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A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells

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Abstract

Traditionally, undifferentiated human embryonic stem cells (hESCs) are maintained on mouse embryonic fibroblast (MEF) cells or on matrigel with an MEF-conditioned medium (CM), which hampers the clinical applications of hESCs due to the contamination by animal pathogens. Here we report a novel chemical-defined medium using DMEM/F12 supplemented with N2, B27, and basic fibroblast growth factor (bFGF) [termed NBF]. This medium can support prolonged self-renewal of hESCs. hESCs cultured in NBF maintain an undifferentiated state and normal karyotype, are able to form embryoid bodies in vitro, and differentiate into three germ layers and extraembryonic cells. Furthermore, we find that hESCs cultured in NBF possess a low apoptosis rate and a high proliferation rate compared with those cultured in MEF-CM. Our findings provide a novel, simplified chemical-defined culture medium suitable for further therapeutic applications and developmental studies of hESCs.

Keywords: Human embryonic stem cells; Self-renewal; Pluripotency; N2B27; bFGF; Chemical-defined medium; Long-term culture; Fibronectin

The first human embryonic stem cell (hESC) line, established from the inner cell mass (ICM) of the blastocyst by Thomson in 1998 [1], proliferates infinitely in culture and maintains pluripotency for multi-lineage differentiation, thus providing an unlimited source of cells for clinical medicine. Traditionally, undifferentiated hESCs are maintained on mouse embryonic fibroblast (MEF) cells or on matrigel in MEF-conditioned medium (CM) [2], which limits the clinical applications due to the potential contamination of pathogens from the animal materials.

Several animal-free culture mediums for hESCs with human feeder layers have already been established [3–8]. However, they require complex procedures, which complicate self-renewal studies and hinder the efficient application of hESCs in clinical medicine. A number of groups have reported their feeder-free culture mediums for hESCs in which FGF and TGFβ family growth factors were adapted and subsequently identified as important self-renewal factors in hESC propagation [9–13]. However, the involvement of knockout serum replacement (KSR) in these culture mediums for hESCs makes them unsuitable for transplantation therapy, because the animal-derived KSR is contaminated with the nonhuman sialic acid Neu5Gc, which elicits an immunoresponse in humans [14]. As a result, a chemical-defined and animal-free medium appropriate for hESC culture is required for clinical applications.

In this study, we used BITS (10 μg/ml BSA and ITS), B27, and N2B27, respectively, to substitute for KSR in the basal DMEM/F12 culture medium. Different

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combinations of several candidate self-renewal factors, such as bFGF, IGF, noggin, PDGF, EGF, Nodal, Wnt3a, TGFβ, and Activin A, were tested and titrated at a variety of concentrations. We found that DMEM/F12 supplemented with N2, B27, and 100 ng/ml basic fibroblast growth factor [termed NBF] is the best combination to maintain the hESCs in an undifferentiated state during long-term culture. The expanded hESCs in NBF culture medium are able to form embryoid bodies in vitro, and differentiate into three germ layers and extraembryonic cells. Furthermore, we found that hESCs cultured in NBF possess low apoptosis rates and high proliferation rates. Our findings provide a novel, simplified, chemical-defined culture medium suitable for further therapeutic applications and developmental studies of hESCs.

Materials and methods

hESC culture and Karyotyping. hESC lines H1 and H9 were obtained from WiCell research institute (Madison, WI). The passage number of H1 cells and H9 cells used in this paper ranged from 47 to 75 and from 42 to 65, respectively. Normal karyotype was confirmed in both cell lines. For Karyotyping, hESCs grown in log phase were harvested and karyotyped by using Giemsa stain (Genzyme). Twenty cells were scored in each case. hESCs were initially maintained on irradiated MEFs in hESC culture medium consisting of 80% DMEM/F12 (Hyclone), 20% knockout serum replacement (KSR), 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (bFGF) (all from Invitrogen). Cells were passaged with 1 mg/ml collagenase IV (Invitrogen) every 5–7 days. For feeder-free culture, cells were transferred to reduced Matrigel (BD Biosciences). Three basal culture mediums with combinations of several growth factors, complemented with DMEM/F12, were examined. The basal culture mediums were: BITS, which includes 10 μg/ml bovine serum albumin (BSA), and insulin transferrin selenium A (ITS), B27, and N2B27. The growth factors included bFGF, IGF, noggin, PDGF, EGF, Nodal, Wnt3a, TGFβ, and Activin A (all from R&D system). The growth factors were added to each culture medium when the culture medium was exchanged. The NBF culture medium contains DMEM/F12 (Hyclone), N-2 supplement (Invitrogen/Gibco) and B-27 supplement (Invitrogen/Gibco), 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM beta-mercaptoethanol, and 100 ng/ml bFGF (all from Invitrogen). We also examined four kinds of human matrices and several matrix combinations to substitute for Matrigel, which included 50 μg/ml human fibronectin, 2 μg/ml human vitronectin, 100μg/ml human collagen IV, and 50 μg/ml human laminin (all from Sigma). Conditioned medium was prepared as described previously [1,15], which was used as a positive control in this study. For human embryoid body (hEB) formation, hESCs passaged with 1 mg/ml collagenase IV (Invitrogen) were cultured in an uncoated, 3.5-cm petri dish in the presence of Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% fetal bovine serum (FBS), 1 mM L-glutamine, 1% non-essential amino acids, 1% ITS, and 0.1 mM β-mercaptoethanol (all from Invitrogen). After 6 days of suspension culture, the embryoid bodies were formed, and the cells were transferred to a plate coated with 5 ng/μl Fibronectin (Sigma). The cells attached to the plate and were cultured for 6 days, then used for further analysis.

Flow cytometric analysis. Cells were dissociated with trypsin/EDTA and resuspended in PBS with 3% horse serum for 15 min at 4 °C. Then the cells were incubated for 30 min at 4 °C with the tumor rejection antigen (Tra)-1-60, Tra-1-81 antibodies, and stage-specific embryonic antigen (SSEA)-4 antibody (Chemicon) and then with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz). Finally, the cells were analyzed by means of a flow cytometer (MoFlo High-Performance Cell Sorter; Dako Cytomation). Flow cytometry data were analyzed with Summit software (Dako Cytomation).

Immunofluorescence. Cells were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min and then blocked in 3% horse serum in PBS at room temperature for 1 h. Cells were incubated with primary antibodies at 4 °C overnight. After 5 washes with PBS, FITC-, or TRITC-conjugated secondary antibody (Santa Cruz) was added and incubated at 37 °C for 1 h.

RT-PCR and quantitative RT-PCR. Total RNA was isolated from cells using TRIzol (Invitrogen) and reverse-transcribed using a reverse transcription system (Promega) according to the manufacturer's protocol. PCR amplification of different genes was performed using EXTaq polymerase (Takara), with a program of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 54–62 °C for 30 s, 72 °C for 30 s, and extension at 72 °C for 10 min. The primers used are shown in the supplemental information.

Quantitative RT-PCR analysis was performed on an ABI PRISM 7300 Sequence Detection System using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR consisted of 12.5 μl SYBR Green PCR Master Mix, 0.8 μl of 10 mM of forward and reverse primers, 10.4 μl water, and 0.5 μl template cDNA in a total volume of 25 μl. Cycling was performed using the default conditions of the ABI 7300 SDS Software 1.3.1:2 min at 50 °C, 10 min at 95 °C, followed by 40 rounds of 15 s at 95 °C, and 1 min at 60 °C. The relative expression of each gene was normalized against β-actin. The primers used for quantitative RT-PCR are shown in the supplemental information.

Annexin V analysis and TUNEL staining. To assess the extent of apoptosis after irradiation, cells were harvested and resuspended in binding buffer (10 mM Hepes, pH 7.4/140 mM NaCl/2.5 mM $CaCl_2$), and were stained with 5 μl of annexin V-FITC (Pharmingen) and 2.5 μg/ml propidium iodide. The cell suspension was incubated for 15 min at room temperature and analyzed by flow cytometry (MoFlo High-Performance Cell Sorter; Dako Cytomation). Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining was carried out using the DeathEnd Fluorometric TUNEL System kit (Promega) according to the manufacturer's instructions.

Growth analysis. hESCs were seeded in 24-well dishes coated with Matrigel at a density of 2×10^4 cells/cm^2 and the medium was changed daily. Cell number was assessed by 3-dimethylthiazol-2,5diphenyltetrazolium bromide (MTT) assay (Promega) according to the instructions of the manufacturer.

Statistical analysis. *t* Tests were performed with GraphPad Prism, version 4.00, for Windows (GraphPad Software, San Diego, CA). Statistical significance was assessed by comparing data from hESCs cultured in different mediums. The differences were considered statistically significant when the *P* value was less than 0.05.

Results

bFGF supports long-term culture of hESCs with N2B27 supplements

In order to develop a chemical-defined culture medium for hESCs, we tested several supplements and a series of growth factor combinations for their ability to support hESC self-renewal (Table 1). hESCs cultured on matrigel in mouse embryonic fibroblast-conditioned medium (MEF-CM) were used as positive controls (Fig. 1G) [2]. In most combinations, we found that 20–90% of hESCs began to differentiate within 4 passages (Fig. 1A–E). After 7 passages there were no undifferentiated colonies in these culture conditions. We further found that only bFGF supplemented with N2B27 supported hESC self-renewal for multiple passages. In these conditions, the self-renewal state of hESCs was indicated by TRA-1-60 expression (data not shown). hESCs in N2B27 supplemented medium

Table 1
Comparison of human embryonic stem cells cultured in different mediums supplemented with different combinations of factors

	Growth
BITS	+
BITS + bFGF(40 ng/ml)	+
BITS + bFGF(80 ng/ml)	++
BITS + bFGF(100 ng/ml)	++
BITS + bFGF(40 ng/ml) + IGF(50 ng/ml)	+
BITS + bFGF(40 ng/ml) + IGF(200 ng/ml)	+
BITS + bFGF(40 ng/ml) + noggin(500 ng/ml)	+
B27	+
B27 + PDGF(10 ng/ml)	+
B27 + bFGF(40 ng/ml)	++
B27 + bFGF(40 ng/ml) + IGF(50 ng/ml)	+
B27 + bFGF(40 ng/ml) + IGF(200 ng/ml)	++
B27 + bFGF(40 ng/ml) + IGF(200 ng/ml) + noggin(400 ng/ml)	++
B27 + bFGF(40 ng/ml) + IGF(200 ng/ml) + PDGF(10 ng/ml) + EGF(20 ng/ml)	++
B27 + bFGF(40 ng/ml) + IGF(200 ng/ml) + PDGF(10 ng/ml) + EGF(20 ng/ml) + noggin(500 ng/ml)	++
N2B27	+
N2B27 + PDGF(10 ng/ml)	+
N2B27 + noggin(500 ng/ml)	+
N2B27 + Activin A (10 ng/ml)	+
N2B27 + Activin A (50 ng/ml)	+
N2B27 + bFGF(20 ng/ml)	+++
N2B27 + bFGF(40 ng/ml)	+++
N2B27 + bFGF(100 ng/ml)	++++
N2B27 + bFGF(40 ng/ml) + noggin(200 ng/ml)	+++
N2B27 + bFGF(40 ng/ml) + Nodal(50 ng/ml)	++
N2B27 + bFGF(40 ng/ml) + Wnt3a(50 ng/ml)	++
N2B27 + bFGF(40 ng/ml) + TGFβ(1 ng/ml)	+
N2B27 + bFGF(40 ng/ml) + Activin A(10 ng/ml)	+
N2B27 + bFGF(40 ng/ml) + Activin A(50 ng/ml)	+
N2B27 + bFGF(40 ng/ml) + IGF(200 ng/ml) + PDGF(10 ng/ml)	++
N2B27 + bFGF(40 ng/ml) + IGF(200 ng/ml) + PDGF(10 ng/ml) + noggin(400 ng/ml)	++
N2B27 + bFGF(40 ng/ml) + IGF(200 ng/ml) + PDGF(10 ng/ml) + EGF(20 ng/ml)	++
N2B27 + bFGF(40 ng/ml) + IGF(200 ng/ml) + PDGF(10 ng/ml) + EGF(20 ng/ml) + noggin(500 ng/ml)	++
N2B27 + IGF(200 ng/ml) + PDGF(10 ng/ml) + EGF(20 ng/ml) + noggin(500 ng/ml)	+
N2B27 + IGF(200 ng/ml) + PDGF(10 ng/ml) + EGF(20 ng/ml)	+

In all cases, cell culture medium was supplemented with DMEM/F12, 1 mM L-glutamine, 1% non-essential amino acids, and 0.1 mM β-mercaptoethanol, and the culture plates were coated with matrigel. The proportion of undifferentiated human embryonic stem cells after ten days is indicated. BSA, bovine serum albumin; ITS, insulin transferrin selenium A; bFGF, basic-fibroblast growth factor; IGF, insulin like growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TGFβ, transforming growth factor β.

together with 40 ng/ml bFGF began to differentiate at passage 16, and hESCs were obviously differentiated at passage 24 (Fig. 1F). Since it has been reported that bFGF supported self-renewal better at high concentrations [11,16], we raised bFGF concentration to 100 ng/ml. We found that hESCs maintained self-renewal for more than 28 passages under this culture condition (Fig. 1H). During the culture period, the cells displayed typical hESC morphology as previously described, such as being tightly packed, and having a high nuclear/cytoplasmic ratio with defined colony borders [16].

hESCs cultured in NBF maintain the undifferentiated state

To further characterize the self-renewal state of hESCs maintained in DMEM/F12 supplemented with N2B27 and 100 ng/ml bFGF (NBF), we analyzed the expression of the hESC surface markers Tra-1-60, Tra-1-81, and SSEA-4 by flow cytometry. For H1 cells (25 passages in NBF), the proportion of Tra-1-60-, Tra-1-81-, and SSEA-4-positive cells was 93%, 96%, and 95%, respectively (Fig. 2A). H9 cells (19 passages in NBF) had a similar proportion of cells expressing stem cell markers (92% for Tra-1-60 and 89% for Tra-1-81) (data not shown). Similar results were obtained with hESCs cultured in the control MEF-CM medium, more than 90% of which expressed high levels of Tra-1-61, Tra-1-81, and SSEA-4 (data not shown). hESCs cultured in NBF medium were stainable with Oct4 and Tra-1-60 (Fig. 2B). RT-PCR confirmed the sustained expression of the hESC self-renewal markers Oct4, Nanog, and Sox2 (Fig. 2C). These results indicate hESCs maintain the undifferentiated state after long-term culture in NBF. As N2B27 is reported to induce neural differentiation [17], we further tested neural marker expression of hESCs cultured in NBF. The hESCs cultured in NBF did not express any neural markers,

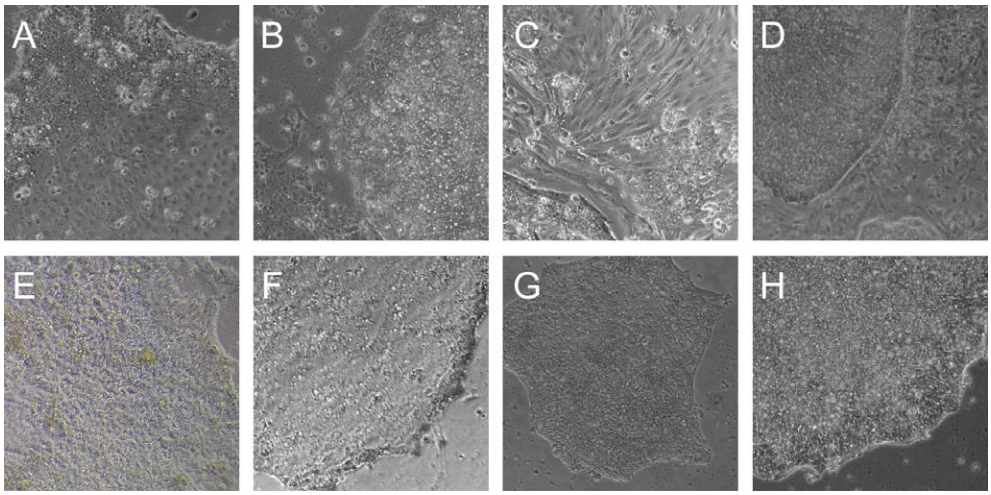

Fig. 1. The morphology of hESCs cultured in different mediums on matrigel. (A) Six days in BITS, (B) two passages (12 days) in BITS and bFGF (100 ng/ml), (C) six days in B27, (D) four passages (24 days) in B27 and bFGF (100 ng/ml), (E) three passages (18 days) in N2B27, (F) 16 passages (96 days) in N2B27 and bFGF (40 ng/ml), (G) six days in MEF-CM, and (H) 25 passages (5 months) in NBF. (Original magnification: 100×.)

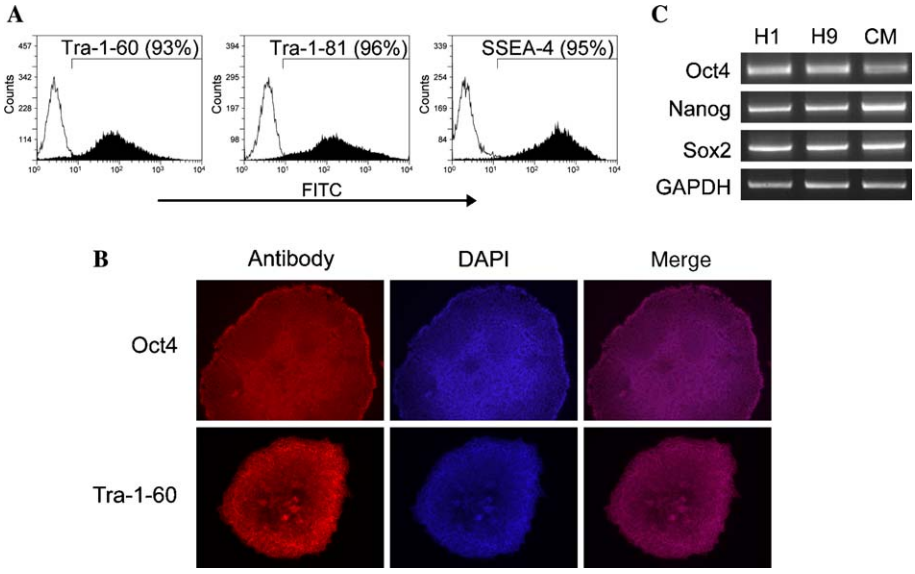

Fig. 2. hESCs maintained an undifferentiated state in NBF. (A) FACS analysis shows that most H1 cells cultured in NBF express Tra-1-60 (left), Tra-1-81 (middle), and SSEA-4 (right). The percentage of positive cells is listed in parentheses. (B) Immunofluorescence staining with antibodies to stem cell markers: Oct4 (top panels) and Tra-1-60 (bottom panels) show H9 (18 passages) cultured in NBF remained in the undifferentiated state. (Original magnification: 100×.) (C) RT-PCR analysis of control cells (CM) or H1 (18 passages) and H9 (13 passages) in NBF. The expression level of pluripotency markers (Oct4, Nanog, and Sox2) and a housekeeping gene (GAPDH) seems to exhibit little difference.

compared with the differentiated hESCs induced by RA (data not shown). These results indicated that hESCs can maintain self-renewal state after long-term culture in NBF medium.

hESCs in NBF display a low apoptosis rate and high proliferation rate while maintaining normal karyotype

Unfavorable culture condition often leads to higher hESC apoptosis rates, resulting in lower rates of proliferation [18–22]. We analyzed the apoptosis rate of H1 cells (19 passages in NBF) using terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining. Compared with hESCs cultured in MEF-CM, there was a lower ratio of apoptotic cells in the NBF medium (Fig. 3A). For H9 cells (17 passages in NBF), the proportion of apoptotic cells in N2B27 medium (9.47%) was also lower than that in MEF-CM (26.25%), as detected by Annexin V-fluorescein isothiocyanate (FITC) staining (Fig. 3B). On the other hand, hESCs in NBF possessed a much higher proliferation rate compared with hESCs in MEF-CM when analyzed by MTT assay (Fig. 3C). These

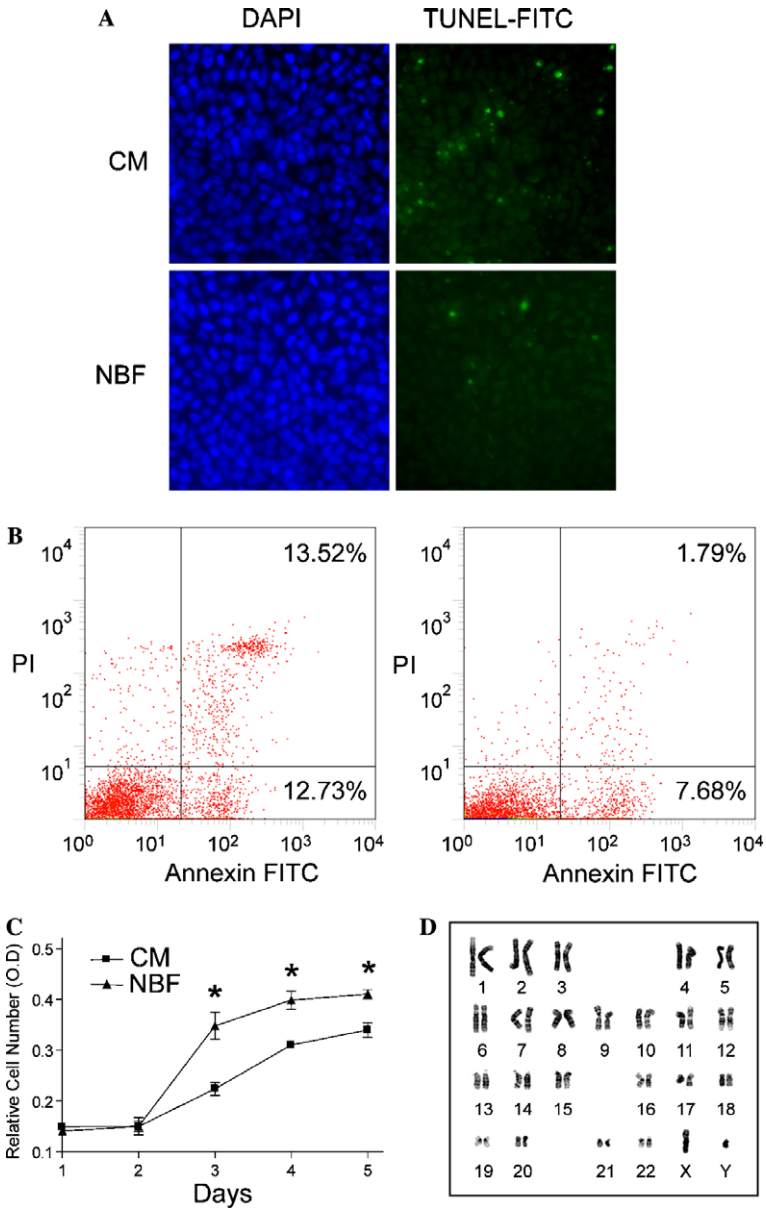

Fig. 3. hESCs in NBF display a low apoptosis rate and high proliferation rate with normal karyotype. (A) TUNEL analysis of H1 cells cultured after 16 passages in NBF (bottom panel) shows a much lower FITC-positive ratio compared to those cultured in CM (top panel). Counterstaining of nuclei in the same colonies with DAPI is shown in the left panels. (Original magnification: 200×.) (B) FACS analysis of apoptotic cells using Annexin V staining. H9 cells were cultured in NBF (right panel) or in CM (left panel). The percentage of positive cells is listed in the gates. (C) Cell number was assessed by 3-dimethylthiazol-2,5diphenyltetrazolium bromide (MTT) assay. Cells (2×10^4 per cm^2) are seeded on plates coated with matrigel and grown in CM or NBF. Bars, standard deviation of $n = 4$ experiments. hESCs in NBF are statistically significant at $^*P < 0.01$ as compared to CM. (D) Giemsa banding analysis of H1 cultured in NBF for 23 passages.

data demonstrate that hESCs in NBF exhibit decreased apoptosis, and increased proliferation rates, which would be expected to facilitate hESC expansion.

Since it is reported that cultured hESCs might undergo genomic alteration [23], we analyzed the karyotype of H1 cells (23 passages in NBF) and H9 cells (16 passages in NBF) by standard G-banding techniques. The results showed that H1 cells in this medium retained a normal karyotype (Fig. 3D). H9 cells were also found to retain a normal karyotype (data not shown).

hESCs cultured in NBF maintain pluripotency

To further evaluate the effects of NBF culture medium on hESCs, we measured the pluripotency of hESCs maintained under these conditions. H1 cells (22 passages in NBF) and H9 cells (19 passages in NBF) readily formed embryoid bodies (EBs) in suspension culture (Fig. 4A). When day 7 EBs were plated for further differentiation, markers of three germ layers (ectoderm, mesoderm, and endoderm) and trophoblasts were found to be highly

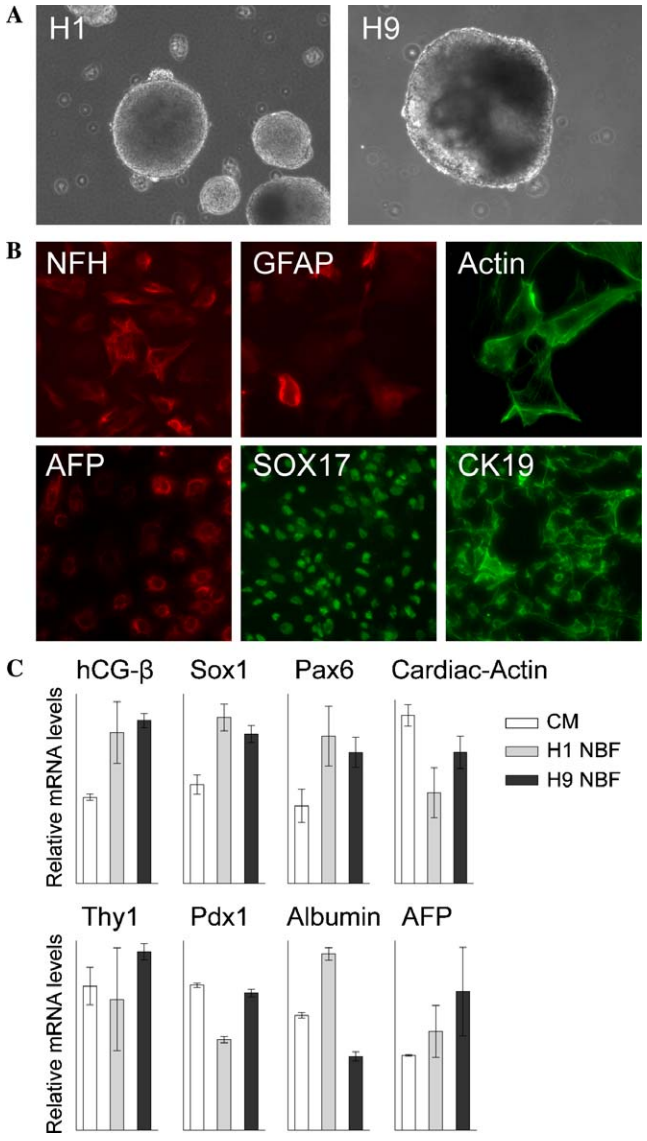

Fig. 4. Differentiation of hESCs cultured in NBF medium. (A) hEB formation. (Original magnification: 100×.) (B) Immunofluorescence staining of differentiated hESCs. NBF cultured hESCs can differentiate into cell types of ectoderm (NFH, GFAP), mesoderm (muscle actin), and endoderm (AFP, SOX17, and CK19). (Original magnification: 200×.) (C) Differentiated cells express markers of ectoderm (Sox 1 and Pax 6), mesoderm (cardiac actin), endoderm (Thy1, PDX1, AFP, and albumin), and trophoblast (hCG-β) derivatives according to the gene expression determined by a quantitative RT-PCR.

expressed by quantitative RT-PCR detection, which were similar to the differentiated cells derived from hESCs cultured in CM (Fig. 4C). The markers we used were: Sox1 and Pax6 for ectoderm, cardiac actin for mesoderm, Thy1, Pdx1, α-fetoprotein (AFP), and albumin for endoderm, and human chorionic gonadotrophin-β (hCG-β) for trophoblast cells. All markers were detected in the differentiated cells. By immunofluorescence staining, we found neurofilament heavy chain (NFH)- or glial fibrillary acidic protein (GFAP)-positive ectoderm cells, muscle actin-positive mesoderm cells, and AFP-, SOX17-, and cytokeratin (CK) 19-positive endoderm cells amongst the cells which had differentiated (Fig. 4B). These results indicate that NBF-cultured hESCs maintained pluripotency and possessed the multi-lineage differentiation potential.

Culture of hESCs in NBF culture medium on different extracellular matrices

As NBF culture medium still includes matrigel, a basement membrane preparation secreted by mouse Engelbreth Holm-Swarm sarcoma cells, and hence does not completely preclude murine protein and undefined components; we tested several kinds of human matrices individually and in combination to substitute for matrigel in NBF medium. We found that collagen IV or vitronectin could not maintain hESCs (Fig. 5A and B) for 7 days, and laminin sustained hESC self-renewal for short period (4 passages) (Fig. 5C). However, fibronectin with or without other matrix maintained hESCs in an undifferentiated state for more than 13 passages (Fig. 5D) (Table 2), and this result was further confirmed by staining of Oct4 and Tra-1-81 (Fig. 5E). And RT-PCR analysis was used to examine the sustained expression of the hESC self-renewal markers Oct4, Nanog and Sox2 (Fig. 5F). Subsequently, we measured the pluripotency of hESCs maintained in NBF on fibronectin, fibronectin and collagen IV, fibronectin and vitronectin, respectively. H1 cells (9 passages) cultured in NBF on fibronectin readily formed EBs (Fig. 5G). hESCs on fibronectin and vitronectin or on fibronectin and collagen IV were also found to form EBs (data not shown). To further confirm the pluripotency of hESCs cultured in these conditions, we analyzed the differentiated derivatives of hESCs by RT-PCR. We found that the markers of all three germ layers and extra embryonic trophoblast could be detected. The markers are hCG-β for extra trophoblast; NFH and Pax6 for ectoderm; Thy1 and cardiac actin for mesoderm; AFP for endoderm (Fig. 5H). These results indicated that hESCs cultured in these conditions retained the undifferentiated state and pluripotency for at least two months.

Discussion

In this study, we developed an animal-free and chemical-defined medium for hESC culture, with bFGF and N2B27 supplemented [termed NBF]. During long-term culture in NBF medium, hESCs maintained a normal karyotype and pluripotency with a low apoptosis and high growth rate.

After comparing N2B27 with BITS, x-vivo (data not shown) and B27, we found that N2B27 supplements were the best chemical-defined substitution for KSR to maintain hESCs. In particular, in N2B27 supplemented medium, hESCs undergo a low apoptosis rate and a high growth rate. In contrast, in traditional culture mediums derived from MEF, hESCs undergo considerable spontaneous apoptosis [18–22], differentiation [24], and chromosomal abnormalities [25–28]. Upon examining the components

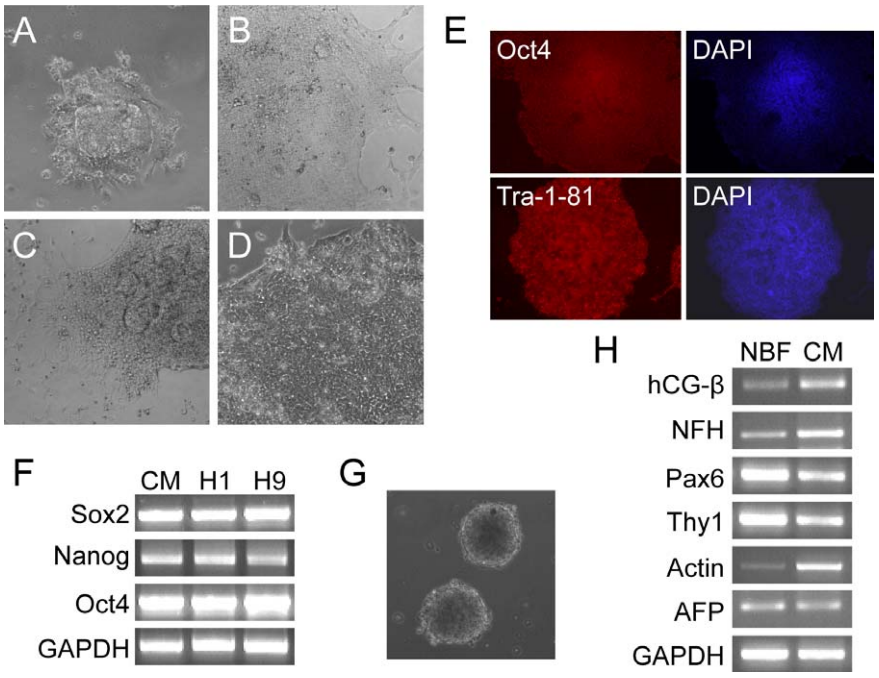

Fig. 5. hESCs cultured in NBF on different human matrices. (A) Six days on vitronectin, (B) six days on collagen IV, (C) four passages (24 days) on laminin, (D) six passages (36 days) on fibronectin, (E) H1 (five passages) cultured on fibronectin displays positive staining of Oct4 (top panel) and Tra-1-81 (bottom panel), (Original magnification: 100×). (F) RT-PCR analysis of control cells (CM) or H1 (9 passages) and H9 (8 passages) in NBF on fibronectin. The expression level of pluripotency markers (Oct4, Nanog and Sox2) and a housekeeping gene (GAPDH) seem to exhibit little difference. (G) hEB formation. (Original magnification: 100×). (H) RT-PCR analysis of the differentiated derivatives from hESCs after nine-passage culture in NBF on fibronectin. hESCs cultured in mouse embryonic fibroblast-conditioned medium (CM) on matrigel were used as positive controls. GAPDH expression was used as the internal control.

Table 2
Comparison of human embryonic stem cells cultured on different coating matrix

	Growth											
Matrix	–	+	+	+	++	++	+++	+++	+++	+++	+++	++++
Fibronectin						♦	♦	♦	♦	♦	♦	
Laminin			♦	♦	♦	♦				♦	♦	
Vitronectin		♦	♦						♦	♦	♦	
Collagen IV	♦			♦				♦			♦	
Matrigel												♦

The proportion of undifferentiated human embryonic stem cells after 10 days is indicated.

in N2B27 supplements (see supplementary Table 1), we found many antioxidants, such as DL-a-tocopherol acetate, DL-a-tocopherol (vitamin E), and superoxide dismutase (SOD), are included. These antioxidants can reduce the free radicals produced by oxygen at the concentration in the normal atmosphere, which might benefit survival, undifferentiated growth and reduce chromosomal abnormalities of hESCs [24,27].

During the development of NBF medium, we found that bFGF played an essential role in long-term hESC maintenance. No combinations of other factors maintained hESCs without bFGF, and a higher concentration of bFGF maintained hESCs longer in our N2B27 supplemented medium. Previously, hESCs were reported to maintain pluripotency in feeder-free conditions containing 40 ng/ml bFGF in the KSR context, and FGF signaling was demonstrated as necessary [12,29] for the sustained self-renewal and pluripotency of hESCs [30,31] in a concentration dependent manner [11,16]. In the course of preparing this manuscript, a similar work reported by Yao et al. also found that hESCs can be maintained in N2B27 and bFGF. However, in their culture medium, only 20 ng/ml bFGF was used together with 0.5 mg/ml BSA. The additional BSA may provide a carrier for bFGF and stabilize bFGF activity, which may account for the lower concentration of bFGF required to maintain hESCs in their culture medium.

Several other factors, such as the TGFβ subfamily of ligands and BMP inhibitors, have been demonstrated to be important to hESC self-renewal [11,13,32–34]. However, we found that they were unnecessary in our chemical-defined NBF medium. For example, the Smad2,3 activity sustained by the Activin/Nodal subfamily of ligands has

been reported to be necessary in hESC self-renewal [32] even when FGF signaling was inhibited [29], possibly for inhibition of neuroectoderm differentiation [34,35]. Unexpectedly, Activin A in our NBF medium dramatically induced hESC differentiation with/without bFGF. We presumed that Activin A contributes to the self-renewal of hESCs only in the context with KSR, which is consistent with other reports that Activin A induced endoderm differentiation of hESCs in serum-free culture medium [36]. The basal requirement for Smad2,3 activity for hESCs cultured in NBF might be met by the autocrine function of Nodal in hESCs [37]. Noggin is also reported to contribute to hESC self-renewal [11,12]. We also test whether noggin could be used to optimize the culture medium. However, we did not observe any contribution of noggin to the NBF medium, possibly because noggin induces neuroectoderm differentiation and only contributes to self-renewal as a BMP inhibitor in the context with KSR [11,38,39]. Because it is also reported that ERK and PI3K might mediate self-renewal signaling [40,41], we also tested the effect of IGF, PDGF, and EGF, several potential stimulators of PI3k and ERK, in hESC self-renewal in N2B27 supplemented medium. However, we failed to substitute for bFGF with those growth factors. It is possible that they were unable to activate PI3K and ERK in hESCs, or some downstream pathways of bFGF other than PI3K and ERK mediate the effect of bFGF. The underlying mechanism of bFGF in hESC self-renewal and the possible roles of other self-renewal factors in distinct contexts will require further investigation.

Most recently, several animal-free and chemical-defined culture mediums have been developed which support undifferentiated growth in hESCs [26,42,43]. It was reported by Ludwig et al. that a combination of factors including bFGF, LiCl, GABA, pipecolic acid, and TGFβ supports a feeder-free culture of hESCs in an atmosphere of low concentration oxygen [26]. However, such culture conditions are very complicated. We and Yao et al. have both simplified the conditions with a chemical-defined medium made up of bFGF with N2B27 supplements, which will facilitate the large-scale expansion of hESCs for clinical applications [43]. However, matrigel was used in the study of Yao et al., which is not absolutely chemical-defined and furthermore does not preclude animal-derived pathogens. To solve this problem, we found fibronectin to be a potential alternative to the animal-derived matrigel in the N2B27 supplemented medium. Interestingly, Lu et al. also found that fibronectin could substitute for matrigel in their chemical-defined medium [42].

This study has demonstrated lower apoptosis rates and higher growth rates in hESCs cultured in NBF medium than in traditional culture medium. It reduces the considerable loss of hESCs in traditional culture medium because of spontaneous apoptosis, and facilitates the large-scale expansion of hESCs. Future study should compare our NBF medium with other formulations of chemical-defined mediums, allowing an optimization of the NBF medium so as to become as completely defined and minimized as possible while supporting long-term hESC culture. This chemical-defined medium will make it possible to more effectively study the molecular mechanism of hESC self-renewal and extends the practical utility of hESC for clinical applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.05.086.

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