splicing yield two mPPAR gamma isoforms. *Proc Natl Acad Sci U S A* 1995; **92**: 7921–7925.
21. Nishiyori A, Tashiro H, Kimura A, *et al*. Determination of tissue specificity of the enhancer by combinatorial operation of tissue-enriched transcription factors. Both HNF-4 and C/EBP beta are required for liver-specific activity of the ornithine transcarbamylase enhancer. *J Biol Chem* 1994; **269**: 1323–1331.
22. Mukai E, Fujimoto S, Sakurai F, *et al*. Efficient gene transfer into murine pancreatic islets using adenovirus vectors. *J Control Release* 2007; **119**: 136–141.
23. Maizel JV Jr, White DO, Scharff MD. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 1968; **36**: 115–125.
24. Dani C, Smith AG, Dessolin S, *et al*. Differentiation of embryonic stem cells into adipocytes in vitro. *J Cell Sci* 1997; **110**: 1279–1285.
25. Tashiro K, Ishii C, Ryoji M. Role of distal upstream sequence in vitamin D-induced expression of human CYP24 gene. *Biochem Biophys Res Commun* 2007; **358**: 259–265.
26. Yasuhara N, Shibazaki N, Tanaka S, *et al*. Triggering neural differentiation of ES cells by subtype switching of importin-alpha. *Nat Cell Biol* 2007; **9**: 72–79.
27. Elefanty AG, Robb L, Birner R, *et al*. Hematopoietic-specific genes are not induced during in vitro differentiation of scl-null embryonic stem cells. *Blood* 1997; **90**: 1435–1447.
28. Mizuguchi H, Koizumi N, Hosono T, *et al*. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther* 2001; **8**: 730–735.
29. Koizumi N, Mizuguchi H, Utoguchi N, *et al*. Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. *J Gene Med* 2003; **5**: 267–276.
30. Rosen ED. The transcriptional basis of adipocyte development. *Prostaglandins Leukot Essent Fatty Acids* 2005; **73**: 31–34.
31. Mueller E, Drori S, Aiyer A, *et al*. Genetic analysis of adipogenesis through peroxisome proliferator-activated receptor gamma isoforms. *J Biol Chem* 2002; **277**: 41925–41930.
32. Chung S, Andersson T, Sonntag KC, *et al*. Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. *Stem Cells* 2002; **20**: 139–145.
33. Sakurai F, Kawabata K, Yamaguchi T, *et al*. Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities. *Gene Ther* 2005; **12**: 1424–1433.
34. Ramezani A, Hawley TS, Hawley RG. Lentiviral vectors for enhanced gene expression in human hematopoietic cells. *Mol Ther* 2000; **2**: 458–469.
35. Okabe M, Ikawa M, Kominami K, *et al*. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 1997; **407**: 313–319.
36. Rust EM, Westfall MV, Samuelson LC, *et al*. Gene transfer into mouse embryonic stem cell-derived cardiac myocytes mediated by recombinant adenovirus. *In Vitro Cell Dev Biol Anim* 1997; **33**: 270–276.
37. Brooks AR, Harkins RN, Wang P, *et al*. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med* 2004; **6**: 395–404.
38. Rufaihah AJ, Haider HK, Heng BC, *et al*. Directing endothelial differentiation of human embryonic stem cells via transduction with an adenoviral vector expressing the VEGF(165) gene. *J Gene Med* 2007; **9**: 452–461.

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Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium

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A major limitation in developing applications for the use of human embryonic stem cells (HESCs) is our lack of knowledge of their responses to specific cues that control self-renewal, differentiation, and lineage selection. HESCs are most commonly maintained on inactivated mouse embryonic fibroblast feeders in medium supplemented with FCS, or proprietary replacements such as knockout serum-replacement together with FGF-2. These undefined culture conditions hamper analysis of the mechanisms that control HESC behavior. We have now developed a defined serum-free medium, hESF9, for the culture of HESCs on a type I-collagen substrate without feeders. In contrast to other reported media for the culture of HESCs, this medium has a lower osmolarity (292 mosmol/liter), L-ascorbic acid-2-phosphate (0.1 μg/ml), and heparin. Insulin, transferrin, albumin conjugated with oleic acid, and FGF-2 (10 ng/ml) were the only protein components. Further, we found that HESCs would proliferate in the absence of exogenous FGF-2 if heparin was also present. However, their growth was enhanced by the addition of FGF-2 up to 10 ng/ml although higher concentrations were deleterious in the presence of heparin.

defined serum-free culture | feeder-free

Human embryonic stem cells (HESCs) were originally derived from the inner cell masses of early embryos explanted onto inactivated mouse embryo fibroblast (MEF) feeder cells, in a medium comprising a 50:50 mix of DMEM and Ham's F12 (DMEM:F12), supplemented with fetal calf serum (FCS) (1, 2). Subsequently, a proprietary basal medium, KO-DMEM supplemented with proprietary 'Knock-Out Serum Replacement' (KSR) (Invitrogen), initially developed for murine ES cells (MESCs), has become widely used both for maintenance and derivation of HESCs. However, 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).

Culture without feeder cells in defined media has proved more problematic, although culture in the absence of feeders but with feeder-conditioned medium is effective (3). Addition of Leukemia Inhibitory Factor (LIF) to FCS-containing medium was sufficient to allow the culture of MESCs without feeders (4, 5). However, LIF does not prevent the differentiation of HESCs (1, 2), although it does activate the STAT3 pathway in these cells (6, 7). Indeed, together with a variety of other differences in their expression of surface antigen markers of the undifferentiated state (8) and capacity for differentiation (9), HESCs appear to differ markedly from MESCs in their responses to activation of signaling pathways associated with FGF and the TGFβ/BMP family of cytokines. For example, activin and Nodal inhibit differentiation of HESCs (10–12), whereas BMP induces their differentiation to trophoblasts (9) or extraembryonic endoderm cells (13). By contrast, BMP signaling inhibits the differentiation of MESCs (14). It is also evident that HESCs have a strong requirement of FGF-2 (15, 16), whereas MESCs do not appear to respond to this growth factor (17). However, pluripotent 'Epiblast Stem Cells' with similar growth factor requirements to HESCs have recently been derived from gastrula stage mouse and rat embryos (18, 19).

Several methods for the culture of HESCs in more defined media, and in the absence of feeders have been reported. Some require culture on Matrigel (Becton-Dickinson) but this contains a variety of extracellular matrix (ECM) components, most likely associated with an ill-defined mixture of growth factors (20–22). Others use fully chemically defined media together with specific ECM attachment factors (23). Nevertheless, there is no consensus as to the optimal formulation, or the nature of the cytokine requirements of HESCs to promote their self-renewal and inhibit their differentiation. One puzzle is the reported requirement for very high concentrations of FGF-2 (up to 100 ng/ml) (24): this suggests that either FGF-2 is operating through an unidentified receptor for which it has a low affinity, or that it is relatively unstable or inefficiently presented to the cells in the culture conditions used.

We have now investigated the culture of HESCs under fully defined culture conditions and have developed a medium that permits their prolonged culture in an undifferentiated state. Although this medium shares a number of features with those described by others, including culture on defined ECM attachment factors, it differs in a number of important respects. In particular, we have used a base medium, ESF (25) that we previously developed for use with MESCs, in contrast to DMEM:F12 commonly used in other formulations (25). This medium also excludes Hepes, but includes heparin, a cofactor for FGF-2, which is required for HESC maintenance.

Results

We first tested the ability of ESF7 medium [supporting information (SI) Table S1], which we had developed for use with MESCs (25), to support the growth of two HESC lines, HUES-1 (Fig. 1*A*) (26) and Shef1 (Fig. 1*B*) (8). These cells were harvested using collagenase, or trypsin/EDTA, respectively, as previously described, from cultures on MEFs, and transferred to type I

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The authors declare no conflict of interest.

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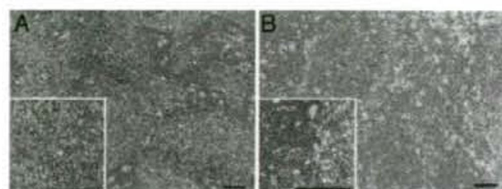

Fig. 1. Phase contrast photomicrograph of HUES-1 (*A*) and Shef 1 (*B*) HESCs cultured on feeders in KSR-based medium. (Scale bar, 200 μm.)

collagen-coated flasks in ESF7 medium. However, the cells died after one day. On further study we found that Hepes is detrimental to even one day of HESC culture in the absence of serum, that LIF provided no advantage in 6-day culture, but that L-ascorbic acid-2-phosphate and FGF-2 enhanced HESC survival and growth over 3 passages (data not shown). From this initial study we developed a variant of the ESF7 formulation, designated hESF8 (Tables S1 and S2). In this medium, on type I collagen-coated flasks, both HUES-1 (Fig. S1*A*) and Shef1 cells (Fig. S1*A*) grew to form densely packed colonies consistent with the cells retaining an undifferentiated HESC morphology.

To determine the optimal concentration of FGF-2 for HESC growth, HUES-1 cells were seeded without feeders, on a type I-collagen substrate, in KO-DMEM/KSR or hESF8 medium containing varying concentrations of FGF-2. In addition, in some experiments, heparin, a cofactor known to stabilize FGF-2, was also added. When HUES-1 cells were cultured in KSR-based medium without feeders on collagen-coated plates, few cells survived and no undifferentiated, alkaline phosphatase-positive colonies were observed with or without FGF-2 in the presence or absence of heparin (data not shown). However, small undifferentiated colonies were observed in hESF8 medium lacking FGF-2, while increasing the dose of FGF-2 up to 50 ng/ml promoted the growth of larger colonies in this medium (Fig. 2*A*). High SSEA-3 expression and low SSEA-1 expression were seen in all cultures, irrespective of the FGF-2 concentration (Fig. 2*B*). However, at 100 ng/ml FGF-2, the colony sizes were again smaller, with apparently differentiated cells identified morphologically, also present.

The addition of heparin to hESF8 medium, in the absence of FGF-2, also promoted HUES-1 cell proliferation in a dose-dependent manner; the greatest effect was seen at 100–200 ng/ml heparin, whereas 1000 ng/ml was markedly deleterious (Fig. 2 *D* and *E*). These cells also retained expression of SSEA-3 with low expression of SSEA-1 (Fig. 2*F*). Finally, we tested the combined

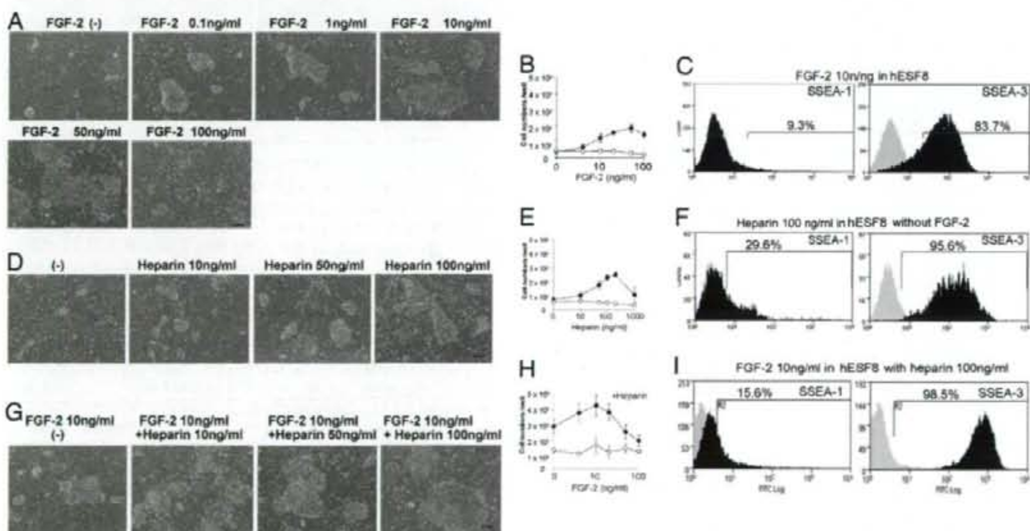

Fig. 2. Effect of FGF-2 and heparin on the HUES-1 cell growth. HUES-1 cells were cultured on type I collagen in hESF8 with varying concentrations of FGF-2 or heparin. (*A*) Phase contrast photomicrograph of HUES-1 cells with various concentration of FGF-2 without heparin. (Scale bar, 200 μm.) (*B*) Number cells harvested after 6 days of culture in KSR-based medium (open circles) or hESF8 (closed circles). Values are the mean ± SD for three measurements. The absence of error bars is due to the small SD in the results. (*C*) FACS profiles for SSEA-1 and SSEA-3 expression of cells cultured with 10 ng/ml FGF-2. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of cells antigen positive. Similar profiles were obtained for cells from cultures grown with all concentrations of FGF-2 from 0 to 100 ng/ml (data not shown). (*D*) Phase contrast photomicrograph of HUES-1 cells cultured without FGF-2 but supplemented with various concentration of heparin. (Scale bar, 200 μm.) (*E*) Number cells harvested after 6 days of culture in KSR-based medium (open circles) or hESF8 (closed circles) with varying concentrations of heparin but no FGF-2. Values are the mean ± SD for three measurements. The absence of error bars is due to the small SD in the results. (*F*) FACS profiles for SSEA-1 and SSEA-3 expression of cells cultured with 100 ng/ml heparin. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of cells antigen positive. Similar profiles were obtained for cells from cultures grown with all concentrations of heparin from 0 to 100 ng/ml (data not shown). (*G*) Phase contrast photomicrograph of HUES-1 cells cultured with various concentration of heparin in hESF8 containing 10 ng/ml FGF-2. (Scale bar, 200 μm.) (*H*) Number cells harvested after 6 days of culture in KSR-based medium (open circles) or hESF8 (closed circles) containing varying concentrations of FGF-2 and 100 ng/ml heparin. Values are the mean ± SD for three measurements. The absence of error bars is due to the small SD in the results. (*I*) FACS profiles for SSEA-1 and SSEA-3 expression of cells cultured hESF8 containing 100 ng/ml heparin and 10 ng/ml FGF-2 (hESF9). Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells. Similar profiles were obtained for cells from cultures grown with all concentrations of FGF-2 from 0 to 100 ng/ml (data not shown).

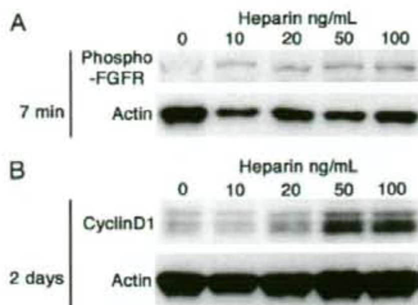

Fig. 3. The effect of heparin on FGF signaling. HUES-1 cells were stimulated with different concentrations of heparin after 48-h starvation of FGF-2 and heparin. (*A*) Seven minutes after heparin addition, the cells were lysed and followed by western blot using an antibody detecting the phosphorylation of FGF receptors. (*B*) After 2 days of culture in the presence of heparin, the cells were lysed and subjected to western blot of cyclin D1. Actin is used as loading control.

effects of heparin and FGF-2. In hESF8 medium containing 100 ng/ml heparin, maximal cell densities were achieved with 10 ng/ml FGF-2, with high SSEA-3 and low SSEA-1 expression; >20 ng/ml FGF-2, maximal cell densities decreased (Fig. 2 *G–I*).

To confirm the effect of heparin on FGF signaling, we analyzed its effect on phosphorylation of the FGF receptor. Rapid phosphorylation of the FGF receptors was induced by heparin in a dose-dependent manner (Fig. 3*A*). Further, expression of cyclinD1 was also induced by heparin, confirming a potential effect on cell cycle regulation (Fig. 3*B*). Based on these observations we supplemented hESF8 medium with 10 ng/ml FGF-2 and 100 ng/ml heparin, and designated this, hESF9.

To determine whether different substrates might affect HESC growth, HUES-1 cells were cultured in hESF9 separately on type I collagen (10 μg/cm^2), fibronectin (5 μg/cm^2), laminin (5 μg/cm^2), or gelatin (10 μg/cm^2). In each case the cells produced typical undifferentiated colonies (Fig. 4 *A–D*) with similar profiles of SSEA-3, TRA-1–60, and SSEA-1 (data not shown). However, subjectively, we judged the colony morphologies more uniform on type I collagen, which we continued to use as the standard substrate in subsequent experiments with hESF9 medium. By contrast, when HUES-1 cells were cultured on Matrigel in hESF9 medium (Fig. 4*E*), or on these ECM components in KSR-based medium (Fig. 4*F*), extensive differentiation was observed.

The hESF9 medium proved capable of supporting the culture of other HESC lines, Shef1, Shef4, Shef5, and H7, after plating on type I collagen. In each case, the expression of the marker antigens SSEA-1, SSEA-3, and TRA-1–60 was similar to cells grown in KSR-based medium on feeders (Fig. 5). Also, we measured the growth rates of Shef1 and Shef5 cells and, as for HUES-1 (Fig. 6). The growth rate and maximum cell densities reached by HUES-1 and Shef1 were higher when the cells were grown in hESF9. However, in the case of Shef5, although the final cell density was higher when grown in hESF9, the initial growth rate was lower.

We subsequently tested long-term culture of HUES-1 to 25 passages and Shef1 to 15 passages on type I collagen in hESF9 medium without feeders. Although a few fibroblastic or neural cells appeared in the cultures at early passages, morphologies of undifferentiated HESC colonies were maintained as those cultured on feeders (Fig. 7*A*). The growth rates of these HESCs after five passages was similar to to those at passage 1 (Fig. 7*B*). The cells retained a normal karyotype (Fig. S2A). They also

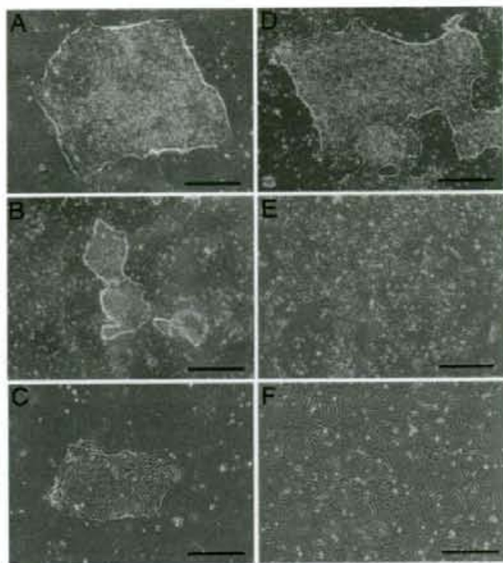

Fig. 4. Effect of ECM on HUES-1 culture. (*A–E*) Phase contrast photomicrographs of HUES-1 cells cultured in hESF9 medium, in flasks coated with collagen (*A*), gelatin (*B*), fibronectin (*C*), laminin (*D*), and Matrigel (*E*). (*F*) Cells were cultured in KSR-based medium on type I collagen. (Scale bar, 200 μm.)

retained expression of SSEA-3, and *OCT3/4*, *NANOG*, *SOX-2* and *REX-1* (Fig. 7*C*, Fig. S2 *B* and *C*). Further, the HUES-1 and Shef1 cells retained the capacity for extensive differentiation as indicated by analysis of ectoderm, mesoderm, and endoderm marker gene expression, *NeuroD*, *Oligo 2*, *MyoD*, *Nkx2.5*, *CD31*, *AFP*, *Cdx2*, *and GATA6* following embryoid body formation (Fig. 7*D*).

Discussion

Our current results indicate that it is possible to culture HESCs in a defined medium, hESF9, in which insulin, transferrin, a low level of fatty acid-free albumin conjugated with oleic acid, and low levels of FGF-2 are the only protein components, together with a substrate composed only of type I collagen. In these

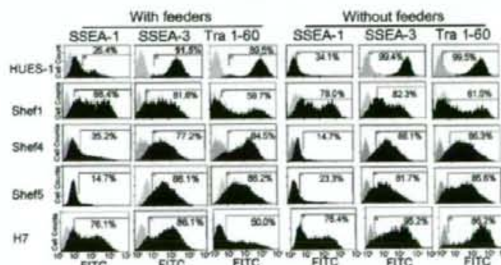

Fig. 5. FACS profiles for SSEA-1, SSEA-3, and TRA-1–60 expression by HUES-1, Shef1, Shef4, Shef5, and H7 HESC lines cultured on type I collagen in hESF9, in comparison with cells grown on feeders in KSR-based medium. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells.

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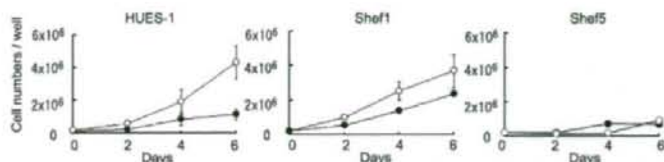

Fig. 6. A comparison of the growth of different HESCs in hESF9 and KSR-based media. HUES-1, Shef1, and Shef5 cells were seeded on feeders in KSR-based medium (closed circles) or on type I collagen in hESF9 (open circles) at a cell density of 1×10^5 cells per well; mean and SD of three experiments. Cell numbers were counted every 2 days.

studies we have grown two HESC lines, HUES-1 and Shef1, through multiple passages while they retained stable expression of undifferentiated markers of HESCs, and a capacity to differentiate. The medium we describe differs in several key respects from media already reported by others.

First, the base medium, ESF (25), which we developed for MESC culture, has a substantially different formulation from DMEM and DMEM:F12, which are widely used in other reports. Thus, the ESF basal medium includes lipoic acid, glutathione, *p*-aminobenzoic acid, which are absent from DMEM:F12. Also, the concentration of biotin, pyridoxine, tyrosine, and phosphate are higher than in DMEM:F12, and the osmolarity of ESF is lower (292 mosmol/liter). Further, we found that Hepes is relatively toxic for HESCs, and we have excluded it. In addition, as in an earlier serum-free medium that we designed for human EC cells (27), we found that inclusion of ascorbic acid was strongly beneficial. However, rather than ascorbic acid itself, which is relatively unstable, we used a long-acting vitamin C derivative, L-ascorbic acid-2-phosphate (28). Ascorbic acid is well known as a scavenger of free oxygen radicals, and its value may relate to these anti-oxidant properties. However, it is not required by MESCs, and another possibility is that the requirement reflects the inability of humans, unlike most other mammals, to synthesize this vitamin.

Second, some other reported media for HESCs have either included particularly high concentrations of FGF-2 (up to 100

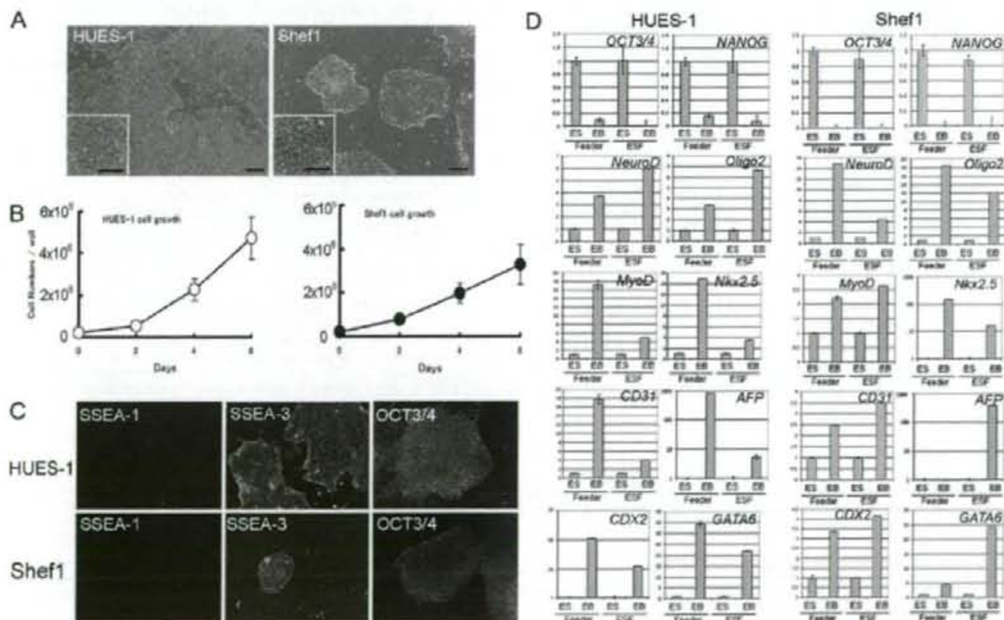

Fig. 7. Long-term culture of HESCs in the defined medium. HUES-1 and Shef1 cells were serially cultured on type I collagen in hESF9. The cells were split at 1:3 every week. (*A*) Phase contrast photomicrograph of HUES-1 at passage 21 and Shef1 cells at passage 14. (Scale bar, 200 μm.) (*B*) Growth of HUES-1 (passage 10) and Shef1 (passage 5) cells in the defined medium. (*C*) Immunohistochemical staining of HUES-1 (passage 24) and Shef1 (passage 14) for SSEA-1, SSEA-3, and OCT3/4. (*D*) Q-PCR analysis of gene expression in HUES-1 and Shef1 on feeder in KSR-based medium (feeder) and HUES-1 (passage 24) and Shef1 (passage 14) on collagen in hESF9 medium (ESF) during *in vitro* differentiation (EB). The name of the gene of differentiation is noted in each bar graph. Expression levels were normalized against GAPDH. The relative level of each gene in undifferentiated cells was defined as "1."

ng/ml) (24), and/or members of the TGF-β family of growth factors (12, 23), and/or Wnt3A (20) and APRIL/BAFF (29). However, for hESF9 we have found that FGF-2 alone, at a concentration of 10 ng/ml, is sufficient. This requirement for low rather than high levels of FGF-2 may be due to our inclusion of heparin. Heparin, a soluble derivative of heparan sulfate, is a well known cofactor for FGF-2, but its use in defined HESC culture media has not been described. In many cases, a requirement for heparin might be satisfied by the production of heparan sulfate by the ES cells themselves, or by their differentiated derivatives. Several reports have strongly indicated a role for FGF signaling in the maintenance of HESCs, in contrast to MESCs, and have suggested that HESCs produce FGF-2 as an autocrine factor (30, 31). Our observation that the addition of heparin in the absence of exogenous FGF results in the phosphorylation of FGF receptors in HESCs is consistent with a role in stabilizing endogenously produced FGF. Likewise, our finding of substantially lower optimal concentrations of exogenous FGF than reported elsewhere might also reflect the stabilizing effect of heparin on this growth factor. Nevertheless, other mechanisms cannot be excluded. For example, heparin has been reported to enhance the activity of Wnt signaling and FGF signaling in HESCs (32).

In the absence of feeders, HESCs require attachment factors to promote their survival and proliferation (8). Matrigel, a basement membrane preparation from the Engelbreth-Holm-Swarm mouse tumor, is often used. 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, NGF, and TGF-β (16). Previous reports have described the use of N2/B27 with 20 ng/ml FGF-2 and Matrigel for feeder free culture of HESC growth (33). Some components such as heparin in Matrigel may support HESC growth with FGF-2. Some authors have replaced Matrigel with purified components, such as type IV collagen, fibronectin, laminin, and vitronectin, alone or in combination (8, 23, 34). In previous studies we found that laminin and fibronectin, but not type I collagen, tended to promote the differentiation of MESCs in defined medium (35). By contrast, in the present study we found that each of these factors was effective in supporting attachment and proliferation of HESCs, although type I collagen appeared the best.

The reasons for the apparent differences in effectiveness of the various formulations for defined HESC culture media are unclear. One possibility is that in some cases the substrate used for attachment of the HESCs, such as Matrigel, has been undefined and might contain extraneous growth factors that confound the analysis. Another possibility is that the lines used by different authors differ in their requirements, either because the undifferentiated stem cells themselves differ intrinsically in their response to, or their autocrine production of specific factors, or because they generate varying amounts of differentiated derivatives that produce factors acting back on the stem cells to promote or inhibit their proliferation. Until specific media formulations are tested by different laboratories on different HESC lines, these issues cannot be easily resolved. An ongoing program of the International Stem Cell Initiative (18) is currently addressing this problem by comparing a number of defined media on different HESC lines in different laboratories.

Materials and Methods

Cells. Stock cultures of HESC lines HUES-1 and Shef1 were maintained in Knockout (KO)-DMEM (Invitrogen) supplemented with Knockout Serum Replacement (KSR, Invitrogen) on inactivated mouse embryo fibroblast feeder cells as described (8, 15, 26). In addition, a subline of H7, H7.s6 (1, 36), and two new HESC lines, Shef4 and Shef5, derived and maintained in our laboratory according to the same protocols as Shef1 were also used. For culture in defined media without feeders, cells were harvested with 0.5 mM EDTA.

Cell Culture Media. FGF-2 was purchased from Peprotech Inc. (Rocky Hill, NZ). Type I collagen was from Nitta Gelatin, Co. (Osaka, Japan). The basal ESF medium was provided by the Cell Science & Technology Institute (Sendai, Japan), according to the formulation described (Table S2 and ref. 25). L-ascorbic acid-2-phosphate was obtained from Wako Pure Chemical Ltd. (Osaka, Japan). All other reagents were from Invitrogen and Sigma Aldrich.

Antigen Expression. Cell surface antigen expression was determined by flow cytometry (37). For *in situ* immunohistochemisty, the cells were fixed with 4% paraformaldehyde and then incubated with first and second antibodies as described in ref. 37. The following monoclonal antibodies to surface marker antigens were prepared and used as described in ref. 37: MC480 (anti-SSEA1) (38), MC631 (anti-SSEA3) (39), and TRA-1–60 (40). Further antibodies to OCT3/4 (1 μg/ml; Santa Cruz) and NANOG (0.4 μg/ml, R&D Systems) were also used.

Reverse Transcription PCR (RT-PCR) and Quantitative RT-PCR. Total RNA was extracted from HESCs using a kit (Qiagen) according to the kit instruction. RT-PCR was performed as described in ref. 41. Q-PCR was carried out using the SYBR Green JumpStartTM Kit on a Bio-Rad iCycler. Primer pairs used are listed in Table S3. Expression levels were all normalized against GAPDH. The relative level of each gene in undifferentiated cells was defined as "1."

Western Blot Analyses. Cells were lysed in SDS lysis buffer [50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, with protease and phosphotase inhibitors Complete and phosSTOP (Roche)]. Antibodies used were anti-phospho-FGF Receptor (AF3285, R&D systems), mouse monoclonal anti-human cyclinD1 DCS-6 (DAKO), and mouse monoclonal anti-actin (Abcam).

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1. Thomson JA, *et al.* (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
2. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. *Nat Biotechnol* 18:399–404.
3. Xu C, *et al.* (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19:971–974.
4. Smith AG, *et al.* (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336:688–690.
5. Williams RL, *et al.* (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336:684–687.
6. Daheron L, *et al.* (2004) LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* 22:770–778.
7. Humphrey RK, *et al.* (2003) Characterization and isolation of promoter-defined nestin-positive cells from the human fetal pancreas. *Diabetes* 52:2519–2525.
8. Draper JS, Moore HD, Ruban LN, Gokhale PJ, Andrews PW (2004) Culture and characterization of human embryonic stem cells. *Stem Cells Dev* 13:325–336.
9. Xu RH, *et al.* (2002) BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 20:1261–1264.
10. Beattie GM, *et al.* (2005) Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells* 23:489–495.
11. James D, Levine AJ, Besser D, Hemmati-Brivanlou A (2005) TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development (Cambridge, UK)* 132:1273–1282.
12. Vallier L, Alexander M, Pedersen RA (2005) Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J Cell Sci* 118:4495–4509.
13. Pera MF, *et al.* (2004) Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. *J Cell Sci* 117:1269–1280.
14. Ying QL, Nichols J, Chambers I, 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.
15. Amit M, *et al.* (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 227:271–278.
16. Hoffman LM, Carpenter MK (2005) Characterization and culture of human embryonic stem cells. *Nat Biotechnol* 23:699–708.

17. Esner M, Pachernik J, Hampl A, Dvorak P (2002) Targeted disruption of fibroblast growth factor receptor-1 blocks maturation of visceral endoderm and cavitation in mouse embryoid bodies. *Internat J of Dev Biol* 46:817–825.
18. Adewumi O, *et al.* (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 25:806–813.
19. Tesar PJ, *et al.* (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448:196–199.
20. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature Med* 10:55–63.
21. Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA (2005) Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods* 2:185–190.
22. Wang G, *et al.* (2005) Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem and Biophys Res Commun* 330:934–942.
23. Ludwig TE, *et al.* (2006) Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* 24:185–187.
24. Levenstein ME, *et al.* (2006) Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells* 24:568–574.
25. Furue 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* 41:19–28.
26. Cowan CA, *et al.* (2004) Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 350:1353–1356.
27. Andrews PW, *et al.* (1994) Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by osteogenic protein-1 (or bone morphogenetic protein-7). *Lab Invest* 71:243–251.
28. Hata R, Senoo H (1989) L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. *J of Cell Physiol* 138:8–16.
29. Lu J, Hou R, Booth CJ, Yang SH, Snyder M (2006) Defined culture conditions of human embryonic stem cells. *Proc Natl Acad Sci USA* 103:5688–5693.
30. Sato N, Sanjuan IM, Heke M, Uchida M, Naef F, Brivanlou AH (2003) Molecular signature of human embryonic stem cells and its comparison with the mouse. *Dev Biol* 260:404–413.
31. Dvorak P, *et al.* (2005) Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. *Stem Cells* 23:1200–1211.
32. Sasaki N, *et al.* (2008) Heparan sulfate regulates self-renewal and pluripotency of embryonic stem cells. *J Biol Chem* 283:3594–3606.
33. Yao S, *et al.* (2006) Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc Natl Acad Sci USA* 103:6907–6912.
34. Amit M, Shariki C, Margulets V, Itskovitz-Eldor J (2004) Feeder layer- and serum-free culture of human embryonic stem cells. *Biol of Reprod* 70:837–845.
35. Hayashi Y, *et al.* (2007) Integrins regulate mouse embryonic stem cell self-renewal. *Stem Cells* 25:3005–3015.
36. Draper JS, *et al.* (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol* 22:53–54.
37. Draper JS, Pigott C, Thomson JA, Andrews PW (2002) Surface antigens of human embryonic stem cells: Changes upon differentiation in culture. *J Anat* 200:249–258.
38. Solter D, Knowles B (1978) Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc Nat Acad Sci (USA)* 75:5565–5569.
39. Shevinsky L, Knowles B, Damjanov I, Solter D (1982) Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. *Cell* 30:697–705.
40. Andrews PW, Banting G, Damjanov I, Arnaud D, Avner P (1984) Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. *Hybridoma* 3:347–361.
41. Matin MM, *et al.* (2004) Specific knockdown of Oct4 and beta2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. *Stem Cells* 22:659–668.

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Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium

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ABSTRACT **Formation of the primitive streak (PS) is the initial specification step that generates all the mesodermal and endodermal tissue lineages during early differentiation. Thus, a therapeutically compatible and efficient method for differentiation of the PS is crucial for regenerative medicine. In this study, we developed chemically defined serum-free culture conditions for the differentiation of embryonic stem (ES) cells into the PS-like cells. Cultures supplemented with Wnt showed induction of expression of a PS marker, the *brachyury* gene, followed by induction of the anterior PS markers *goosecoid* and *foxa2*, a posterior PS marker, *evx1*, and the endoderm marker *sox17*. Similar differentiation of PS by Wnt was also observed in human ES cells. Moreover, we revealed that the activation of the Wnt canonical pathway is essential for PS differentiation in mouse ES cells. These results demonstrated that Wnt is an essential and sufficient factor for the induction of the PS-like cells *in vitro*. These conditions of induction could constitute the initial step in generating therapeutically useful cells of the definitive endoderm lineage, such as hepatocytes and pancreatic endocrine cells, under chemically defined conditions.— Nakanishi, M., Kurisaki, A., Hayashi, Y., Warashina, M., Ishiura, S., Kusuda-Furue, M., Asashima, M. Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium. *FASEB J.* 23, 000–000 (2009)**

Key Words: mesendoderm · endoderm · regenerative medicine · brachyury · sox17

During gastrulation, a specific subgroup of epiblast cells ingresses into the primitive streak (PS) and subsequently generates the mesoderm and definitive endoderm (1, 2). PS contains the progenitors of these cells at the early- and mid-PS stages of gastrulation (3–5). Therefore, PS formation is an essential specification step in the generation of all the mesodermal and endodermal tissue lineages, including the pancreas, liver, and heart.

Recent studies using knockout mice suggested that Wnt signaling is indispensable for the differentiation of the PS. In *Wnt3a*-homozygous knockout embryos, epiblast cells that are ingressing into the PS are diverted to a neuroectodermal fate rather than forming paraxial mesodermal cells (6). Double-homozygous mutants of coreceptors for the transduction of Wnt signals *Lrp5;Lrp6* also fail to establish the PS (7). Moreover, ablation of β-catenin in embryonic endoderm changes cell fate from endoderm to precardiac mesoderm (8). These reports support the importance of Wnt signaling in the differentiation of the PS, mesoderm, and endoderm *in vivo*.

In addition to Wnt signaling, transforming growth factor-β (TGF-β) signaling is important for PS differentiation. Mouse embryos deficient for one of the transforming growth factor-β members, *Nodal*, fail to form both the mesoderm and the definitive endoderm after implantation (9). Moreover, animals that lack one allele of *Smad2* and *Smad3* exhibit defects in the definitive endoderm (10). Loss of *Smad3* in the context of one wild-type allele of *Smad2* results in impaired production of the anterior axial mesendoderm, while *Smad2-Smad3* double-homozygous mutants completely lack mesoderm and fail to gastrulate (11). Collectively, these results suggest that dose-dependent TGF-β signaling *via* Smad2/3 mediates cell fate in the early stage of mesoderm and endoderm formation.

In vitro differentiation of PS from both human and mouse embryonic stem (ES) cells using activin A, which is a member of the TGF-β superfamily, has been reported (12–15). In contrast, the role of Wnt signaling in the differentiation of ES cells to PS is not well-documented,

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although Wnt signaling has been shown to maintain pluripotency in human and mouse EScells (16).

In this study, we developed a simple culturing method using a chemically defined medium (ESF) to induce specifically the PS-like cells from mouse ES cells. Previously, we established that this medium could be used to propagate mouse ES cells without feeder cells (17, 18). Here, we demonstrate that treatment with Wnt efficiently induces directed induction of tissue culture analogs of the PS from both mouse and human ES cells. Furthermore, we show that Wnt selectively activates canonical signaling during PS differentiation of ES cells. Our results verified that Wnt signaling is essential and sufficient for PS differentiation from ES cells *in vitro.* Moreover, our method could be used as a basic protocol for the preparation of endodermal and mesodermal tissues under chemically defined conditions.

MATERIALS AND METHODS

Cell culture

The mouse ES cell D3 line (American Type Culture Collection, Manassas, VA, USA) was routinely cultured in a tissue culture dish coated with type I collagen (Nitta Gelatin Inc., Osaka, Japan) in a humidified atmosphere of 5% CO_2 at 37°C in a chemically defined medium, designated ESF7, as described previously (17, 18). For subculturing, mouse ES cells were dissociated with 0.2 mg/ml EDTA in PBS and seeded at 1.7×10^3 cells/cm^2 every 4 days.

For the induction of PS differentiation, mouse ES cells were dissociated with 20 μg/ml EDTA · 4Na in PBS and seeded onto tissue culture dishes coated with laminin (Sigma, St. Louis, MO, USA) at $0.75–2.5 \times 10^4$ cells/cm^2. The ES cells were cultured in a chemically defined differentiation medium A [ESF basal medium (Cell Science & Technology Institute, Sendai, Japan) that contains 10 μg/ml insulin, 5 μg/ml apo-transferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, and 0.5 mg/ml BSA (Sigma)] supplemented with the indicated concentration of Wnt-3a (R&D Systems, Minneapolis, MN, USA) or activin A. Besides these supplements, recombinant mouse Dkk1 (R&D Systems) was supplemented where indicated. The culture medium was renewed every 2 days.

Human ES cells (KhES-1) were obtained from the Institute for Frontier Medical Science, Kyoto University (Kyoto, Japan), with approval for human ES cell use granted by the Minister of Education, Culture, Sports, Science, and Technology of Japan. The Review Board of the University of Tokyo approved this research. The entire study was conducted in accordance with the Declaration of Helsinki. Human ES cells were maintained on a feeder layer of mouse embryonic fibroblasts pretreated with 10 μg/ml mitomycin C (Sigma) for 3 h. Human ES cells were maintained in Dulbecco modified Eagle medium (DMEM)/F-12 supplemented with 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 2 mM L-glutamine, penicillin/streptomycin, 20% knockout serum replacement (KSR; Invitrogen, Carlsbad, CA, USA) and 5 ng/ml bFGF (Upstate Biotechnology, Lake Placid, NY, USA) in a humidified atmosphere of 3% CO_2 and 97% air at 37°C. ES cells were partially dissociated with human ES cell dissociation solution [0.25% trypsin and 0.1 mg/ml collagenase IV (Gibco, Carlsbad, CA, USA) in PBS that contained 1 mM $CaCl_2$ and 20% KSR] and subcultured every 3 or 4 days.

For the induction of PS-like cells, human ES colonies were partially dissociated into clumps with the above-mentioned human ES cell dissociation solution; this was followed by gentle trituration. The human ES clumps (300 cells/clump) were then cultured for 3 days in a laminin-coated tissue culture test plate (2–2.5 clumps/cm^2) in hESF-dif medium (Nipro, Osaka, Japan) that was supplemented with 10 μg/ml insulin, 5 μg/ml apo-transferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, 0.5 mg/ml BSA (Sigma), and 50 ng/ml of Wnt-3a (R&D Systems) or activin A. The chemical components of hESF-dif are same as those of mouse ESF except HEPES. HEPES is excluded from hESF-dif medium.

Embryoid body assay for mouse ES cells

We performed two kinds of embryoid body (EB) assays in this study. For the differentiation of mouse ES cells into an ectodermal lineage (see Fig. 3), mouse ES cells treated with Wnt-3a for 3 days as described above or with undifferentiated ES cells were dissociated with 10 μg/ml trypsin and 20 μg/ml EDTA · 4Na in PBS and cultured in round-bottom low-cell-binding plates (Nunc, Roskilde, Denmark) (2000 cells/50 μl) using a fetal bovine serum (FBS) -containing differentiation medium B [DMEM supplemented with 15% FBS (Gibco) and penicillin-streptomycin] for 24 h to prepare EBs. The EBs were then transferred to gelatin-coated 24-well test plates and cultured for 12 days in differentiation medium B, which was replenished every 4 days.

For the experiment shown in Fig. 1*f*, mouse ES cells were dissociated with 10 μg/ml trypsin and 0.2 μg/ml EDTA in PBS and cultured in round-bottom low-cell-binding plates (2000 cells/50 μl) using the chemically defined differentiation medium A without Wnt-3a or activin for 24 h. The next day, 150 μl of differentiation medium A was added to each well, and the EBs were further cultured for 2 days.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated from duplicated samples using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany), and 500–2000 ng of RNA was used for reverse-transcription with the SuperScript First-Strand System (Invitrogen). PCRs were carried out using 1/50–1/100 dilutions of the cDNA per reaction, 500 nM of the forward and reverse primers, and the Quantitect SYBR Green master mix (Qiagen). The following PCR conditions were used: 95°C for 15 min, followed by 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Alternatively, Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer's instructions. Real-time PCR was performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, *gapdh*. The primer sequences used in this study are described in **Table 1**.

Immunostaining

ES cells were seeded onto laminin-coated Lab-Tek chamber slides (Nunc). The cells were washed with PBS and fixed in cold acetone for 5 min. After washing with PBS, the cells were blocked with 3% BSA in PBS for 30 min at room temperature and incubated with the primary antibody for 16 h at 4°C. The cells were washed twice with PBS, incubated with secondary antibody that was labeled with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) for 1 h at room temperature, and mounted in VectaShield Hardset Mounting Medium with 4′,6′-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). The cells were observed under a fluorescence microscope and photographed with the ORCA-3 CCD camera controlled by

TABLE 1. *Oligodeoxynucleotide primers used for real-time PCR*

Gene	Organism	Primers (forward/reverse; 5′ to 3′)
brachyury	Mouse	TACCCCAGCCCCTATGCTCA/GGCACTCCGAGGCTAGACCA
brachyury	Human	GCAAAAGCTTTCCTTGATGC/ATGAGGATTTGCAGGTGGAC
evx1	Mouse	AACTGGCAGCAGCCTTAAAC/CGTGGCTCATCATGTAGGTG
foxa2	Mouse	AGTTAAAGTATGCTGGGAGCCGTGA/TTCATGTTGCTCACGGAAGAGTAGC
foxa2	Human	CTGGAGCAGCTACTATGC/CCGCGCTCATGTTGC
gapdh	Mouse	GCTACACTGAGGACCAGGTTGTC/AGCCGTATTCATTGTCATACCAGG
gapdh	Human	CTCTGCTCCTCCTGTTCGAC/ACGACCAAATCCGTTGACTC
goosecoid	Mouse	ABI TaqMan gene expression assays (assay ID: Mm00650681_g1)
sox1	Mouse	GTCATGTCCGAGGCCGAGAA/AGCAGCGTCTTGGTCTTGCG
sox7	Mouse	GCCACGGCCACGTATTACAA/TGACCTCTTGCCACCAAGGA
sox17	Mouse	AGGGCCAGAAGCAGTGTTACACA/TCTCGTGTAGCCCCTCAACTGTTC

the Luminavision software (Mitani, Fukui, Japan). The primary antibodies used were goat anti-brachyury (4 μg/ml; R&D Systems), mouse anti-β-catenin (1.25 μg/ml; BD Biosciences, Franklin Lakes, NJ, USA), mouse anti-E-cadherin (2.5 μg/ml; BD Biosciences), mouse anti-GSC (10 μg/ml; Abnova, Taipei, Taiwan), goat anti-HNF-3β/FOXA2 (2 μg/ml; R&D Systems), and goat anti-SOX17 (5 μg/ml; Neuromics, Edina, MN, USA).

For immunofluorescent staining of the differentiated EBs, they were fixed in 4% paraformaldehyde in PBS for 25 min. After washing with PBS, the fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min, blocked with 3% BSA in PBS for 30 min, and incubated with the mouse anti-β3-tubulin monoclonal antibody (Chemicon, Temecula, CA, USA) diluted in 1% BSA, 0.1% Tween-20 in PBS for 16 h at 4°C, followed by 2 washes with PBS. Then, the samples were incubated with the secondary antibody labeled with Alexa Fluor 594 (Invitrogen) diluted in 1% BSA, 0.1% Tween- 20 in PBS for 1 h at room temperature. After washing with PBS, cell nuclei were stained with DAPI for 7 min. The EBs were observed under a fluorescence microscope and photographed with AquaCosmos (Hamamatsu Photonics, Hamamatsu, Japan) connected to the ORCA-3 CCD camera.

Immunoblotting

Cells were lysed in a minimal volume of ice-cold lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1%

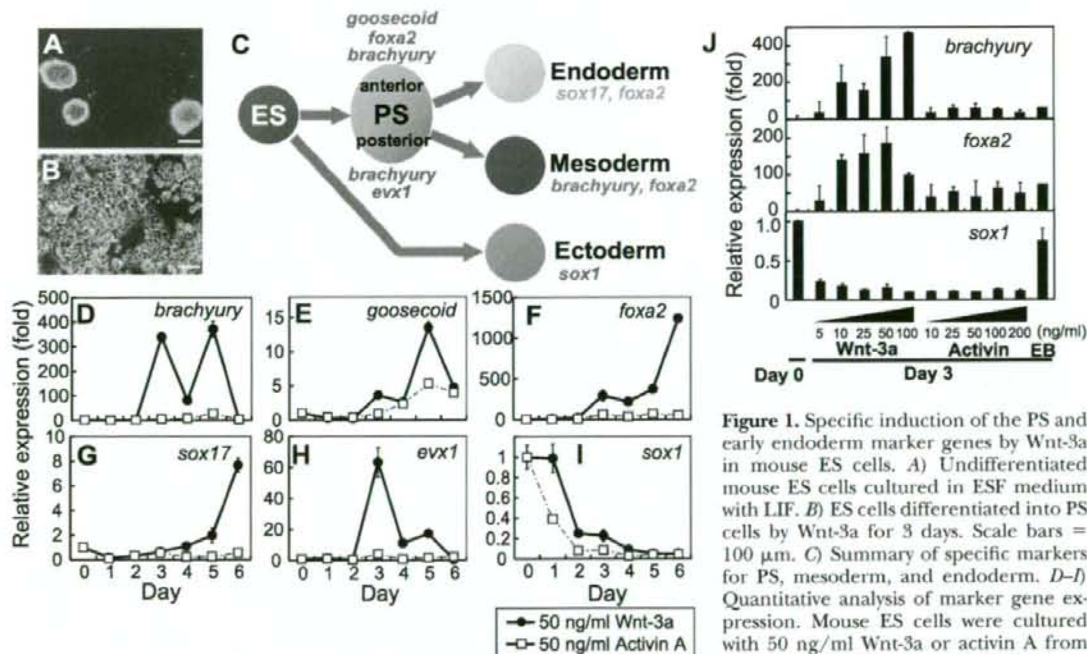
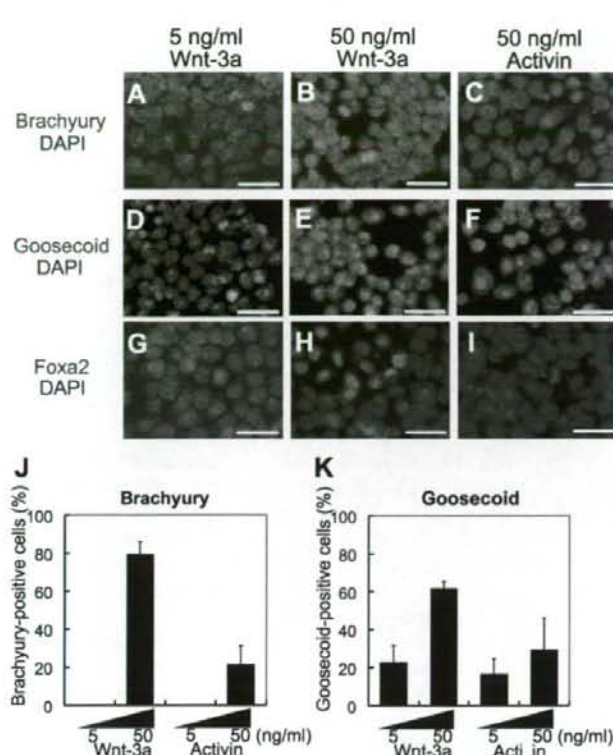


Figure 1. Specific induction of the PS and early endoderm marker genes by Wnt-3a in mouse ES cells. *A*) Undifferentiated mouse ES cells cultured in ESF medium with LIF. *B*) ES cells differentiated into PS cells by Wnt-3a for 3 days. Scale bars = 100 μm. *C*) Summary of specific markers for PS, mesoderm, and endoderm. *D–I*) Quantitative analysis of marker gene expression. Mouse ES cells were cultured with 50 ng/ml Wnt-3a or activin A from day 0 to day 6. Duplicates of the cultures were harvested at the indicated times, and the expression levels of the indicated genes were analyzed by quantitative RT-PCR. Values were normalized to *gapdh* mRNA, and the control values were arbitrarily set to day 0 (undifferentiated ES cells). *J*) Titration of Wnt-3a and activin concentration for the differentiation to PS-like cells. Duplicate cultures were harvested on day 3, and the expression levels of the indicated genes were analyzed by quantitative RT-PCR as described above. EB indicates the spontaneously differentiated cells by forming EB in a chemically defined serum-free differentiation medium for 3 days as described in Materials and Methods.



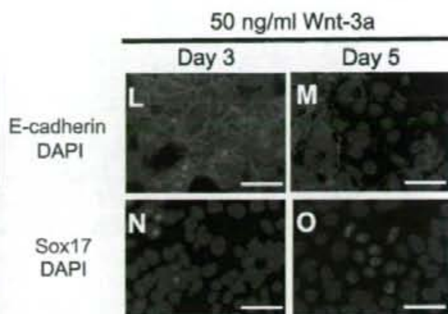


Figure 2. Proportions of PS marker-positive cells in the Wnt-3a treated cell population. *A–I*) Immunofluorescence staining of mouse ES cells treated with 5 ng/ml (left panels) or 50 ng/ml (middle panels) Wnt-3a or 50 ng/ml activin A (right panels) from day 0 to day 3. Cells were immunostained (red) with antibodies against Brachyury (*A–C*), Goosecoid (*D–F*), or Foxa2 (*G–I*). Nuclei were stained with DAPI (blue). *J, K*) Bar graphs show ratios of Brachyury-positive (*J*) and Goosecoid-positive (*K*) cells to the total number of cells. *L–O*) Immunofluorescence staining (red) with anti-E-cadherin (*L, M*) or anti-Sox17 (*N, O*) antibody of ES cells treated with 50 ng/ml Wnt-3a from day 0 to day 3 (*L, N*) or day 5 (*M, O*). Nuclei are stained with DAPI (blue). Scale bars = 10 μm.

Nonidet P-40, protease inhibitor cocktail (Roche, Basel, Switzerland), PhosSTOP (Roche), 1 mM Na_3VO_4, 25 mM NaF, and 25 mM β-glycerophosphate]. After rotating at 4°C for 1 h, the supernatant was collected by centrifugation at maximum speed for 15 min at 4°C. The proteins were separated by SDS-PAGE and detected by Western blotting with horseradish peroxidase (HRP) -conjugated anti-mouse or anti-goat immunoglobulin G (IgG) as the secondary antibody. For signal detection, SuperSignal West Femto substrate (Pierce, Rockford, IL, USA) was applied, and the membranes were visualized with the LAS-1000plus lumino-image analyzer (Fuji Film, Tokyo, Japan).

The following primary antibodies were used in this study. Mouse anti-actin was purchased from Sigma, and mouse anti-β-catenin was from BD Biosciences. Mouse anti-GSK3 was obtained from Upstate Biotechnology. Rabbit anti-phospho-GSK-3 S9/S21 and rabbit anti-phospho-PKC (pan) were from Cell Signaling (Danvers, MA, USA). Rabbit anti-c-Jun, rabbit anti-phospho-c-Jun, and rabbit anti-PKC were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RESULTS

Mouse ES cells were cultured in the chemically defined serum-free medium, ESF7. Under these conditions, ES colonies formed without feeder cells (**Fig. 1*A***). Using this culture medium, we examined the effects of Wnt-3a and activin on the differentiation of ES cells to PS *in vitro*. Treatment of mouse ES cells with Wnt-3a induced efficient differentiation into flattened monolayer cells (Fig. 1*B*). The expression levels of marker genes for the PS, mesoderm, and endoderm in these cells were analyzed by quantitative reverse transcriptase-PCR (RT-PCR). The specific markers used in this study are summarized in Fig. 1*C*. In the ES cells, Wnt-3a induced up to 370-fold the expression of the PS- and mesoderm-specific transcription factor, *brachyury* (19, 20), with induction starting on day 3 (Fig. 1*D*). Activin treatment also induced the expression of *brachyury* mRNA, albeit at an extremely lower level and more slowly. The expression of the anterior PS-enriched transcription factor *goosecoid* (21, 22) was induced by Wnt-3a treatment, and activin A treatment again showed weaker induction of this gene (Fig. 1*E*). Another anterior PS marker *foxa2* (23–25) was dramatically induced up to 1200-fold by Wnt-3a, and an endoderm marker *sox17* was also induced (Fig. 1*F, G*). The expression levels of *foxa2* and *sox17* peaked on Day 6. However, activin A did not show significant effect on the expression of *foxa2* and *sox17*. As shown in Fig. 1*H*, Wnt-3a transiently induced early posterior PS marker, *evx1* (26). In contrast to the PS markers, the expression levels of the ectodermal marker *sox1* and the primitive endoderm marker *sox7* were down-regulated by Wnt-3a or activin A before the onset of *brachyury* and *foxa2* induction (Fig. 1*I*, data not shown). These results indicate that Wnt, but not activin, specifically induces the PS-like cells under chemically defined culture conditions.

To eliminate the possibility that these effects were compared under nonoptimal conditions, titration of these ligands was verified by quantitative RT-PCR (Fig. 1*J*). The expression of *brachyury* mRNA was highest when the

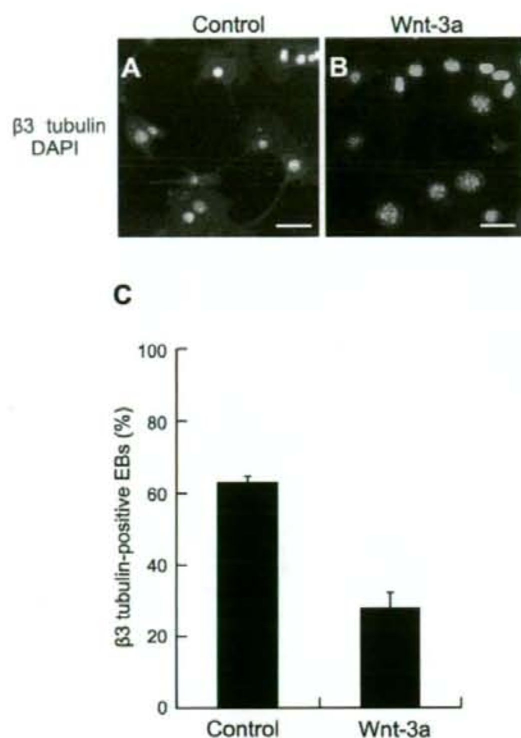


Figure 3. Reduced potential of Wnt-3a-treated mouse ES cells to differentiate into non-PS ectodermal lineages. EBs generated from undifferentiated mouse ES cells or ES cells cultured with 50 ng/ml Wnt-3a for 3 days were spontaneously differentiated. After 12 days of culture, EBs were immunostained with an antibody against β3-tubulin (red). Nuclei were stained with DAPI (blue). *A*) β3-Tubulin-positive neurons in the EBs formed from the undifferentiated ES cells. *B*) Differentiated cells from Wnt-3a-treated ES cells. *C*) Ratio of β3-tubulin-positive cells. The ratios are expressed as the percentage of β3-tubulin-positive EBs to the total number of EBs. Scale bars = 10 μm.

ES cells were treated with 100 ng/ml of Wnt-3a. In contrast, 25–50 ng/ml of activin induced the highest expression of the *brachyury* gene. The expression induced by activin, however, was much weaker than that by Wnt-3A. Higher concentrations of Wnt3a and activin than these resulted in decreased expression of this gene. When the titration was performed with the *foxa2* or *sox1* genes, the optimal concentration was similar to those with *brachyury* gene, and 50–100 ng/ml of Wnt-3a produced the highest effect. The optimal concentration of activin was also 50–100 ng/ml for these genes. These results indicated that the concentration of Wnt-3a and activin (50 ng/ml) used for the analyses, as shown in Fig. 1*D–I*, was appropriate, and the effects of these factors on the differentiation into PS were properly analyzed. In addition, we compared the efficiency of differentiation by Wnt-3a treatment and random differentiation by EB formation in a chemically defined medium. Treatment with Wnt-3a increased the expression of the PS marker genes to greater extent than random EB differentiation did (Fig. 1*J*, right edge); this further supported the fact that Wnt-3a is indeed a specific inducer of PS formation.

To examine further the differentiated PS-like cells, Wnt-3a- or activin A-treated ES cells were immunostained with specific antibodies for Brachyury, Goosecoid, and Foxa2 at day 3 of differentiation. Intense immunostaining for Brachyury was detected in both the nuclei and cytoplasm of the cells (**Fig. 2*B***). When the strongly immunopositive cells (Fig. 2*B*) were counted, 80% of the Wnt-3a (50 ng/ml) -treated ES cells were found to be Brachyury-positive (Fig. 2*J*). The anterior PS marker Goosecoid was detected mainly in the cell nuclei after treatment with 50 ng/ml Wnt-3a (Fig. 2*E*), and 61% of the cells were Goosecoid-positive (Fig. 2*K*). However, treatment with either activin A or a low concentration of Wnt-3a (5 ng/ml) resulted in very weak expression of Brachyury (Fig. 2*A, C, J*). Similarly, the number of Goosecoid-positive cells was small in ES cells treated with a low concentration of Wnt-3a (5 ng/ml) or activin A (50 ng/ml) (Fig. 2*D, F, K*). In the absence of Wnt or activin A, the ES cells did not attach to the laminin-coated dish and did not grow at all (data not shown). These data further confirmed that Wnt efficiently induces PS differentiation, including the anterior PS, in mouse ES cells.

In addition to the strong expression of *brachyury* and *goosecoid* in the PS and the axial mesoderm *in vivo* (19–22), E-cadherin also expresses in the PS during development, although the expression level decreases during the mesoderm differentiation (27). In our system, E-cadherin was detected in all the cells treated with 50 ng/ml Wnt-3a on day 3 (Fig. 2*L*), and 30–40% of cells became E-cadherin-negative on day 5 (Fig. 2*M*). Concurrently with this change, an early differentiation marker of definitive endoderm, Sox17, started to express on day 5 (Fig. 2*N, O*). These data further support our finding that Wnt-3a induces within 3 days a significant number of cells that correspond to the tissue culture analogs of the PS in developing mouse embryos, which subsequently differentiate into the mesoderm and endoderm.

To evaluate the developmental potential of Wnt-3a-induced PS-like cells, mouse ES cells treated with 50 ng/ml of Wnt-3a for 3 days were allowed to form EBs and to differentiate spontaneously in serum-containing medium for 12 days. Control ES cells differentiated into a significant number of β3-tubulin-positive axon-extending neurons (**Fig. 3*A***), demonstrating the ability to differentiate into the ectodermal lineages. In contrast, EBs differentiated from Wnt-3a-treated ES cells contained few β3-tubulin-positive cells (Fig. 3*B*). In the quantification of this immunofluorescence analysis, β3-tubulin-positive cells were detected in 63% of the control EBs, whereas the number of β3-tubulin-positive EBs was significantly decreased to 27% after Wnt-3a-treatment (Fig. 3*C*). These results indicate that Wnt-3a-treated ES cells have a markedly reduced ability to differentiate into non-PS ectodermal lineages.

Next, we analyzed the signal specificity of PS differentiation induced by Wnt-3a. Since the initial 2 days of culture at any concentration of Wnt-3a did not induce

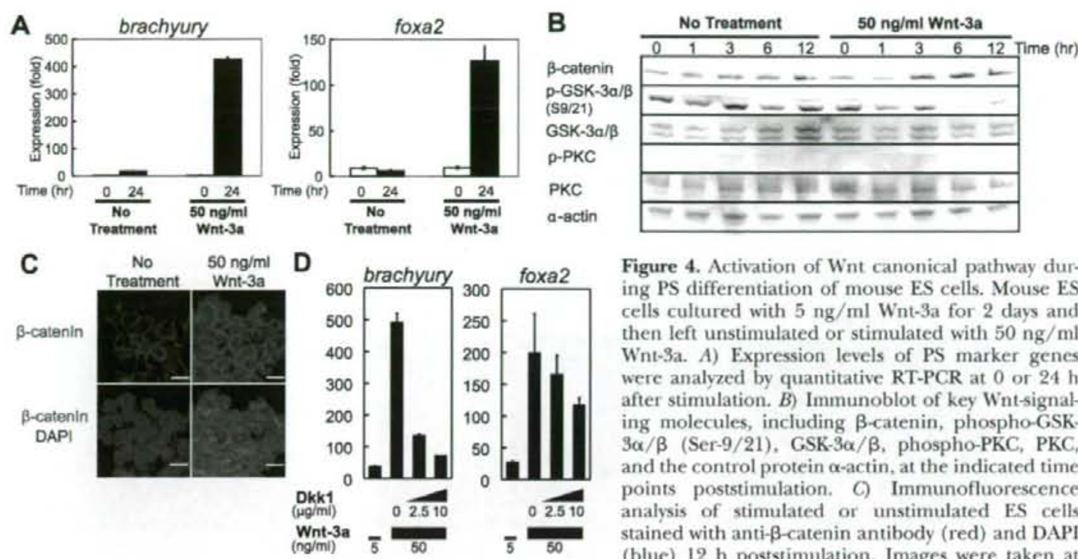


Figure 4. Activation of Wnt canonical pathway during PS differentiation of mouse ES cells. Mouse ES cells cultured with 5 ng/ml Wnt-3a for 2 days and then left unstimulated or stimulated with 50 ng/ml Wnt-3a. *A*) Expression levels of PS marker genes were analyzed by quantitative RT-PCR at 0 or 24 h after stimulation. *B*) Immunoblot of key Wnt-signaling molecules, including β-catenin, phospho-GSK-3α/β (Ser-9/21), GSK-3α/β, phospho-PKC, PKC, and the control protein α-actin, at the indicated time points poststimulation. *C*) Immunofluorescence analysis of stimulated or unstimulated ES cells stained with anti-β-catenin antibody (red) and DAPI (blue) 12 h poststimulation. Images were taken at the same exposure length. Scale bars = 20 μm. *D*) Specific inhibition of Wnt-3a-dependent PS differentiation by Dkk1. Expression levels of *brachyury* and *foxa2* were analyzed by quantitative RT-PCR 24 h after stimulation with 50 ng/ml Wnt-3a alone or with the combination of the indicated concentration of Dkk1.

the expression of PS markers (Fig. 1, and data not shown), we analyzed the activation of Wnt signaling pathways after 2 days of preculturing of the ES cells in the presence of 5 ng/ml of Wnt-3a. Treatment of the ES cells with 50 ng/ml Wnt-3a after the preculture period markedly increased the expression levels of the PS marker genes *brachyury* and *foxa2* within 24 h (**Fig. 4*A***). Under these conditions, phosphorylation of GSK-3α/β almost disappeared 6–12 h after Wnt-3a stimulation. Concomitantly, there was a significant increase in the level of β-catenin protein (Fig. 4*B*), whereas the control cells retained the initial levels of β-catenin and GSK-3α/β phosphorylation. Immunofluorescence analysis clearly showed the accumulation of β-catenin protein in ES cells 12 h after Wnt-3a stimulation (Fig. 4*C*). We also examined the activation of PKC and c-Jun, which are implicated in the signal transduction of noncanonical Wnt signaling (28, 29). However, no significant activation of PKC or c-Jun was detected (Fig. 4*B*, and data not shown). Dkk1 has been used as an inhibitor of the Wnt canonical signaling pathway (30). Treatment of ES cells with Dkk1 strongly inhibited the expression of the *brachyury* and *foxa2* genes induced by exogenous Wnt-3a (Fig. 4*D*), indicating that PS differentiation of ES cells by Wnt-3a is dependent on Wnt canonical signaling.

Finally, to determine whether Wnt signaling could also induce PS differentiation in human ES cells in a chemically defined serum-free medium, we cultured human ES cells for 3 days in medium that was supplemented with 50 ng/ml Wnt-3a, and analyzed the specific marker genes for PS by quantitative RT-PCR (**Fig. 5*A*, *B***). For the differentiation of human ES cells, a commercially available hESF-dif medium for human ES cells was used. KSR and bFGF were not included in the differentiation medium. Although the effects of activin in human ES cells were superior to those in mouse ES cells, Wnt-3a induced a significantly higher expression of several PS-specific markers. In addition to these quantitative data, immunofluorescence analysis was performed using specific antibodies for PS markers. The result of this analysis showed that significant numbers of immunopositive cells expressing Brachyury, Goosecoid, and Foxa2 were observed in the flattened cells that grew out from the clump of ES cells (Fig. 5*C*–*E*). In most of these cells, Brachyury and Goosecoid were colocalized to the nucleus (data not shown). Semiquantitative analysis by counting immunopositive cells revealed that Wnt-3a-treatment induces quite efficient differentiation of human ES cells into PS-like cells (29–53%) as shown in Fig. 5*F*–*H*. On the basis of these data, we conclude that Wnt-3a is also an effective and specific inducer of PS differentiation in human ES cells when cultured in a chemically defined medium.

DISCUSSION

In this study, we demonstrate that mouse ES cells cultured in a serum-free chemically defined medium, ESF7, and treated with Wnt-3a produce a highly enriched culture of anterior and posterior PS. Recently, there have been reports that Wnt-3a can induce the differentiation of the posterior PS and mesoderm from ES cells (15, 31, 32). However, *in vitro* induction of the anterior streak or definitive endoderm has not been achieved with Wnt (12–15). In our unique culture system, robust expression of PS marker genes, including anterior PS markers, was readily observed in Wnt-3a-treated cells. Initially a whole PS and the mesoderm marker *brachyury* were induced, followed by expression

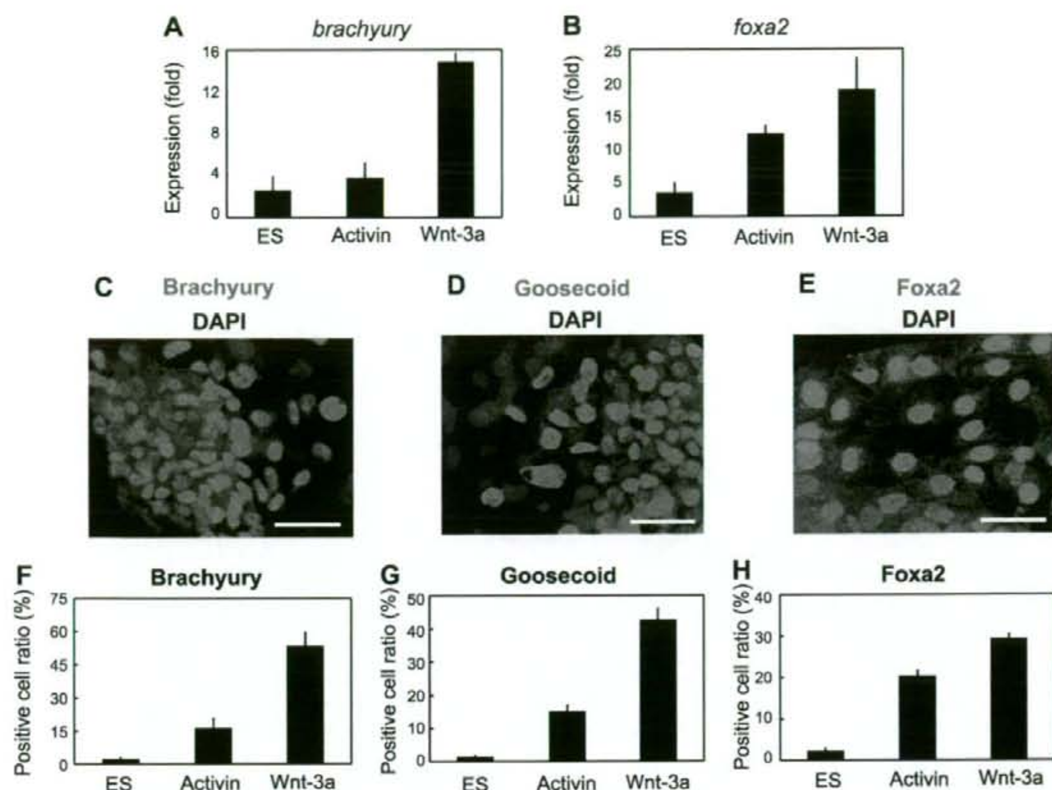


Figure 5. Directed differentiation of human ES cells into PS by Wnt treatment. *A, B*) Quantitative analysis of marker gene expression of human ES cells treated with 50 ng/ml of Wnt-3a or activin A for 3 days. Duplicate cultures were analyzed by quantitative RT-PCR. *C–E*) Immunofluorescent staining of Wnt-3a treated human ES cells. Cells treated withWnt-3a for 3 days were immunostained with antibodies against Brachyury (*C*), Goosecoid (*D*), and Foxa2 (*E*). Nuclei were stained with DAPI (blue). Scale bars = 50 μm. *F–H*) Quantitative analysis of the immunofluorescent staining in *C–E*. Immunopositive cells in Wnt-3a-treated cells were counted in 5 independent fields.

of the anterior PS markers *goosecoid* and *foxa2*. The endoderm marker *sox17* was induced within 6 days. In contrast, genes not expressed in the PS *in vivo*, such as the ectoderm marker *sox1* and the primitive endoderm marker *sox7*, were suppressed by Day 3. These results indicate that Wnt-3a induces directed differentiation into PS-like cells, including its anterior subdivision, which could be expected to further differentiate into both the mesoderm and definitive endoderm.

Immunofluorescence analysis revealed that a large proportion of the Wnt-3a-treated cells expressed both Brachyury and Goosecoid. Moreover, E-cadherin, which is expressed in the PS but not in the mesoderm, was detected in all the cells treated with Wnt-3a for 3 days. The expression of these markers in the Wnt-3a-treated Day 3 cells was consistent with the expression patterns in the anterior PS. Furthermore, Wnt-3a treatment significantly reduced the potential of ES cells to differentiate into non-PS lineages, such as neural ectodermal cells. These results support our conclusion that Wnt-3a treatment commits ES cells to the PS lineage in the chemically defined medium.

We also analyzed the Wnt signaling pathways during the anterior and posterior PS differentiation. In this study, we demonstrate that Wnt-3a specifically inhibits the phosphorylation of GSK-3α/β and induces a significant increase in the level of β-catenin protein in ES cells cultured in our chemically defined medium. Moreover, a Wnt canonical pathway-specific inhibitor, Dkk1, abrogated Wnt-dependent induction of *brachyury* and *foxa2* gene expression. A recent study suggested the importance of canonical signaling in the differentiation of mesoderm from ES cells using complex EB formation, although in that study, Wnt activity alone was not sufficient to induce the mesoderm-specific gene expression (32). Our data clearly demonstrate for the first time that Wnt-3a is sufficient for the induction of both the anterior and posterior PS, from which the definitive endoderm and mesoderm are produced, and that Wnt canonical signaling is essential for this differentiation.

Previous studies have shown that high doses of activin promote the differentiation of endoderm from ES cells (12, 15). However, under our culture conditions, cultivation with activin alone had little effect on the expres-

sion of *sox17*, which suggests that the ability of activin to induce the PS and endoderm is quite limited in our system. This discrepancy may reflect differences in the induction methods used. Some of the previous studies used EB formation as the initial step in the differentiation into PS (13, 15). EB formation causes changes in the cellular microenvironment, such as high concentrations of locally produced cytokines and various interactions between different cell types within the EB structures (33–36). These complex conditions may enable unexpected crosstalk between different signaling pathways. In addition, serum (14) and serum replacement supplements of undisclosed compositions (12) were used to induce mesendodermal differentiation from ES cells. In contrast to these previous studies, we used a completely defined medium that does not contain any inducing factors, with the exception of minimal supplements, such as insulin and transferrin. Our results suggest that unknown factors derived from serum or serum replacement or complex conditions, such as cell-cell interactions in the EB, are required for endoderm induction by activin.

In this study, we demonstrate that Wnt treatment also induces PS differentiation in human ES cells. These results suggest that Wnt is a common inducing factor for both the anterior and posterior PS in mammalian ES cells. Our method can be an innovative approach to obtain enriched cultures of PS cells in a serum-free chemically defined medium. Our method does not entail procedures that might affect reproducibility, such as EB formation, coculturing with other cells, or gene targeting. Although the potential of these induced cells for the generation of terminally differentiated PS derivatives is currently under investigation, further studies are likely to show that this method is an essential step in generating therapeutically useful cells derived from the PS, such as cardiomyocytes, vascular endothelial cells, hepatocytes, and pancreatic β-cells.

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REFERENCES

1. Tam, P. P., and Behringer, R. R. (1997) Mouse gastrulation: the formation of a mammalian body plan. *Mech. Dev.* **68,** 3–25
2. Loebel, D. A., Watson, C. M., De Young, R. A., and Tam, P. P. (2003) Lineage choice and differentiation in mouse embryos and embryonic stem cells. *Dev. Biol.* **264,** 1–14
3. Kinder, S. J., Tsang, T. E., Wakamiya, M., Sasaki, H., Behringer, R. R., Nagy, A., and Tam, P. P. (2001) The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. *Development* **128,** 3623–3634
4. Lawson, K. A., Meneses, J. J., and Pedersen, R. A. (1991) Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113,** 891–911
5. Wells, J. M., and Melton, D. A. (1999) Vertebrate endoderm development. *Annu. Rev. Cell Dev. Biol.* **15,** 393–410
6. Yoshikawa, Y., Fujimori, T., McMahon, A. P., and Takada, S. (1997) Evidence that absence of Wnt-3a signaling promotes neuralization instead of paraxial mesoderm development in the mouse. *Dev. Biol.* **183,** 234–242
7. Kelly, O. G., Pinson, K. I., and Skarnes, W. C. (2004) The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* **131,** 2803–2815
8. Lickert, H., Kutsch, S., Kanzler, B., Tamai, Y., Taketo, M. M., and Kemler, R. (2002) Formation of multiple hearts in mice following deletion of beta-catenin in the embryonic endoderm. *Dev. Cell* **3,** 171–181
9. Brennan, J., Lu, C. C., Norris, D. P., Rodriguez, T. A., Beddington, R. S., and Robertson, E. J. (2001) Nodal signalling in the epiblast patterns the early mouse embryo. *Nature* **411,** 965–969
10. Liu, Y., Festing, M., Thomon, J. C., Hester, M., Rankin, S., El-Hodiri, H. M., Zorn, A. M., and Weinstein, M. (2004) Smad2 and Smad3 coordinately regulate craniofacial and endodermal development. *Dev. Biol.* **270,** 411–426
11. Dunn, N. R., Vincent, S. D., Oxburgh, L., Robertson, E. J., and Bikoff, E. K. (2004) Combinatorial activities of Smad2 and Smad3 regulate mesoderm formation and patterning in the mouse embryo. *Development* **131,** 1717–1728
12. Yasunaga, M., Tada, S., Nishikawa, S. T., Nakano, Y., Okada, M., Jakt, L. M., Nishikawa, S., Chiba, T., Era, T., and Nishikawa, S. I. (2005) Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat. Biotechnol.* **23,** 1542–1550
13. Kubo, A., Shinozaki, K., Shannon, J. M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H. J., and Keller, G. (2004) Development of definitive endoderm from embryonic stem cells in culture. *Development* **131,** 1651–1662
14. D'Amour, K. A., Agulnick, A. D., Eliazer, S., Kelly, O. G., Kroon, E., and Baetge, E. E. (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat. Biotechnol.* **23,** 1534–1541
15. Gadue, P., Huber, T. L., Paddison, P. J., and Keller, G. M. (2006) Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 16806–16811
16. Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A. H. (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* **10,** 55–63
17. Furue, M., Okamoto, T., Hayashi, Y., Okochi, H., Fujimoto, M., Myoishi, Y., Abe, T., Ohnuma, K., Sato, G. H., Asashima, M., and Sato, J. D. (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
18. 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
19. Wilkinson, D. G., Bhatt, S., and Herrmann, B. G. (1990) Expression pattern of the mouse T gene and its role in mesoderm formation. *Nature* **343,** 657–659
20. Inman, K. E., and Downs, K. M. (2006) Localization of Brachyury (T) in embryonic and extraembryonic tissues during mouse gastrulation. *Gene Expr. Patterns* **6,** 783–793
21. Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B., and Robertson, E. J. (1994) A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120,** 1919–1928
22. Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R., and Bradley, A. (1999) Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* **22,** 361–365
23. Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M., and Darnell, J. E. Jr. (1994) The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78,** 575–588
24. Monaghan, A. P., Kaestner, K. H., Grau, E., and Schutz, G. (1993) Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119,** 567–578

25. Kaestner, K. H., Hiemisch, H., Luckow, B., and Schutz, G. (1994) The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. *Genomics* **20,** 377–385
26. Dush, M. K., and Martin, G. R. (1992) Analysis of mouse Evx genes: Evx-1 displays graded expression in the primitive streak. *Dev. Biol.* **151,** 273–287
27. Stemmler, M. P., Hecht, A., Kinzel, B., and Kemler, R. (2003) Analysis of regulatory elements of E-cadherin with reporter gene constructs in transgenic mouse embryos. *Dev. Dyn.* **227,** 238–245
28. Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S., and Nishida, E. (2002) JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep.* **3,** 69–75
29. Kuhl, M., Sheldahl, L. C., Malbon, C. C., and Moon, R. T. (2000) Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in *Xenopus*. *J. Biol. Chem.* **275,** 12701–12711
30. Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S. A. (2001) Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat. Cell Biol.* **3,** 683–686
31. Doble, B. W., Patel, S., Wood, G. A., Kockeritz, L. K., and Woodgett, J. R. (2007) Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev. Cell* **12,** 957–971
32. Lindsley, R. C., Gill, J. G., Kyba, M., Murphy, T. L., and Murphy, K. M. (2006) Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* **133,** 3787–3796
33. Liu, Y., Asakura, M., Inoue, H., Nakamura, T., Sano, M., Niu, Z., Chen, M., Schwartz, R. J., and Schneider, M. D. (2007) Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 3859–3864
34. Vittet, D., Buchou, T., Schweitzer, A., Dejana, E., and Huber, P. (1997) Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6273–6278
35. Aumailley, M., Pesch, M., Tunggal, L., Gaill, F., and Fassler, R. (2000) Altered synthesis of laminin 1 and absence of basement membrane component deposition in (beta)1 integrin-deficient embryoid bodies. *J. Cell Sci.* **113**(Pt. 2), 259–268
36. Bader, A., Gruss, A., Hollrigl, A., Al-Dubai, H., Capetanaki, Y., and Weitzer, G. (2001) Paracrine promotion of cardiomyogenesis in embryoid bodies by LIF modulated endoderm. *Differentiation* **68,** 31–43

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日本におけるヒト ES、iPS 細胞研究標準化：その1

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要旨　　1998年にヒト胚性幹（ES）細胞が樹立され、発生過程におけるメカニズムの解明や再生医療応用などの目的で、国際的にはさかんに研究が進められている。一方、日本においては種々の問題から、多くの研究者がヒト ES 細胞を使用して研究を進められているという現状ではない。2007年にヒト人工多能性幹（iPS）細胞が開発され、日本においてもにわかにヒト幹細胞の培養に携わる研究者が増加してきた。ヒト iPS 細胞は、ヒト ES 細胞様の形質を有しており、ES 細胞用のプロトコールのほとんどを iPS 細胞に応用することが可能である。この総説では、ヒト ES についての基本的な培養について紹介する。

キーワード：　ヒト ES 細胞、ヒト iPS 細胞、フィーダー細胞

序　　文

1998年 Thomson らにより[1]、ヒト ES 細胞が樹立されて10年の月日が過ぎようとしている。当時、Thomson 教授からヒト ES 細胞の供与をうけた多くの研究者がその培養ができず、たびたび Thomson 教授に再送を求めるため、ジャクソン研究所でヒト ES 細胞のトレーニングが始まったと聞く。この話は、ヒト ES 細胞の培養が従来の細胞培養とはかなり異なっていることを意味するのではないだろうか。現在、ヒトES 細胞は、国際的には発生過程におけるメカニズムの解明や再生医療研究などにさかんに使用されている。一方、日本においては2004年に中辻らのグループにより[2,3]ヒト ES 細胞株が樹立されたが、種々の問題から多くの研究者がヒト ES 細胞を使用して研究を進めているという現状ではない。2006年に山中らのグループにより、マウス人工多能性幹（iPS）細胞の開発が発表された[4]。多くの研究者達はヒト iPS 細胞の開発までにはまだまだ時間がかかるだろうと予想していた。しかし、その予想を大きく裏切って、2007年にヒト iPS 細胞の作成が発表された[5]。その後は次々と世界中から新しいヒト iPS 細胞作成の報告されている[6-9]。日本国内においても、各研究室で新規の iPS 細胞の作製が行われ、当 JCRB 細胞バンク（厚生労働省研究資源事業）に新規のヒト iPS 細胞が寄託されて、現在、分譲の準備を行っている。ヒト ES 細胞は株間の差が大きいことが知られている。一つの研究室で樹立された細胞であっても、株間によって増殖速度や分化傾向も異なり[10]、また、継代方法を変える必要がある場合

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も多い。英国シェフィール大学 Andrews 教授がリーダーとして推進している International human ES cell initiatives（ISCI）プロジェクトでは、日本を含めた世界11カ国の研究者らが共同で60株近くのヒトES細胞株の特徴を比較し、ヒトES細胞研究の標準化が進められている[11,12]。ヒトiPS細胞はヒトES細胞様の特徴を有しており、同じ細胞株から作製されたiPS細胞であっても、クローン間の差が認められる。ES細胞を用いた培養プロトコールのほとんどをiPS細胞に応用することが可能である。ヒトES細胞研究が一般的でなかった日本においてヒトiPS細胞を用いた研究を行うためには、ヒトES細胞を基準とした標準化研究を行う必要があるだろう。まず、ヒトES、iPS細胞ついて論文に記載されないような内容も含めて基礎的な培養方法について紹介する。

1．フィーダー細胞

ES細胞を未分化状態に保持するために、一般的にはマウス胎児由来初代培養線維芽細胞（mouse embryonic fibroblast: MEF）がフィーダー細胞として用いられている[13]。線維芽細胞といっても純粋な線維芽細胞ではなく、実際には多くの細胞が混ざっている。マウスの系統は、各種細胞の樹立者が使用したものを使うのが望ましい。MEFはマイトマイシンCあるいはγ線照射により有糸分裂を不活性化して使用する。最近は、各社からマイトマイシンあるいはγ線照射処理済みのMEFが供給されている（表1）。各自で初代培養を行ってMEFを作成すると安価なように思えるが、ES細胞の未分化性を維持できるかどうかなどのチェックを含めると、ロット管理にかなり手間がかかる。MEFではなく、樹立されたSTO細胞株を使用する場合もある[14]。STO細胞（ATCC, CRL-1503）は、Dr. Alan Bernsteinにより分離されたSIMマウス線維芽細胞のチオグアニンおよびウアバイン抵抗性亜系で、奇形癌腫（EC）やマウスES細胞のフィーダーとして用いられている。STO細胞は容易に細胞を増やすことが可能であり、ほぼMEFと同様に問題なく培養できる場合もあるが、ES細胞との相性の問題があったり、4日間以上の培養には耐えられないなど、難しい点もある。しかし、相性よく培養できる場合には、取り扱いが簡単で安価である。ネオマイシン抵抗性（neor）発現ベクターおよびLIF発現ベクターを安定的に組み込んだSTO細胞（SNL細胞、あるいはSNL 76/7 STO細胞、ECACC 07032801）は、山中らのグループによるヒトiPS細胞培養に使用されている。また、動物由来細胞を使用せず、ヒト化培養条件を目指して様々なヒト組織由来細胞が試みられている[15,16]。

2．培　養　液

Thomsonらにより樹立された当時はDulbecco's

表1　JCRBにおいて使用しているフィーダー細胞の種類

細　胞　名	処　　理	入手先	カタログ番号	細胞数 /25 cm^2
Primary mouse embryo fibroblast				
Strain CF-1	マイトマイシン処理済	ミリポア	PMEF-CF*	2.4×10^5
Hygro Resistant Strain C57/BL6	マイトマイシン処理済	ミリポア	PMEF-HL*	2×10^5
ICR	マイトマイシン処理済	リプロセル	RCHEFC003*	2×10^5
STO 細胞		ATCC	CRL-1503	
SNL 76/7 STO 細胞		ECACC	07032801	

* のあるものは、当バンクで使用可能であることを確認済み。

modified Eagle's medium、あるいはDMEMとF12を1:1に混合したDM/F12、と20%牛血清が使用されていた[13]。しかし、血清成分にはヒトES細胞の分化因子も含まれていることから改良され、近年は、線維芽細胞増殖因子（FGF-2）とKnockOut Serum Replacement™（KSR, Invitrogen）とKnockOut DMEM（Invitrogen）が使用されている[17]。DM/F12、あるいはKnockOut DM/F12（Invitrogen）も基礎培地として使用されている（表1）。KSRは、serum-freeとされているが、動物由来成分を含み、ロット差があるためにロットチェックが必要となる。また、解凍して2週間以上たったものは品質が保証されていない。HEPESを含まない培地を使用することが多い。HEPESはもともと一般的な培養細胞に対して毒性があり、ロット差も多いことが知られている。一方、HEPESを使用しない場合には緩衝作用が弱くなり、pHの変化も大きくなる。幹細胞は、弱酸性には強いがアルカリ性には弱いので、注意が必要である[18]。

3. 細胞分散法

ヒトES、iPS細胞の培養において細胞分散法がもっとも難しいと言えるだろう。シングルセルにしてしまうと、ほとんどの細胞がアポトーシスにより生存できないため、コロニーを50～100個ぐらいの細胞集団にして継代を行う[1,19]。継代時の細胞分散は、機械的方法と酵素による方法に大別される[13]。酵素による方法は、均一に細胞分散でき、簡便である。一方、分散方法によっては分化しなくなる、あるいは、染色体異常となるなど、細胞分散方法による様々な現象がヒトES細胞研究者の間では知られている。特に、酵素処理によってシングルセルが多くでてしまうような操作により、染

表2　ヒトES用培地[25,26]

最終濃度	ストック	GIBCO番号
80% Knockout DMEM (あるいは DMEM/F12)*		10829-018 (Sigma D6421)
20% GIBCO Knockout SR		10828-028
1% non-essential amino acid soltuion	100 x MEM non-essential amino acid soltuion	11140-035
1 mM L-glutamine	0.146 g in 10 ml PBS	21-51-016
0.1 mM β-mercaptoethanol	14.3 M β-mercaptoethanol	Sigma M-7154
4 mg/ml human bFGF**	2 μg/ml in PBS with 0.1% BSA	13256-029

【手順】

1. L-glutamine / β-mercaptoethanol 液の作成
   10 ml の CMF-PBS に、0.146 g の L-glutamine を 15 ml チューブに入れる。
   7 μl の β-mercaptoethanol を入れてよく混ぜる。
2. フィルターユニットに、以下を入れる。

Knockout DMEM	160 ml
GIBCO Knockout SR	40 ml
L-glutamine / β-mercaptoethanol 液	2 ml
100 x non-essential amino acid solution	2 ml
human bFGF	400 μl

3. フィルターする。
4. 4 ℃にて保存し、1 週間以内に使用する。

*：JCRB 細胞バンクでは HEPES を含まない GIBCO11965、11765 を混ぜて使用している。
**：研究室によって使用濃度は少しずつ異なっているので、実際に培養を開始する際には、その細胞に添付されているプロトコールを参照のこと。

表３．JCRB におけるヒト ES、iPS 細胞の継代の手順

	手　順	時　間
準備	25 cm^2 フラスコ[1]に0.1%ゼラチン溶液を 2 ml ずつ入れる[2]。	
	37°Cインキュベーターに静置。	30 min
	MEF 用培地を作成[3]。	
	ゼラチン液を吸引。PBS にて洗浄。	
	各フラスコに MEF 用培地を 4 ml ずつ入れる。	
	15 ml チューブに MEF 培地を 9 ml 入れる。	
	N_2 に入れたまま MEF をクリーンベンチ近くに持ってくる。	
	バイアルの蓋をクリーンベンチ内で開けてバイアルの N^2 を抜く。	
	37°Cウォーターバスに入れて溶解。半分以上凍った状態でクリーンベンチ内へ移動する。MEF 培地でピペッティングしながら溶解。MEF 浮遊液を 15 ml チューブに入れる。	できるだけ短い時間で行う。
	1000 rpm 遠心	2 min
	新しい MEF 用培地に MEF を浮遊させる。	
	MEF 細胞浮遊液を 1 ml ずつ各フラスコに入れる。	
	MEF を CO_2 インキュベーターに入れて、培養。	24 h
	MEF 用培地からES用培地（FGF-2 なし）に交換し、培養[4]。	24 h
継代	ヒト ES、iPS 細胞を培養しているフラスコの培地を吸引。	
	1 unit/ml Dispase[5]（Roche/ 解凍後 3 日以内に使用）を 1 ml 入れる。	
	37°C・CO_2 インキュベーターに入れてインキュベーション。	3~10 min[6]
	Dispase を吸引。	
	hES 培地 10 ml を入れて、10 ml ピペットをつけたピペットエイド（強にする）で培地を吹きかけるようにしてコロニーをはがす（できるだけ回数を少なくする。2 回程度のピペッティングでコロニーがはがれないような場合は、セルスクレーパーを使用してコロニーをはがす）。	
	顕微鏡でコロニーの分散状態を確認する。	
	15 ml チューブに細胞浮遊液を入れて、300 rpm にて遠心（大きいコロニーのみを回収する）。	2 min
	新しい hES 培地を入れて細胞浮遊液とする（ピペッティングはしない）。	
	MEF の培地を吸引。	
	各フラスコに細胞浮遊液を入れる（Slit の割合は株による）	
	顕微鏡でコロニーの分散状態を確認。	
	CO_2 インキュベーターに入れて培養。	24 h
培地交換	接着率が悪い株の場合、翌日の培地交換は行わない場合もある	(24 h)
	培地交換に必要な hES 培地をチューブに分取し、FGF-2 を入れる。	
	37°Cウォーターバスで培地を温める。	5 min
	細胞の状態を顕微鏡でチェック。	
	温めた培地をクリーンベンチに持ってくる。	
	フラスコの培地を吸引。	
	温めた培地を入れる。	
	細胞の状態をチェック[8]。	
	CO_2 インキュベーターに入れて、培養。	
	基本的に毎日培地交換を行う[7]。	

① メーカーによって細胞の生着率や継代時のディスパーゼの処理時間なども変わってくる。当バンクではコーニングを使用している。
② 成育医療センター樹立 iPS 細胞は、C57/BL6 マウスの MEF を使用して樹立されている。市販のものでは、Hygro Resistant Strain C57/BL6（ミリポア）が使用可能であることを確認している。MEF バイアル 1 本を30枚の 25 cm^2 フラスコに播種している。ただし、ロット差があるため、新しいロットの際には、密度を変えて播種してチェックする必要がある。
③ high glucose, L-gluthaminem、15%牛胎児血清（ES グレード）含有 DMEM
④ MEF は播種してから24時間後では十分に広がっていないため、2 日後以降に使用する方が望ましい。継代する前に、事前にヒト ES 用培地に交換をしておき、MEF をヒト ES 培地になじませておくとよい。
⑤ Dispase の活性は解凍後 3 日以後は急激に低下する。細胞分散の処理時間を一定にするためには、用事解凍して使用するのが望ましい。また、提示されている酵素活性が同様であっても、会社によって微妙にその活性は異なる。当セルバンクでは Roch・Dispase 1 unit/ml で使用できることは確認している。
⑥ Dispase に対する感受性は株によって異なる。新しいロットのディスパーゼあるいは新しい細胞株の場合には、まず、3 分37°Cで処理して顕微鏡でコロニーの状態を確認する。ES、iPS 細胞のコロニーのエッジが光って、少しだけ丸くカールしていたら、すぐにディスパーゼを除く。コロニーが巻きあがるようにカールしている場合には処理時間が長すぎる。その場合、簡単に揺らしただけでコロニーがはがれてしまう可能性があるため、ディスパーゼは吸引せずに、培地を加えて遠心してディスパーゼを除く。2～3 回培地で洗った方が生着率が良い。
⑦ 培地の調整でよく間違えるのが 2-ME の濃度である。2-ME の濃度が高い場合、培地交換してすぐに細胞が死んでいく。
⑧ 平日は毎日培地交換を行うが、週末は土曜日か日曜日のどちらか 1 回のみにしている。ただし、その場合、コンフルエントでない状態にしておく必要がある。