SUPPLEMENTARY INFORMATION

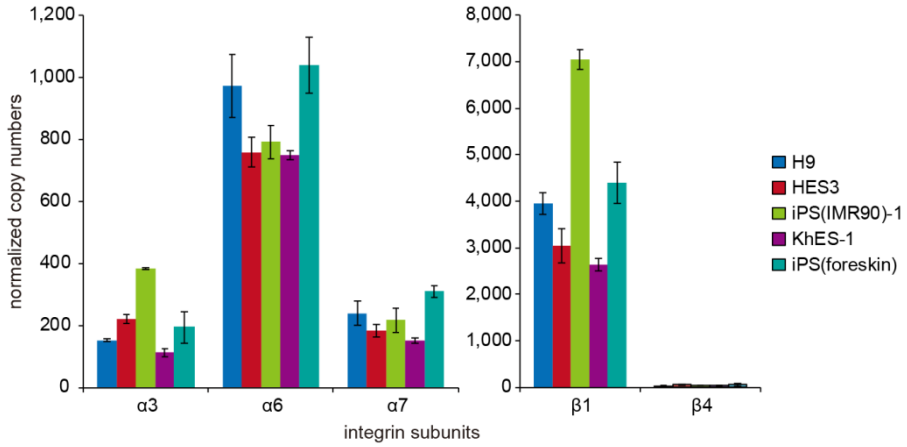
Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells

Takamichi Miyazaki, Sugiko Futaki, Hirofumi Suemori, Yukimasa Taniguchi, Masashi Yamada, Miwa Kawasaki, Maria Hayashi, Hideaki Kumagai, Norio Nakatsuji, Kiyotoshi Sekiguchi & Eihachiro Kawase

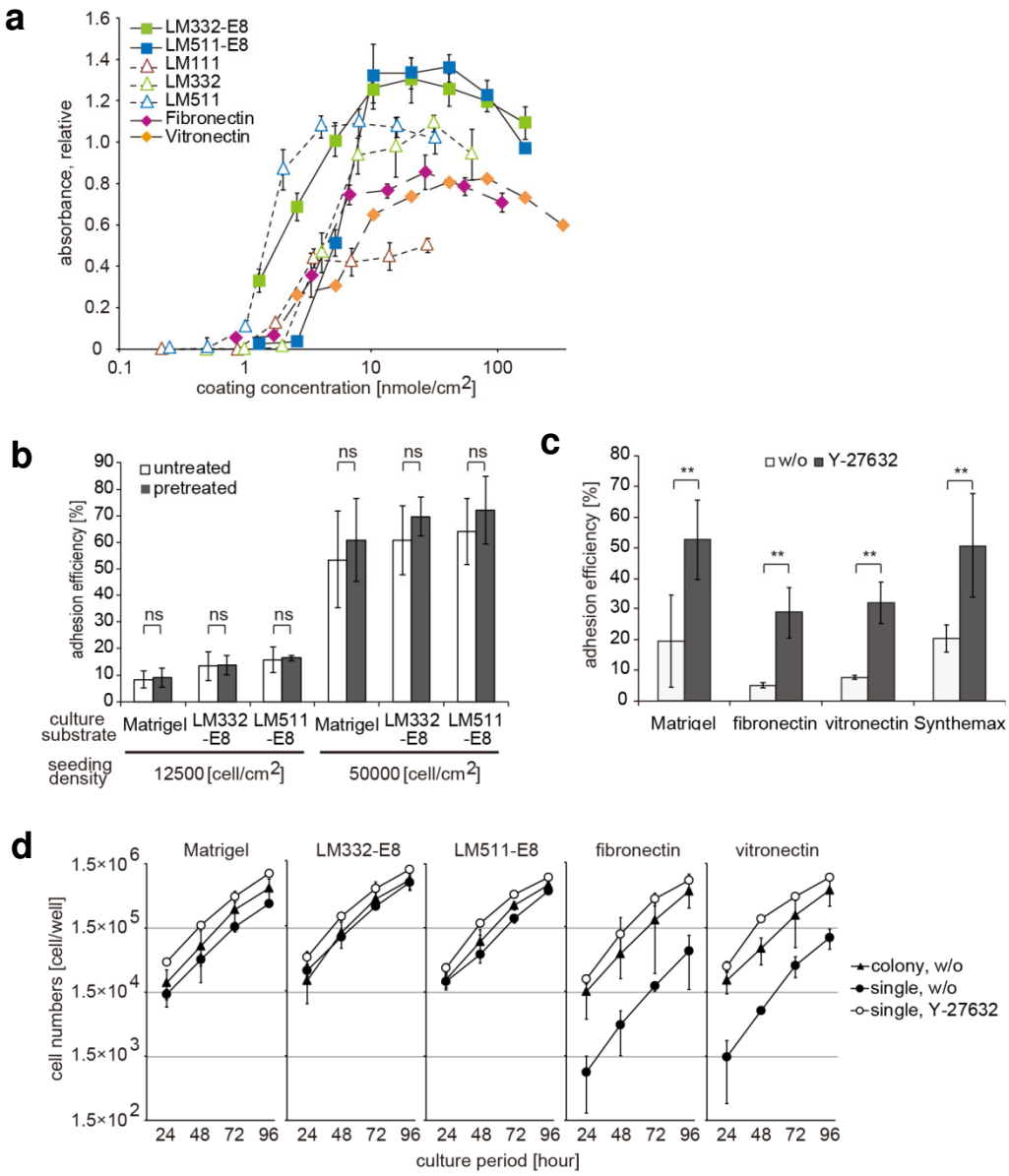
This file includes

- Supplementary Figure S1-S7
- Supplementary Movie legend
- Supplementary Table S1 and S2
- Supplementary Methods

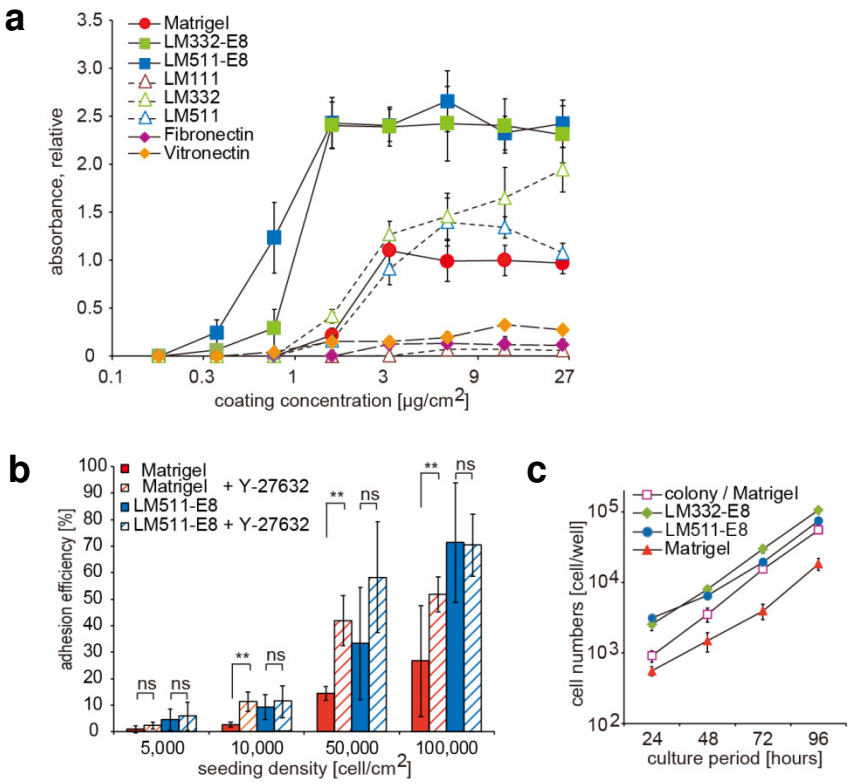
SUPPLEMENTARY FIGURES



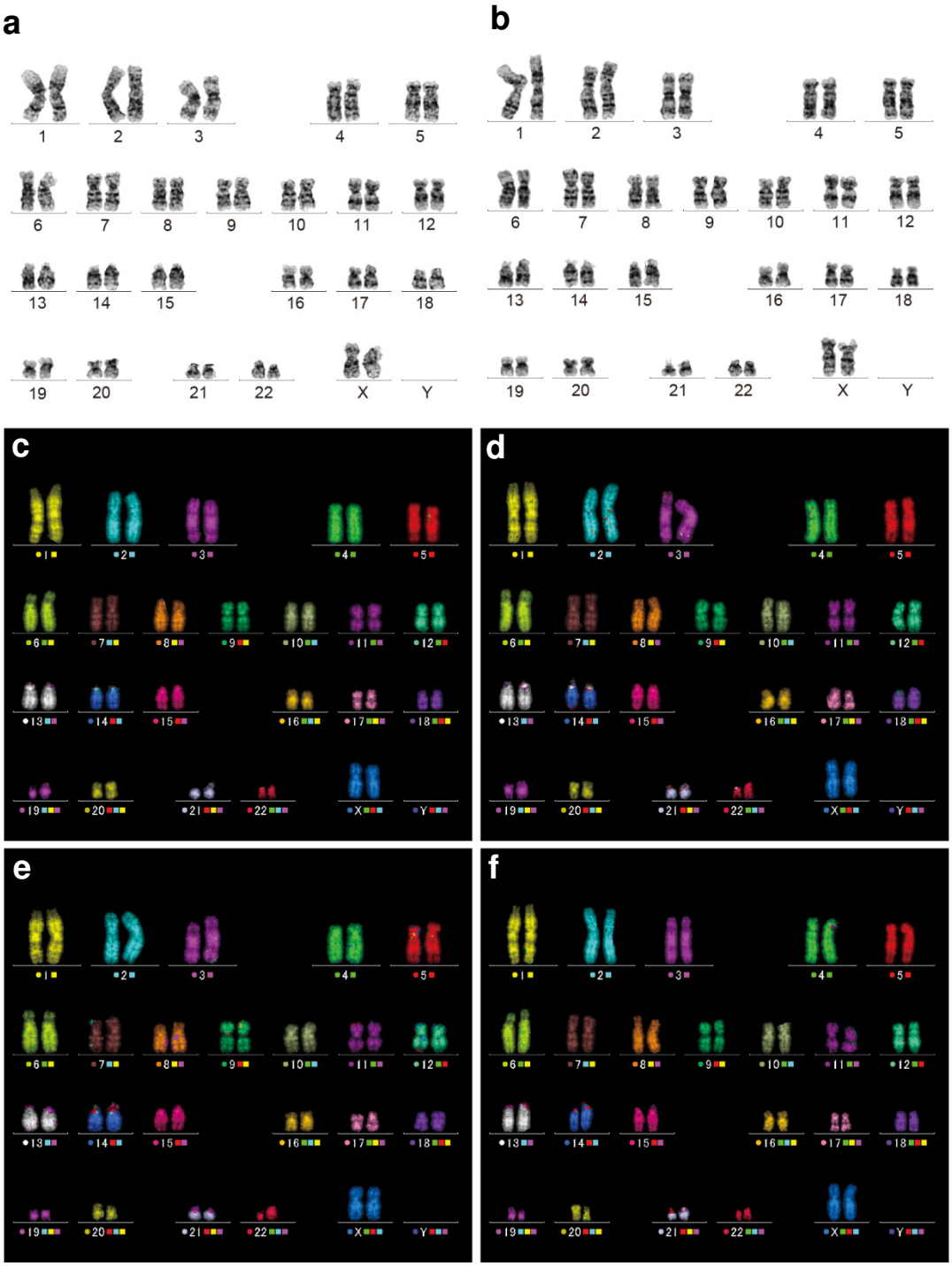
Supplementary Figure S1. Integrin expression on hESCs and hiPSCs.
α6 and β1 integrins were abundantly expressed in two hiPSC lines (iPS(IMR90)-1 and iPS (foreskin)), as well as in three hESC lines (H9, HES3, and KhES-1). Total RNA from hESCs and hiPSCs cultured on MEF feeder cells was used for qPCR analysis. Copy numbers were normalized against qPCR reactions using plasmids, with the cDNA of each integrin subunit used as a template. Total RNA was isolated using an RNeasy Mini Kit, and cDNAs were synthesized from 100 ng RNA using an ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. To detect integrins, qPCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems) and an ABI 7000 real-time PCR system (Applied Biosystems). The amplification levels of individual integrin subunits were normalized against those of the standard cDNAs encoding individual integrin subunits, and expressed as copy numbers per 1 ng total RNA used for reverse transcription. The primer sets for integrin subunits are described in a previous report [19]. All experiments were performed in triplicate, and the results were expressed as the means ± SD.



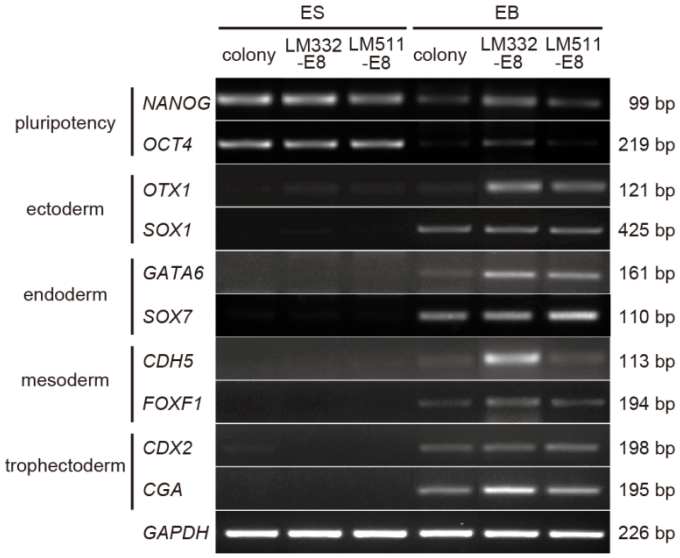
Supplementary Figure S2. Additional validation of LM-E8s for H9 hESC attachment.
(a) Dose-response adhesion curves of H9 hESCs on a molar basis. (b) Validation of Y-27632 treatment for hESC culture on LM-E8s. Completely dissociated H9 hESCs with Y-27632 pretreatment before dissociation (pretreated) or without pretreatment (untreated) were incubated for 6 hours with Y-27632. (c) Adhesion efficiency of dissociated H9 hESCs on previously reported ECMs. H9 hESCs at 5×10^4 cells/cm^2 were incubated for 6 hours with or without Y-27632. (d) Short-term curves of H9 hESCs on various ECMs. H9 hESCs as colonies or single cells were seeded at 5×10^4 cells/cm^2 with or without Y-27632, and the cell numbers were counted every 24 hours. Note that once adhered, H9 hESCs showed similar proliferation regardless of culture substrates, cluster sizes, and Y-27632 treatment. Data represent the means ± S.D. of three experiments. **: $P < 0.05$, Student's t-test.



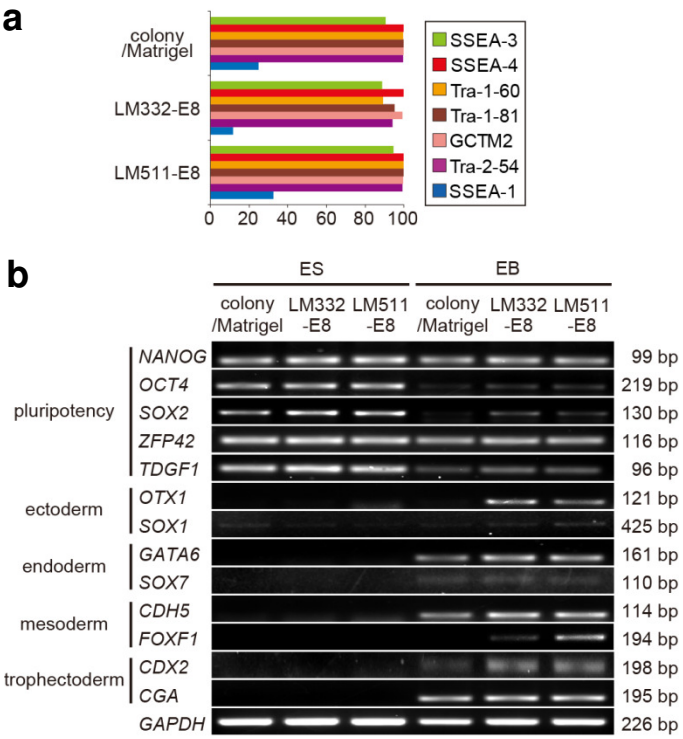
Supplementary Figure S3. Validation of LM-E8s for hiPSC attachment.
(**a**) Dose-response adhesion curves of iPS(IMR90)-1 cells on various ECMs. (**b**) Density-dependent adhesion of dissociated iPS(IMR90)-1 cells seeded on LM511-E8 in mTeSR1 medium. Completely dissociated iPS(IMR90)-1 cells showed higher rates of adhesion on LM511-E8 compared with that on Matrigel. Dissociated cells were incubated on LM511-E8 in mTeSR1 medium for 6 hours with or without Y-27632. Data represent the means ± S.D. of three experiments (n = 3). **: $P < 0.05$, ns: non-significant, Student's t-test. (**c**) Short term growth curves of iPS(IMR90)-1 cells on LM-E8s in mTeSR1 medium. Dissociated iPS(IMR90)-1 cells on LM-E8s proliferated well, and their growth rates did not differ from that of the colony culture. Data are the means ± S.D. of three individual experiments (n = 3).



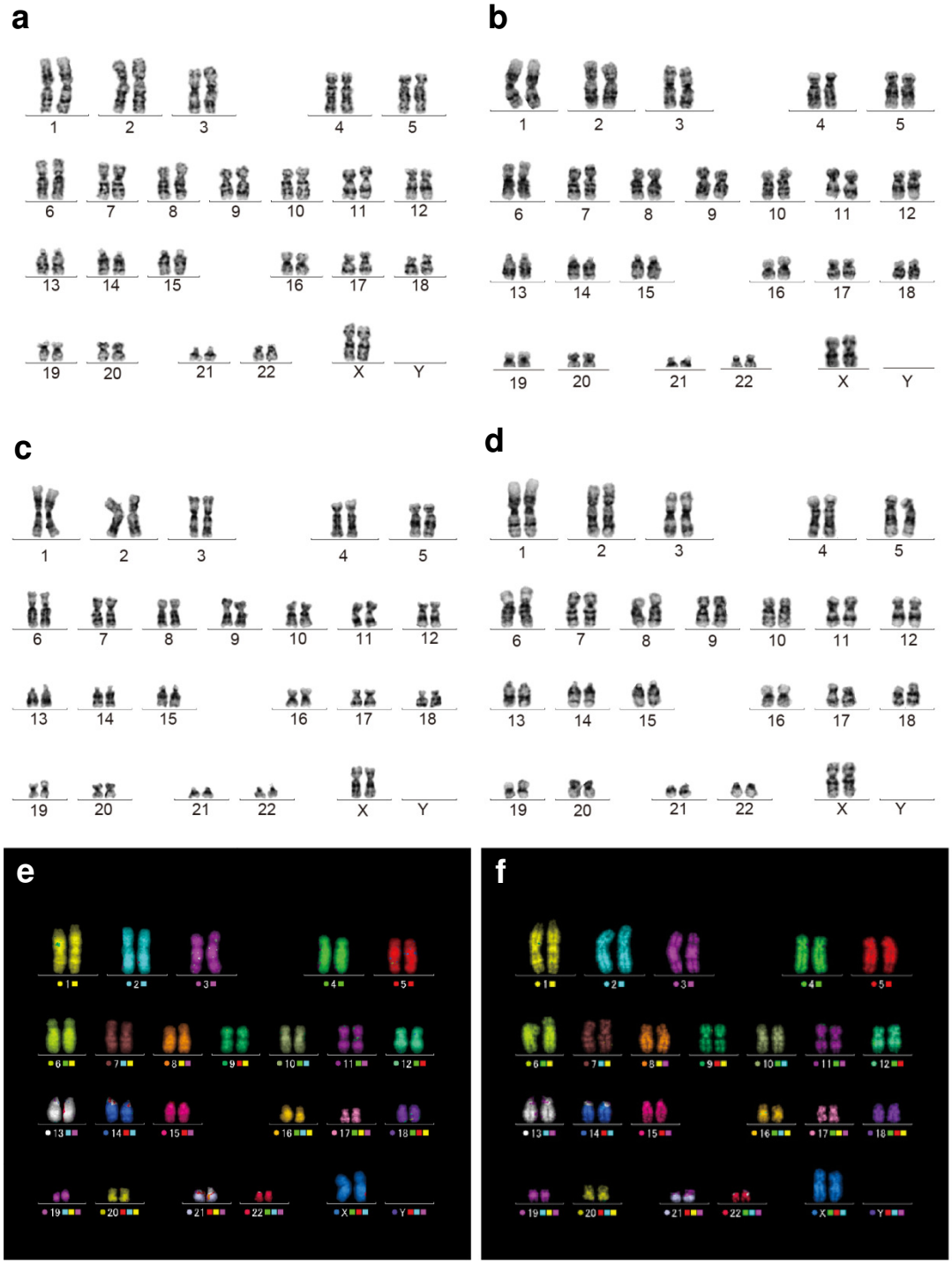
Supplementary Figure S4. H9 hESCs and iPS(IMR90)-1 cells with normal karyotypes following complete dissociation and culture in mTeSR1 medium. (**a,b**) G-banding analysis and (**c–f**) M-FISH analysis. H9 hESCs cultured on LM332-E8 (**a**) and LM511-E8 (**b**) (after 10 passages) had a normal karyotype, 46, XX. H9 hESCs cultured on LM332-E8 (**c**) and LM511-E8 (**d**) for 30 passages had a normal karyotype, 46, XX. iPS(IMR90)-1 cells cultured on LM332-E8 (e) and LM511-E8 (f) for 20 passages had a normal karyotype, 46, XX.



Supplementary Figure S5. RT-PCR analysis of differentiation marker genes after 10 days of embryoid body development. iPS(IMR90)-1 cells were cultured on LM332-E8 or LM511-E8 in mTeSR1 medium for 10 passages after complete dissociation, or on Matrigel after colony dissociation. Cells were allowed to form embryoid bodies for 10 days.



Supplementary Figure S6. LM-E8s sustain the pluripotency of 253G1 iPSCs in xeno-free TeSR2 medium after complete dissociation. (**a**) Flow cytometric profiles of 253G1 iPSCs on LM-E8s for 15 passages after complete dissociation. Numbers indicate the percentages of cells that were positive for cell surface markers. (**b**) RT-PCR analysis of marker genes for the differentiation of embryoid bodies. 253G1 iPSCs were cultured in TeSR2 medium for 10 passages on LM-E8s after complete dissociation, and the cells were allowed to form embryoid bodies for 10 days.



Supplementary Figure S7. Normal karyotypes were present in H9 hESCs after 30 passages and 253G1 hiPSCs after 10 passages following complete dissociation and culture in xeno-free TeSR2 medium. (**a-d**) G-banding analysis. (**e, f**) and M-FISH analysis. H9 hESCs cultured on LM332-E8 (**a, e**) or LM511-E8 (**b, f**) had normal karyotypes, 46, XX. 253G1 hiPSCs cultured on LM332-E8 (c) or LM511-E8 (d) had normal karyotypes, 46, XX.

Supplementary Movies

Time-lapse recording at the initial attachment of dissociated H9 hESCs onto LM332-E8 (**Movie S1**), LM511-E8 (**Movie S2**), Matrigel (**Movie S3**), fibronectin (**Movie S4**), and vitronectin (**Movie S5**). Movies represent 24 hours from cell seeding.

Supplementary Table S1a. Results of two-way analysis of variance for multiple comparison assays

	Matrigel			LM332-E8			LM511-E8			LM511(EQ)		
Source	*F*	*P*	F_{crit}	*F*	*P*	F_{crit}	*F*	*P*	F_{crit}	*F*	*P*	F_{crit}
Anti-integrin α6	327.13	<0.001	4.49	48.53	<0.001	4.49	9.68	0.007	4.49	23.72	<0.001	4.49
Anti-integrin β1	11.35	0.004	4.49	21.03	<0.001	4.49	29.40	<0.001	4.49	0.24	0.629	4.49
Anti-integrin α6 and Anti-integrin β1	12.85	0.002	4.49	3.04	0.100	4.49	0.46	0.508	4.49	0.65	0.432	4.49

F indicates the F value. *P* indicates the P value. F_{crit} indicates the critical value of F. A difference with $P < 0.05$ at $F_{crit} < F$ was considered significant.

Where *P* is higher than 0.05 and *F* is lower than F_{crit} (e.g. LM332-E8, LM511-E8, and LM511(EQ)), the significant difference was estimated by Tukey's test.

Supplementary Table S1b. Results of the studentized range statistic

	LM332-E8	LM511-E8	LM511(EQ)
Anti-integrin α6	6.33	8.68	1.30
Anti-integrin β1	8.70	7.52	5.68
Anti-integrin α6 and Anti-integrin β1	11.55	15.62	5.36

Where the statistics are higher than 5.22 (level of significance, 0.05; number of treatment, 4; degrees of freedom, 5), the differences were considered as significant ($P < 0.05$).

Supplementary Table S2. List of primers used for semi-quantitative RT-PCR

Genes	Forward primer 5'-3'	Reverse primer 5'-3'	Product (bp)	Annealing (°C)
NANOG	CTGCTGAGATGCCTCACACG	TGCCTTTGGGACTGGTGGA	99	60
OCT4	TCTCGCCCCCTCCAGGT	GCCCCACTCCAACCTGG	219	60
SOX2	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG	130	60
ZFP42	AGAAACGGGCAAAGACAAGAC	GCTGACAGGTTCTATTTCCGC	116	60
TDGF1	ACAGCACAGTAAGGAGCTAAAC	CGTCCGTAGAAGGAGGGAGG	96	60
OTX1	CACTAACTGGCGTGTTTCTGC	AGGCGTGGAGCAAAATCG	121	60
SOX1	GCGGAAAGCGTTTTCTTTG	TAATCTGACTTCTCCTCCC	425	60
GATA6	TGTGCAATGCTTGTGGACTC	AGTTGGAGTCATGGGAATGG	161	60
SOX7	GCCAAGGACGAGAGGAAAC	CTCTTCTGGGACAGCGTCA	110	60
CDH5	GATCAAGTCAAGCGTGAGTCG	AGCCTCTCAATGGCGAACAC	114	60
FOXF1	ACAGCGGCGCCTCTTATATC	CTCCTTTCGGTCACACATGC	194	60
CDX2	CCGAACAGGGACTTGTTTAGAG	CTCTGGCTTGGATGTTACACAG	198	60
CGA	GTTTCTGCATGTTCTCCATTC	GTGGACTCTGAGGTGACGT	195	60
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226	60

SUPPLEMENTARY METHODS

Semi-Quantitative RT-PCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen), and cDNA was synthesized from 1–2 μg RNA using an Omniscript™ RT Kit (Qiagen) according to the manufacturer's instructions. PCR conditions were optimized to facilitate semi-quantitative comparison of samples within the log phase of amplification. Gene-specific primers were designed based on published sequences (Supplementary Table 2). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Differentiation assay

For the direct differentiation assay, hESCs or hiPSCs were plated onto multiwell chamber slides (Nunc) coated with Matrigel. For mesodermal differentiation, cells were cultured for 14 days in DMEM/F12 supplemented with 10% FBS, 2 mM glutamine, 0.1 mM nonessential amino acids, and 0.1 mM 2-mercaptoethanol. For endodermal differentiation, cells were cultured with 1% FBS and 100 ng/ml activin for 7 days. For neural differentiation, cells were treated with 100 ng/ml recombinant mouse noggin (R&D systems) in N2-B27 medium without FGF-2 for 10 days. Differentiated cells were then confirmed by immunofluorescence staining as described below.

Embryoid body formation was analysed as follows. Cells treated with a CTK solution, consisting of 1 mg/ml collagenase IV (Invitrogen), 0.25% trypsin (Invitrogen), 1 mM $CaCl_2$ and 20% knockout serum replacement (KSR), were detached as clumps using a cell scraper and then cultured as a suspension in petri dishes with human ESC medium without FGF-2. The medium was changed every 2 days. After 10 days, total RNA was extracted using an RNeasy Mini Kit, and the expression levels of differentiation markers were estimated by semi-quantitative RT-PCR as described above. For teratoma formation, approximately 5×10^6 cells were injected into the testes of severe combined immunodeficient mice. After 8 weeks, teratomas were surgically dissected from mice and fixed with 4% paraformaldehyde in PBS. The samples were embedded in paraffin, sectioned at 5 μm and processed for H&E staining.

Immunofluorescence staining

Differentiated cells were prepared as described above. To detect OCT4 expression, cells that had been cultured on LM-E8s or Matrigel for 20 passages were seeded onto 8-well culture slides (BD Falcon, 354118) coated with LM-E8s or Matrigel, and then cultured for 3 days. Cells were then fixed with 4% paraformaldehyde in PBS for 15 minutes. After three washes in PBS, cells were permeabilized with PBT (0.1% Triton X-100 in PBS) for 10 minutes, and blocked with blocking buffer (10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in PBT) for 1 hour. Cells were then incubated with the primary antibody (diluted in 1% BSA/PBT) for 1 hour at RT. After three washes in PBT, cells were incubated with the secondary antibody (diluted in 1% BSA/PBT) for 1 hour at RT, and counterstained with DAPI (0.2 μg/ml final concentration) for 1 minute. Slides were washed three times in PBS and mounted with 90% glycerol in PBS, and fluorescence was examined using a Zeiss imaging system with AxioVision software (Zeiss). Primary antibodies against the following markers were used: AFP (1:200; Sigma), Brachyury (1 μg/ml; Santa Cruz), and βIII tubulin (5 μg/ml; Sigma). Anti-mouse IgG/Alexa 546 conjugated (1 μg/ml; Molecular Probes) and anti-goat IgG/Alexa 546 conjugated (1 μg/ml; Molecular Probes) were used as secondary antibodies.

Immunoblotting

Prior to cell seeding, dissociated H9 hESCs were incubated for 1 hour at 37°C as a suspension in mTeSR1 medium. Cells were then seeded at 5×10^4 cells/cm^2 in culture vessels coated with 1.5 μg/cm^2 of each substrate. After 1 hour of incubation, cells were rinsed with ice-cold PBS and lysed in a buffer containing 0.5% (w/v) SDS, 5% (v/v) glycerol, 5 mM EDTA, 10 mM NaF, 10 mM sodium diphosphate, 2 mM Na_3VO_4, and 10 mM Tris-HCl, pH 7.5. Then the protein concentrations of the lysates were determined using a BCA protein assay kit (Pierce). Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene fluoride membranes (Millipore) in 0.1 M Tris base, 0.192 M glycine and 20% (v/v) methanol using a semi-dry electrophoretic transfer cell (Bio-Rad). Membranes were treated at RT for 1 hour with a blocking buffer containing 5% (w/v) non-fat milk powder in 0.1% (w/v) Tween 20/TBS (T-TBS), and then incubated with antibodies in the blocking buffer at 4°C overnight. After three washes in T-TBS, membranes were incubated with peroxidase-coupled secondary antibodies diluted in the blocking buffer at

RT for 1 hour. The membranes were then washed with T-TBS, and visualized using an ECL chemiluminescence system (GE Healthcare). The following antibodies used were: anti-phospho Akt (Cell Signaling, #4058), anti-Akt (Cell Signaling, #9272), anti-phospho-FAK (Biosource, #44-624), anti-FAK (Transduction Laboratories, #F15020), anti-phospho-p44/42 MAPK (ERK1/2) (Cell Signaling, #9106), anti-p44 MAPK (ERK1) (Santa Cruz, #sc-94), anti-phospho-myosin light chain 2 (Cell Signaling, #3671), and anti-myosin light chain (Sigma, #M4401).