
684 signalling cascade triggers transition of pluripotent embryonic
685 stem cells from self-renewal to lineage commitment. *Develop-*
686 *ment* 134, 2895–2902.
- 687 Li, J., Wang, G., Wang, C., Zhao, Y., Zhang, H., Tan, Z., Song, Z.,
688 Ding, M., Deng, H., 2007. MEK/ERK signaling contributes to the
689 maintenance of human embryonic stem cell self-renewal.
690 *Differentiation* 75, 299–307.
- 691 Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R.
692 E., Schulze, E.N., Song, H., Hsieh, C.L., Pera, M.F., Ying, Q.L.,
693 2008. Germline competent embryonic stem cells derived from rat
694 blastocysts. *Cell* 135, 1299–1310.
- 695 Morrison, G.M., Oikonomopoulou, I., Migueles, R.P., Soneji, S., Livigni,
696 A., Enver, T., Brickman, J.M., 2008. Anterior definitive endoderm
697 from ESCs reveals a role for FGF signaling. *Cell Stem Cell* 3, 402–415.
- 698 Nichols, J., Jones, K., Phillips, J.M., Newland, S.A., Roode, M.,
699 Mansfield, W., Smith, A., Cooke, A., 2009. Validated germline-
700 competent embryonic stem cell lines from nonobese diabetic
701 mice. *Nat. Med.*
- 702 Niwa, H., Ogawa, K., Shimosato, D., Adachi, K., 2009. A parallel
703 circuit of LIF signalling pathways maintains pluripotency of
704 mouse ES cells. *Nature* 460, 118–122.
- 705 Pece, S., Chiariello, M., Murga, C., Gutkind, J.S., 1999. Activation of
706 the protein kinase Akt/PKB by the formation of E-cadherin-
707 mediated cell-cell junctions. Evidence for the association of
708 phosphatidylinositol 3-kinase with the E-cadherin adhesion
709 complex. *J. Biol. Chem.* 274, 19347–19351.
- Pera, M.F., Andrade, J., Houssami, S., Reubinoff, B., Trounson, A.,
710 Stanley, E.G., Ward-van Oostwaard, D., Mummery, C., 2004.
711 Regulation of human embryonic stem cell differentiation by BMP-
712 2 and its antagonist noggin. *J. Cell Sci.* 117, 1269–1280.
713
- Sapkota, G., Alarcon, C., Spagnoli, F.M., Brivanlou, A.H., Massague,
714 J., 2007. Balancing BMP signaling through integrated inputs into
715 the Smad1 linker. *Mol. Cell* 25, 441–454.
716
- Schmierer, B., Hill, C.S., 2007. TGFbeta-SMAD signal transduction:
717 molecular specificity and functional flexibility. *Nat. Rev. Mol.*
718 *Cell Biol.* 8, 970–982.
719
- Sperger, J.M., Chen, X., Draper, J.S., Antosiewicz, J.E., Chon, C.H.,
720 Jones, S.B., Brooks, J.D., Andrews, P.W., Brown, P.O., Thomson,
721 J.A., 2003. Gene expression patterns in human embryonic stem
722 cells and human pluripotent germ cell tumors. *Proc. Natl. Acad.*
723 *Sci. USA* 100, 13350–13355.
724
- Stavridis, M.P., Lunn, J.S., Collins, B.J., Storey, K.G., 2007. A
725 discrete period of FGF-induced Erk1/2 signalling is required for
726 vertebrate neural specification. *Development* 134, 2889–2894.
727
- Sumi, T., Fujimoto, Y., Nakatsujii, N., Suemori, H., 2004. STAT3 is
728 dispensable for maintenance of self-renewal in nonhuman
729 primate embryonic stem cells. *Stem Cells* 22, 861–872.
730
- Takei, S., Ichikawa, H., Johkura, K., Mogi, A., No, H., Yoshie, S.,
731 Tomotsune, D., Sasaki, K., 2009. Bone morphogenetic protein-4
732 promotes induction of cardiomyocytes from human embryonic
733 stem cells in serum-based embryoid body development. *Am. J.*
734 *Physiol. Heart Circ. Physiol.* 296, H1793–H1803.
735
- Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., Rossant, J.,
736 1998. Promotion of trophoblast stem cell proliferation by FGF4.
737 *Science* 282, 2072–2075.
738
- Wang, X., Lin, G., Martins-Taylor, K., Zeng, H., Xu, R.H., 2009.
739 Inhibition of caspase-mediated anoikis is critical for basic
740 fibroblast growth factor-sustained culture of human pluripotent
741 stem cells. *J. Biol. Chem.* 284, 34054–34064.
742
- Watanabe, S., Umehara, H., Murayama, K., Okabe, M., Kimura, T.,
743 Nakano, T., 2006. Activation of Akt signaling is sufficient to
744 maintain pluripotency in mouse and primate embryonic stem
745 cells. *Oncogene* 25, 2697–2707.
746
- Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M.,
747 Wataya, T., Takahashi, J.B., Nishikawa, S., Muguruma, K., Sasai,
748 Y., 2007. A ROCK inhibitor permits survival of dissociated human
749 embryonic stem cells. *Nat. Biotechnol.* 25, 681–686.
750
- Xu, R.H., Chen, X., Li, D.S., Li, R., Addicks, G.C., Glennon, C.,
751 Zwaka, T.P., Thomson, J.A., 2002. BMP4 initiates human
752 embryonic stem cell differentiation to trophoblast. *Nat. Bio-*
753 *technol.* 20, 1261–1264.
754
- Xu, R.H., Peck, R.M., Li, D.S., Feng, X., Ludwig, T., Thomson, J.A.,
755 2005. Basic FGF and suppression of BMP signaling sustain undiffer-
756 entiated proliferation of human ES cells. *Nat. Methods* 2, 185–190.
757
- Yao, Y., Li, W., Wu, J., Germann, U.A., Su, M.S., Kuida, K., Boucher,
758 D.M., 2003. Extracellular signal-regulated kinase 2 is necessary
759 for mesoderm differentiation. *Proc. Natl. Acad. Sci. USA* 100,
760 12759–12764.
761
- Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., Smith, A., 2003a.
762 Conversion of embryonic stem cells into neuroectodermal
763 precursors in adherent monoculture. *Nat. Biotechnol.* 21,
764 183–186.
765
- Ying, Q.L., Nichols, J., Chambers, I., Smith, A., 2003b. BMP
766 induction of Id proteins suppresses differentiation and sustains
767 embryonic stem cell self-renewal in collaboration with STAT3.
768 *Cell* 115, 281–292.
769
- Ying, Q.L., Wray, J., Nichols, J., Battle-Morera, L., Doble, B.,
770 Woodgett, J., Cohen, P., Smith, A., 2008. The ground state of
771 embryonic stem cell self-renewal. *Nature* 453, 519–523.
772
- Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., Thomson, J.A.,
773 2001. In vitro differentiation of transplantable neural precursors
774 from human embryonic stem cells. *Nat. Biotechnol.* 19,
775 1129–1133.
776

777

Please cite this article as: Na, J., et al., Inhibition of ERK1/2 prevents neural and mesodermal differentiation and promotes human embryonic stem cell self-renewal, *Stem Cell Res.* (2010), doi:10.1016/j.scr.2010.06.002

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

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Received: 25 March 2010 / Accepted: 5 April 2010 / Published online: 29 April 2010 / Editor: J. Denry Sato
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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

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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

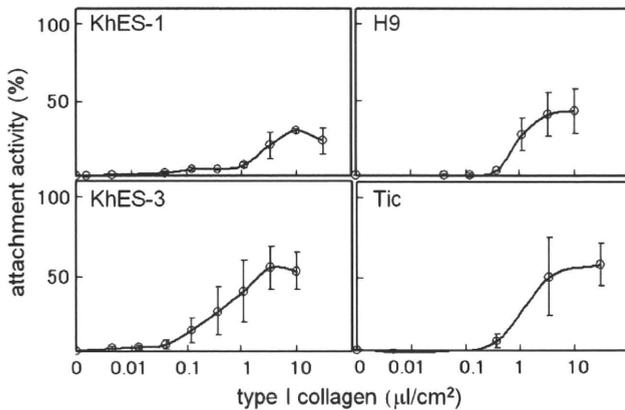


Figure 1. Attachment and proliferation of hES and iPS cells on type I collagen. The attachment of hES/iPS cells to ECM components was measured by the procedures followed by Fassler and Meyer (1995). Briefly, a 96-well microplate (Corning Costar, Corning, NY) was coated with each adhesion molecule at 37°C for 3 h. hES/iPS cells were seeded at confluent density (3×10^6 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

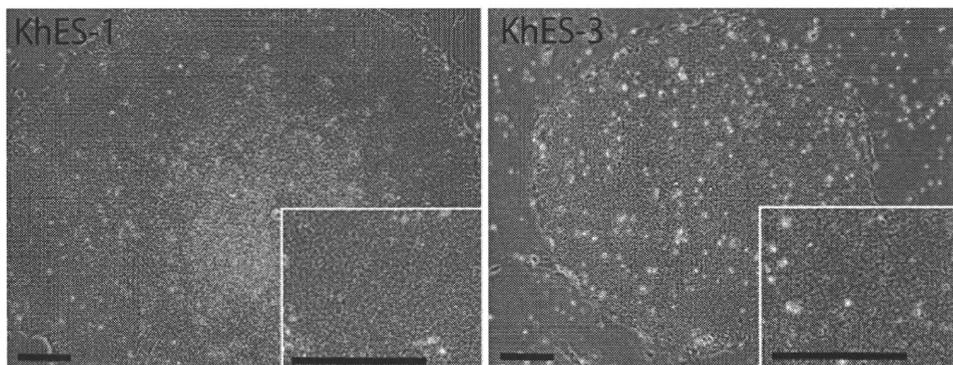


Figure 2. hES cells cultured on fibronectin in hESF9 medium. Phase-contrast microphotographs of KhES-1 at passage 16 and KhES-3 at passage 6 cultured on fibronectin in hESF9 medium. After 2 d, the KhES-1 cells and KhES-3 cells (from Kyoto University) cultured on MEF in KSR-based medium were passaged 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.