CELL BIOLOGY. For the article “Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium,” by Miho K. Furue, Jie Na, Jamie P. Jackson, Tetsuji Okamoto, Mark Jones, Duncan Baker, Ryu-Ichiro Hata, Harry D. Moore, J. Denry Sato, and Peter W. Andrews, which appeared in issue 36, September 9, 2008, of Proc Natl Acad Sci USA (105:13409–13414; first published August 25, 2008; 10.1073/pnas.0806136105), the authors note that in Fig. 7D, Q-PCR data were inadvertently duplicated. This error does not affect the conclusions of the article. The corrected figure and its legend appear below.

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.”

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BIOPHYSICS. For the article “Single-molecule studies of group II intron ribozymes,” by Miriam Steiner, Krishanthi S. Karunati-laka, Roland K. O. Sigel, and David Rueda, which appeared in issue 37, September 16, 2008, of Proc Natl Acad Sci USA (105:13853–13858; first published September 4, 2008; 10.1073/pnas.0804034105), the authors note that Miriam Steiner should have been included among those credited with designing the research. The corrected author contributions footnote appears below.

Author contributions: M.S., R.K.O.S., and D.R. designed research; M.S. and K.S.K. performed research; M.S., R.K.O.S., and D.R. analyzed data; and M.S., R.K.O.S., and D.R. wrote the paper.

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CORRECTIONS
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

Hum. 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. L4) (26) and Shefi (Fig. 1B) (8). These cells were harvested using collagenase, or trypsin/EDTA, respectively, as previously described, from cultures on MEFs, and transferred to type 1...
collagen-coated flasks in ESF7 medium. However, the cells died after one day. On further study we found that Heps 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. S1A) and Shef1 cells (Fig. S1A) 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. 2A). High SSEA-3 expression and low SSEA-1 expression were seen in all cultures, irrespective of the FGF-2 concentration (Fig. 2B). 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. 2D and E). These cells also retained expression of SSEA-3 with low expression of SSEA-1 (Fig. 2F). Finally, we tested the combined...
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. 3A). Further, expression of cyclin D1 was also induced by heparin, confirming a potential effect on cell cycle regulation (Fig. 3B). 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 \( \mu \)g/cm\(^2\)), fibronectin (5 \( \mu \)g/cm\(^2\)), laminin (5 \( \mu \)g/cm\(^2\)), or gelatin (10 \( \mu \)g/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. 4E), or on these ECM components in KSR-based medium (Fig. 4F), 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. 7A). The growth rates of these HESCs after five passages was similar to those at passage 1 (Fig. 7B). The cells retained a normal karyotype (Fig. S2A). They also retained expression of SSEA-3, and OCT3/4, NANOG, SOX-2, and REX-1 (Fig. 7C, Fig. S2B 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. 7D).

**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 conditions, hESCs maintained their ability to support the culture of other HESC lines, Shef1, Shef4, Shef5, and H7 for a period of more than 25 passages.

**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.

**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 \( \mu \)m.)

**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.
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. 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 $1 \times 10^5$ cells per well; mean and SD of three experiments. Cell numbers were counted every 2 days.

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) The growth of HUES-1 (passage 10) and Shef1 cells (passage 5) 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) those during in vitro differentiation (EB). The name of the gene of differentiation are noted in each bar graph. Expression levels were all 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 signals [e.g. ref. 32].

In the absence of feeder cells, 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 embryonic fibroblast feeder cells as described (8, 15, 26). In addition, a subline of H7, H7.6 (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, NJ). Type I collagen was from Nitta Gelatin, Co. (Osaka, Japan). The basal ES 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 immunohistochemistry, 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 markers of 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 Taq ReadyMix (Bioblock Scientific, Strasbourg) or the PowerSyper™ SYBR Green SuperMix (Thermo Fisher Scientific). Primer pairs 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 phosphatase inhibitors Complete and phosphoSTOP (Roche). Antibodies used were anti-phospho-FGF Receptor (AF2385, R&D systems), mouse monclonal anti-human cyclinD1 DCS-6 (DAKO), and mouse monclonal anti-actin (Abcam).

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