Supplementary Information

A thermoresponsive and chemically-defined hydrogel for long term culture of human embryonic stem cells

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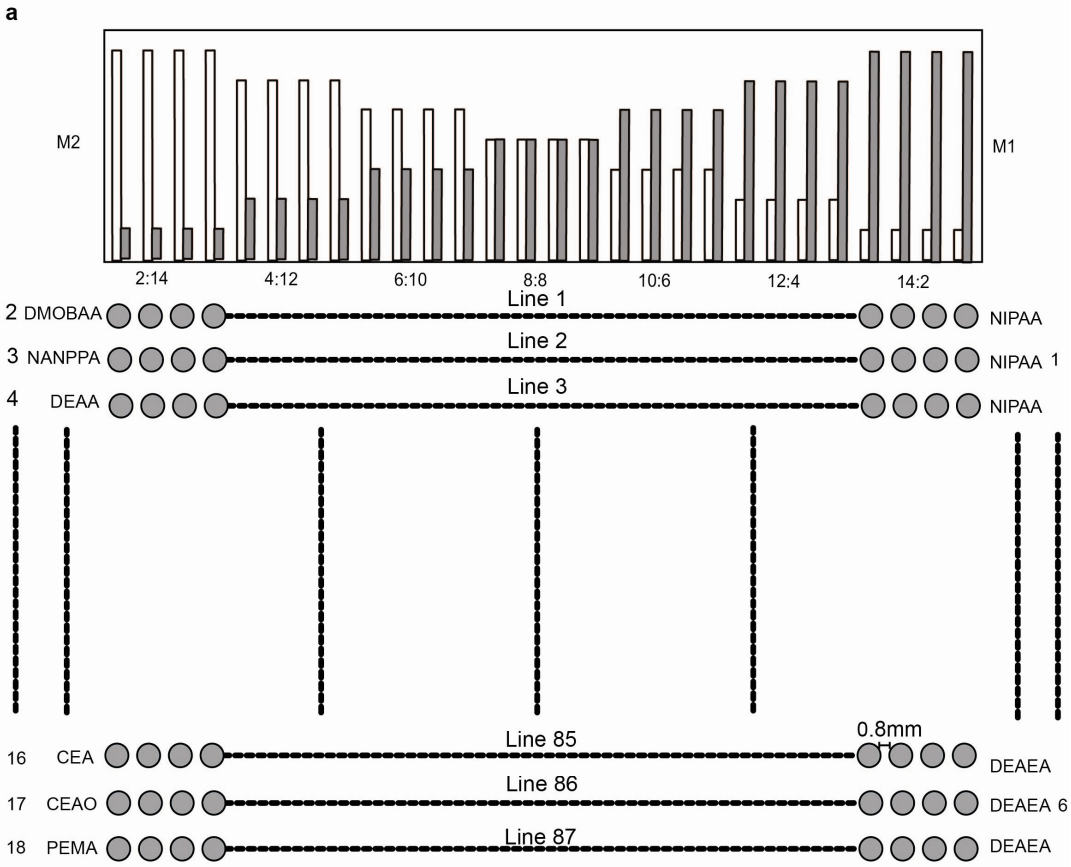
Supplementary Figure S1. Preparation of polymer arrays.

a



b

Monomers		Structure	Con. in H_2O (%wt)[a)]	Monomers		Structure	Con. in H2O (%wt)	Monomers		Structure	Con. in H_2O (%wt)
X	MBA		0.7	7	DMAA		14	14	PEG6MA		14
1	NIPAA		14	8	AAm		14	15	AAH		10 (pH7)
2	DMOBAA		14	9	EOA		7	16	CEA		12 (pH7)
3	NANPPA		14 (pH7)	10	AEtMA-Cl		14	17	CEAO		14 (pH7)
4	DEAA		2.8	11	HBA		7	18	PEMA		7 (pH6)
5	DMAEMA		14 (pH7)	12	HEMA		7				
6	DEAEA		14 (pH7)	13	HPMA		7				

Supplementary Figure S1. Preparation of polymer arrays.

a, Representation of the process of fabricating a polymer microarray (75 mm x 26 mm) with 2436 spots (300-400 mm) made up of monomers printed in various combinations of 3 monomers (monomer 1; M1) and monomer 2; M2) at different M1 to M2 ratios with the cross-linker (MBA), to create 609 different polymers, with each polymer printed in quadruplicate. b, Chemical structures, abbreviations and concentrations of the 18 monomers (1-18) and cross-linker (MBA; X) used to create the polymer arrays.

Supplementary Figure S2. Growth of RCM1 hESC on polymer arrays.

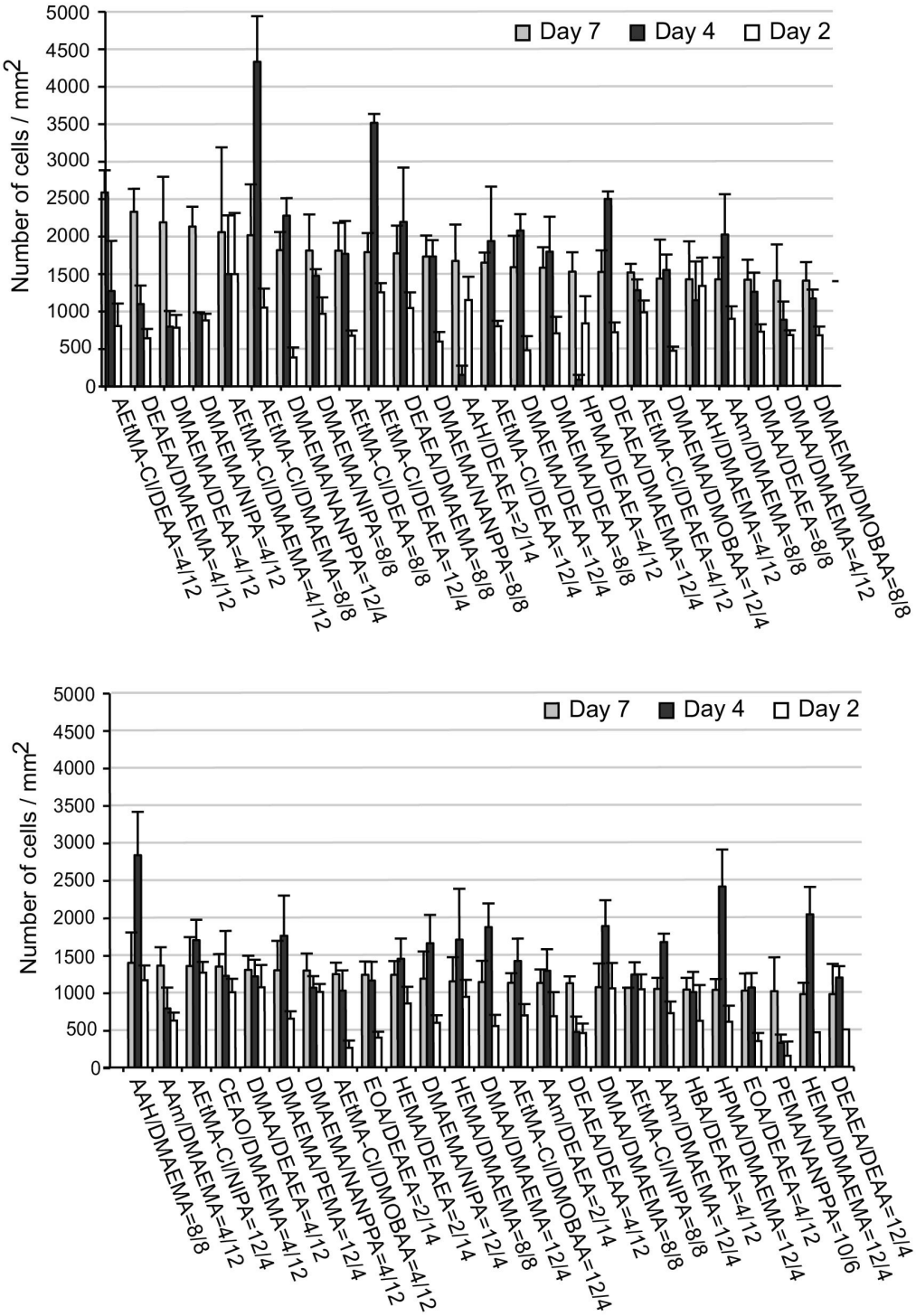
M1	M2	Cell Adhesion						
		02:14	04:12	06:10	08:08	10:06	12:04	14:02
DMOBAA	NIPA	1	2	2	2	1	2	2
NANPPA		3	3	3	3	3	3	3
DEAA		2	3	3	2	2	2	2
DMAEMA		3	3	3	3	3	3	3
DEAEA		3	3	3	3	3	3	3
DMAA		0	0	1	1	1	2	1
AAm		0	0	0	2	2	2	1
EOA		1	1	2	2	2	3	3
AEtMA-Cl		2	3	3	3	3	3	3
HBA		0	0	1	1	2	1	1
HEMA		0	1	1	2	2	2	1
HPMA		2	2	2	2	2	2	2
PEG6MA		1	2	2	1	1	1	1
AAH		1	1	0	0	0	0	1
CEA		1	1	0	1	0	0	1
CEAO		1	0	0	0	0	1	0
PEMA		1	1	1	1	1	1	1
NANPPA	DMOBAA	1	1	1	1	1	1	1
DEAA		2	2	2	2	2	2	2
DMAEMA		3	3	3	3	3	3	2
DEAEA		1	3	3	3	3	3	3
DMAA		2	2	2	3	2	2	2
AAm		2	2	2	2	2	2	1
EOA		3	3	3	3	2	2	2
AEtMA-Cl		3	3	3	3	3	3	3
HBA		2	2	1	2	2	2	2
HEMA		3	2	3	2	2	1	2
HPMA		2	2	2	1	1	1	1
PEG6MA		1	1	1	1	1	1	1
AAH		1	0	1	1	0	1	1
CEA		0	0	1	0	0	1	1
CEAO		0	0	0	0	0	0	0
PEMA		1	1	1	1	1	1	1
DEAA	NANPPA	2	3	3	2	3	3	2
DMAEMA		2	3	3	3	3	3	3
DEAEA		3	3	3	3	3	2	2
DMAA		3	2	2	2	2	2	2
AAm		3	3	0	2	1	1	0
EOA		3	3	2	2	1	1	1
AEtMA-Cl		2	3	3	2	2	2	2
HBA		2	2	2	1	1	1	1
HEMA		3	3	2	2	2	0	0
HPMA		3	3	3	2	2	1	1
PEG6MA		1	2	1	1	1	1	1
AAH		3	3	0	0	0	0	0
CEA		2	2	1	0	1	0	0
CEAO		3	2	1	0	1	0	0
PEMA		2	2	1	1	3	1	1

M1	M2	Cell Adhesion						
		02:14	04:12	06:10	08:08	10:06	12:04	14:02
DMAEMA	DEAA	3	3	3	3	3	3	3
DEAEA		3	3	3	3	2	2	1
DMAA		2	3	3	3	2	2	2
AAm		2	1	1	1	2	1	1
EOA		2	3	3	3	2	2	2
AEtMA-Cl		3	3	3	3	3	3	3
HBA		2	2	2	2	1	1	0
HEMA		3	2	2	2	1	1	1
HPMA		2	2	2	2	2	1	1
PEG6MA		2	2	2	1	1	1	1
AAH		1	2	1	2	1	1	1
CEA		3	2	1	1	1	1	1
CEAO		2	2	2	1	1	1	1
PEMA		1	2	1	1	1	1	1
DEAEA	DMAEMA	3	3	3	3	3	3	2
DMAA		3	3	3	3	3	3	2
AAm		3	3	3	3	3	3	2
EOA		3	3	3	3	3	3	1
AEtMA-Cl		3	3	3	3	3	2	2
HBA		3	3	3	3	3	2	1
HEMA		3	3	3	3	3	3	2
HPMA		3	3	3	3	3	3	2
PEG6MA		3	3	3	3	2	2	1
AAH		3	3	3	3	3	1	1
CEA		3	3	3	3	2	1	1
CEAO		3	3	3	2	1	1	1
PEMA		3	3	2	1	1	1	1
DMAA	DEAEA	3	3	3	3	3	2	2
AAm		2	3	2	2	2	2	1
EOA		3	3	3	2	2	2	1
AEtMA-Cl		3	3	3	3	3	3	3
HBA		3	3	2	2	1	1	1
HEMA		3	2	2	2	2	1	1
HPMA		3	2	2	2	2	2	2
PEG6MA		3	3	1	1	1	0	0
AAH		2	2	1	1	1	1	0
CEA		1	0	0	1	1	1	1
CEAO		2	1	0	1	1	1	1
PEMA		1	1	1	3	2	1	1
EOA	PEMA	1	1	3	1	2	2	3
AEtMA-Cl		1	1	2	2	3	3	1

0	Less than 10% area of a spot covered with cells;
1	10% to 40% area of a spot covered with cells;
2	40% to 70% area of a spot covered with cells;
3	70% to 100% area of a spot covered with cells;

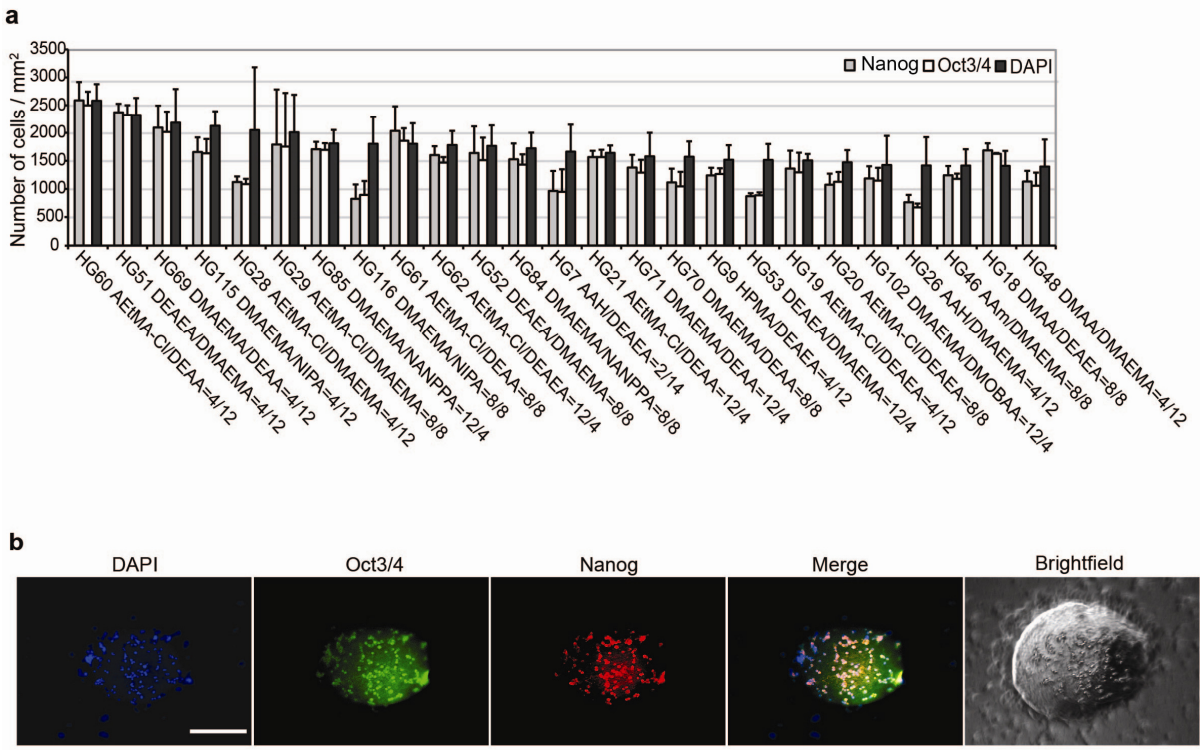
Supplementary Figure S2. Growth of RCM1 hESC on polymer arrays.
The ability of RCM1 hESC to grow on each of the 609 different polymers was assessed 24h after culture of cells on a polymer array slide. Cells were stained with DAPI, and the percentage of each spot covered by cells was quantified manually using a 4 tier scoring system (0-3). Each score is the average of quadruplicate spots, rounded to the nearest integer. The table is a representative example of 2 independent experiments. Polymers highlighted by a red box were selected for further screening.

Supplementary Figure S3. Cell growth support by the top 50 polymers.



Supplementary Figure S3. Cell growth support by the top 50 polymers.
Average number of RCM1 hESC bound to the top 50 polymers after 2, 4 and 7 days of culture (one slide for each day, with each polymer spotted nine times, and DAPI-positive cells counted using an IMSTAR microscope system). Bars represent mean±SD of the nine spots. The 25 polymers that supported growth best at day 7 were selected for further analysis.

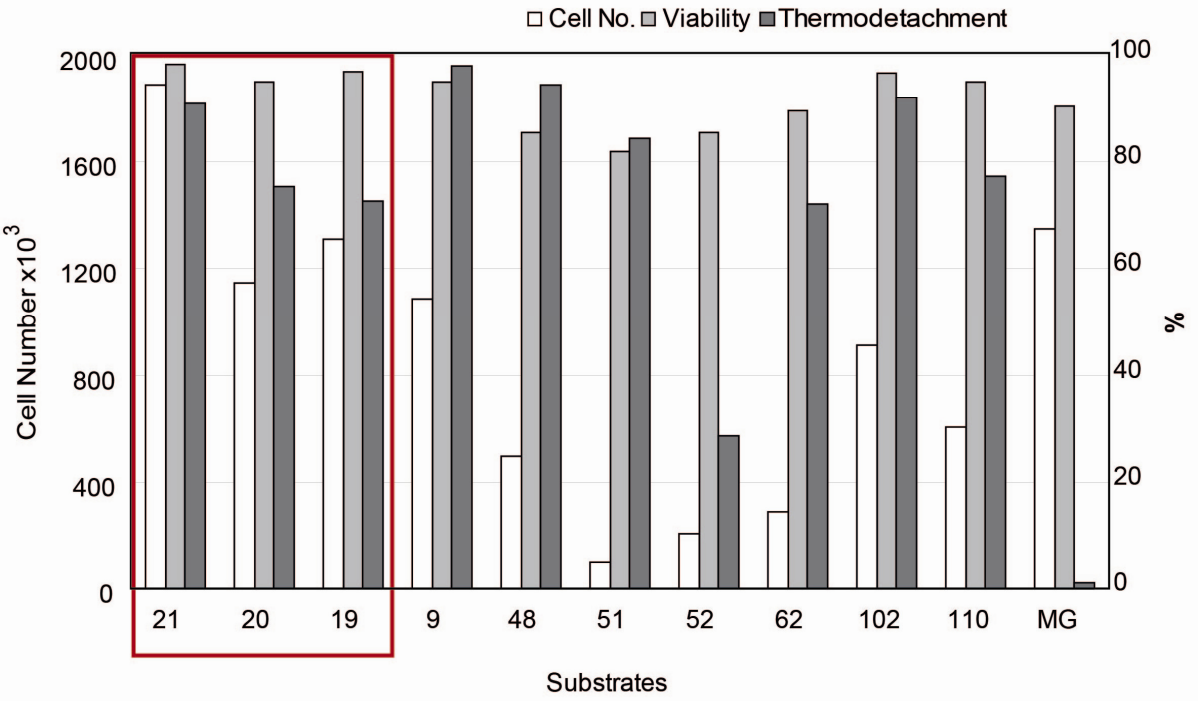
Supplementary Figure S4. Growth and stem cell marker expression of RCM1 hESC cultured on the top 25 polymers.



Supplementary Figure S4. Growth and stem cell marker expression of RCM1 hESC cultured on the top 25 polymers.

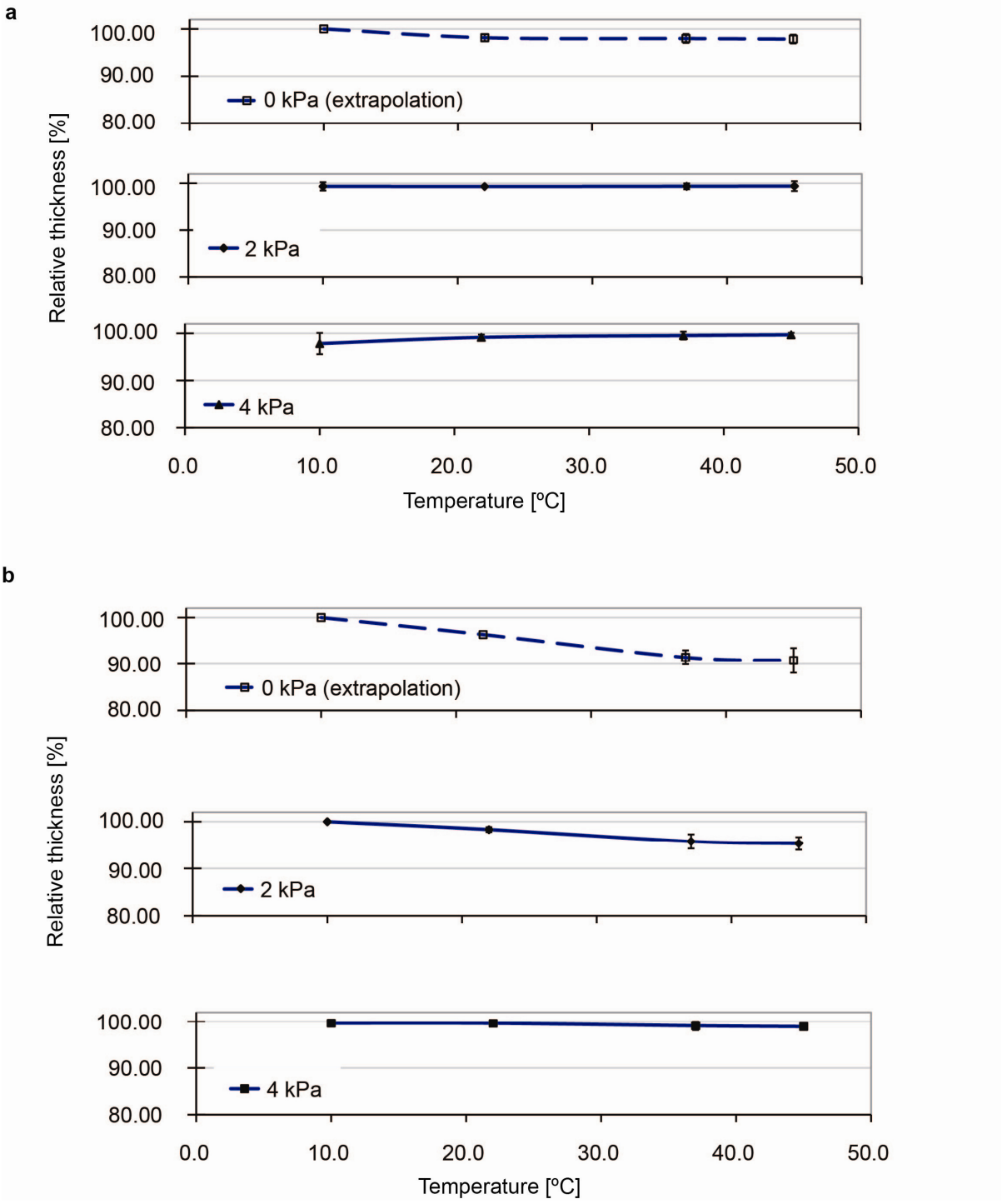
a, The number of RCM1 hESC positive for Nanog, Oct3/4 and DAPI per mm^2 after 7 days of culture on the top 25 polymers, as quantified by a high-throughput IMSTAR imaging system. Bars represent mean ± SD of nine spots per polymer. b, Representative example of Nanog (red), Oct3/4 (green) and DAPI (blue) expression and bright field images of RCM1 hESC cultured for 7 days on a polymer (HG51 DEAEA/DMAEMA (4:12)). Scale bar equals 100μm.

Supplementary Figure S5. Selection of polymer candidates for further analyses.



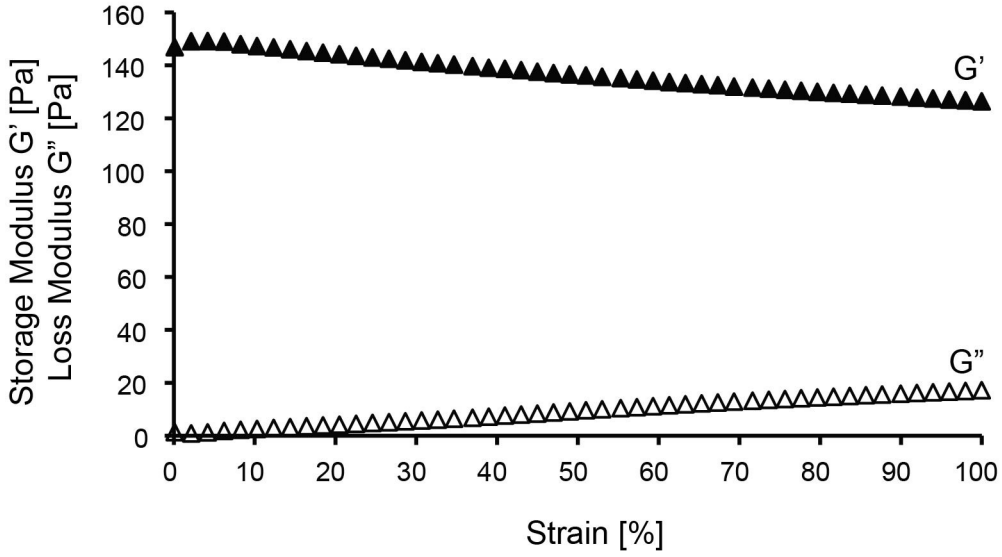
Supplementary Figure S5. Selection of polymer candidates for further analyses. Quantification of total cell number (haematocytometer counts), percent cell viability (trypan blue exclusion) and percent thermo-detachment at 15°C of RCM1 hESC cultured for 7 days on glass coverslips coated with the top 10 polymers or Matrigel™ (MG) and placed in 24-well culture plates. This facilitated selection of a family of three well-performing polymers (red box) for further analyses to assess their ability to support pluripotency and self-renewal of hESCs.

Supplementary Figure S6. Physical properties of two hydrogels (HG19 and HG20) that are related in monomer composition to HG21.



Supplementary Figure S6. Physical properties of two hydrogels (HG19 and HG20) that are related in monomer composition to HG21.
a, HG19 composed of the same monomers as HG21 but in a different ratio, shows a change in relative thickness at lower temperatures in response to actual (2 and 4kPa) and extrapolated (0 kPa) compressive forces, confirming swelling of HG19 at lower temperatures. b, HG20, composed of the same monomers as HG21 and HG19, but in a different ratio, shows a similar change in relative thickness at lower temperatures under the same conditions as HG19 and HG21.

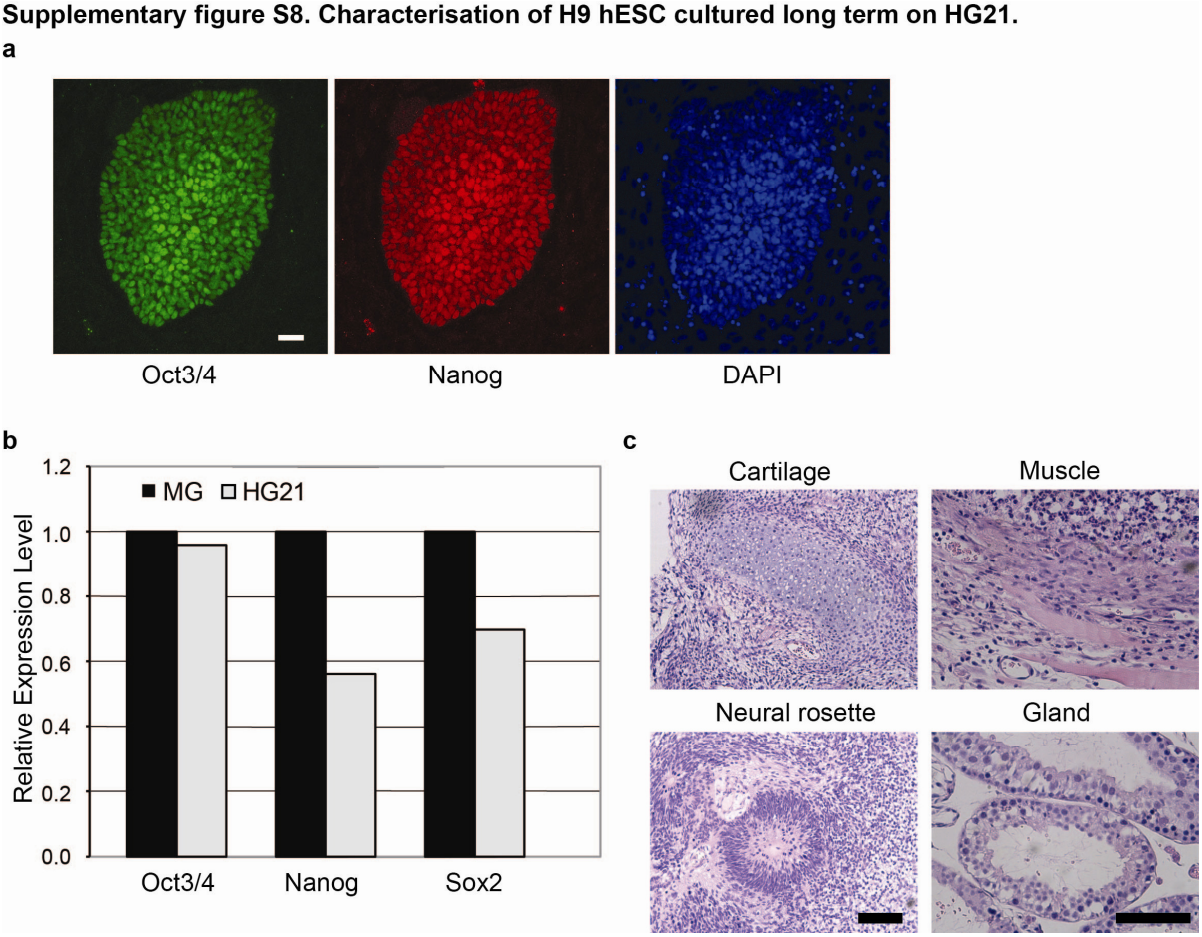
Supplementary Figure S7. Rigidity of HG21.



Supplementary Figure S7. Rigidity of HG21.

The mechanical proprties of HG21 showing the storage modulus (G') and the loss modulus (G'') versus strain %. Rheology analysis was used to examine the rigidity of hydrogels. During the measuement, an oscillatory shear strain was imposed on HG21 at a frequency of 1Hz and the resulting oscillatory shear stress was determined. The results indicate (G') was much greater than (G'') and independent of frequency (less than 1Hz, data not shown). Analysis of HG21 thus showed that the storage modulus was much greater than the loss modulus, a typical property of a hydrogel network, implying HG21 is a soft gel.

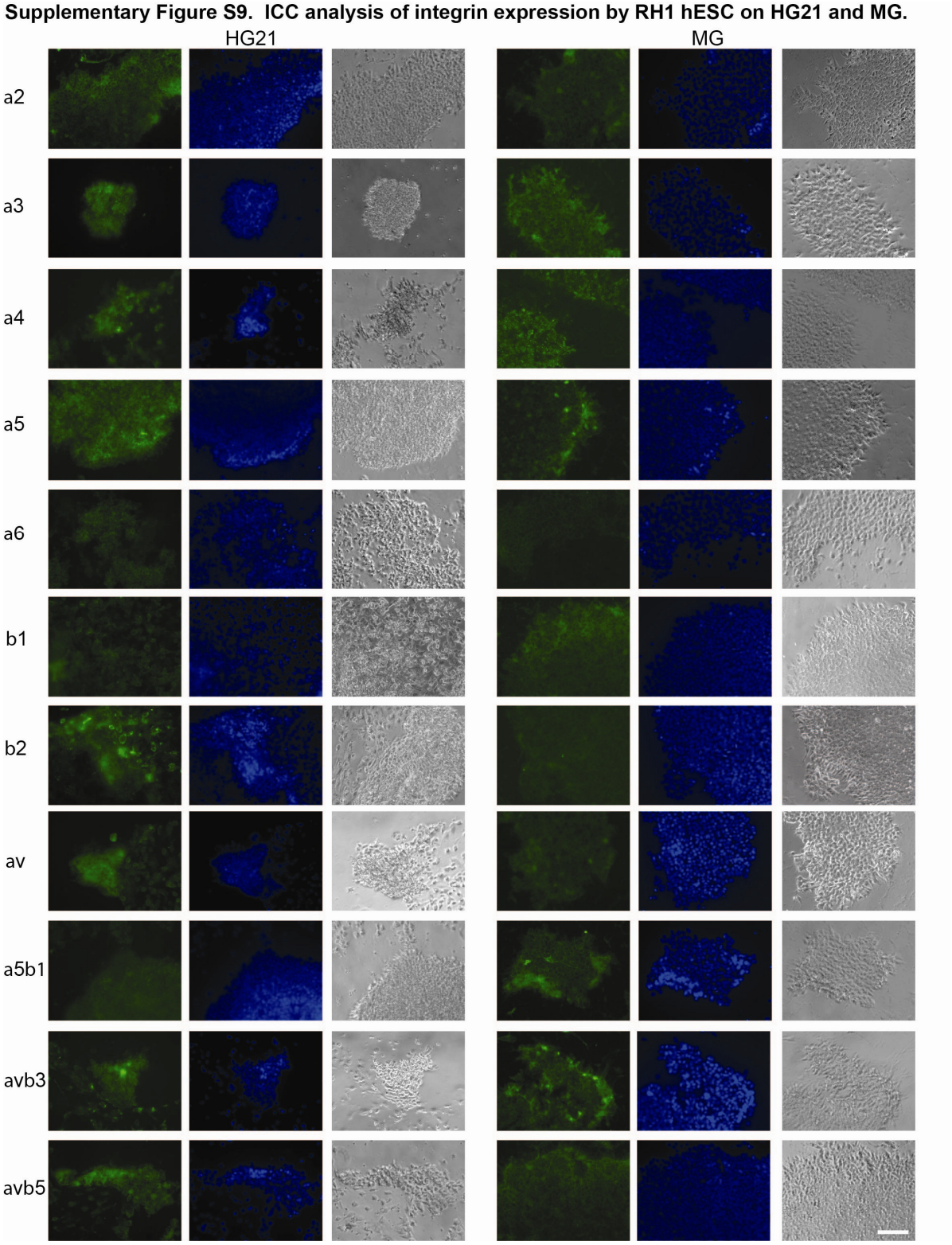
Supplementary figure S8. Characterisation of H9 hESC cultured long term on HG21.



Supplementary figure S8. Characterisation of H9 hESCs cultured long term on HG21.

a, H9 hESCs cultured on HG21-coated cover slips for 6 passages retain expression of transcription factors Oct3/4 and Nanog as assessed by immunocytochemistry. b, H9 hESCs cultured on HG21-coated coverslips for 6 passages retain RNA expression of transcription factors Oct3/4, Nanog and Sox2. RNA levels are expressed as fold change compared with the expression of respective genes in H9 hESC grown on Matrigel ^{TM} (MG) for 6 passages. c, Hematoxylin & eosin staining of sections of teratomas formed in testes of fox chase SCID beige mice following injection of H9hESC cultured on HG21 for 9 passages shows that the teratomas contain derivatives of all three germ layers: Mesoderm (cartilage and muscle), ectoderm (neural rosette) and endoderm (glandular structures). Scale bars equal 100mm.

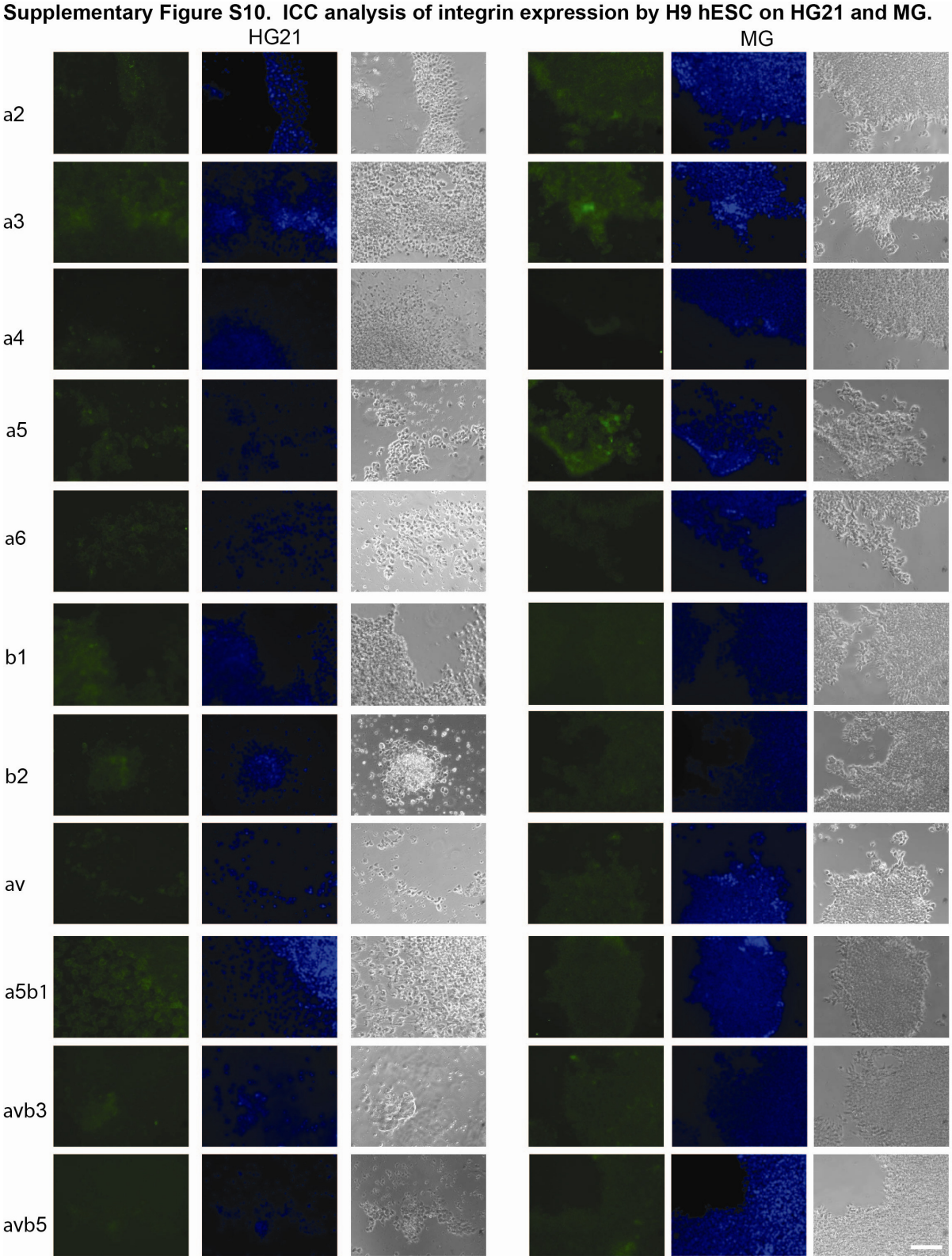
Supplementary Figure S9. ICC analysis of integrin expression by RH1 hESC on HG21 and MG.



Supplementary Figure S9. ICC analysis of integrin expression by RH1 hESC on HG21 and MG.

Representative images of cell adhesion molecules (left column), DAPI stained nuclei (centre column) and phase contrast micrograph (right column) of stained RH1 hESC colonies cultured on HG21 (left set) and Matrigel ™ (MG; right set). Scale bar equals 100 µm.

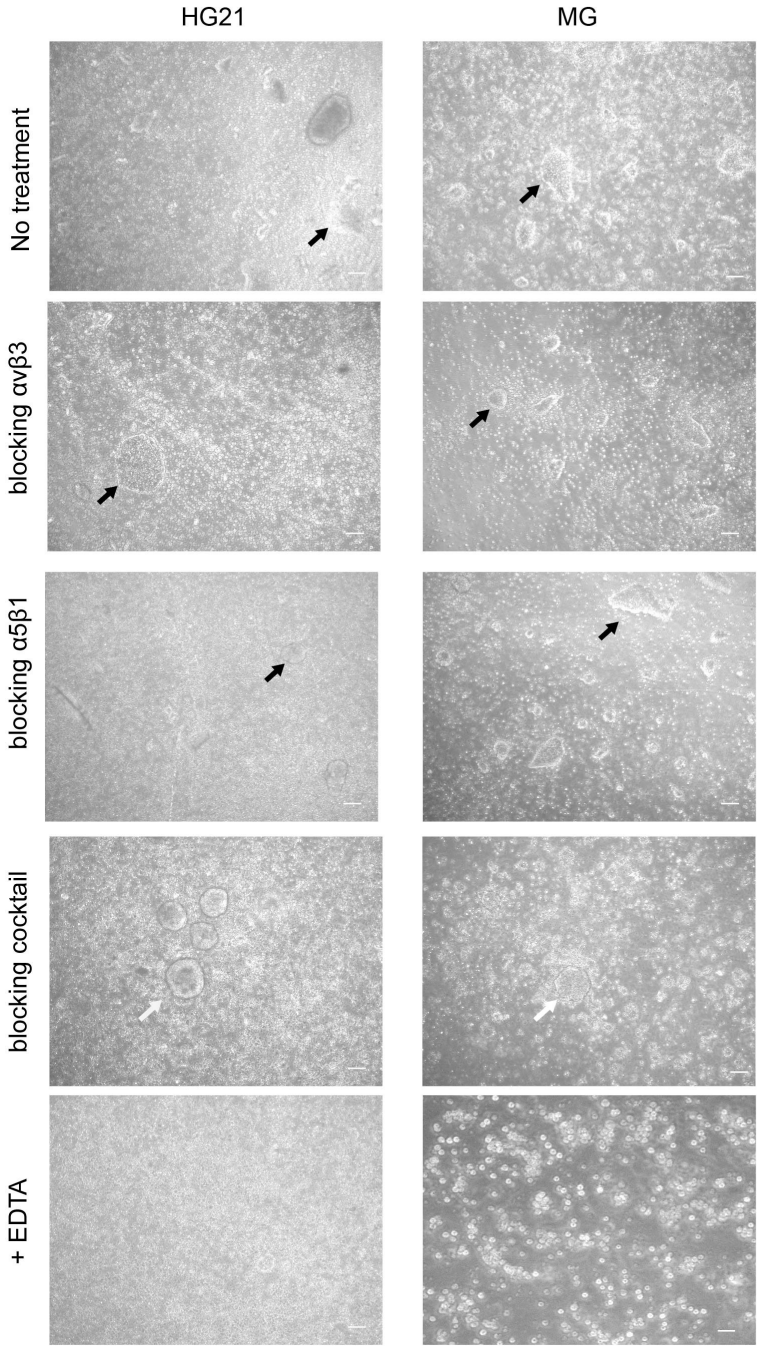
Supplementary Figure S10. ICC analysis of integrin expression by H9 hESC on HG21 and MG.



Supplementary Figure S10. ICC analysis of integrin expression by H9 hESC on HG21 and MG.

Representative images of cell adhesion molecules (left column), DAPI stained nuclei (centre column) and phase contrast micrograph (right column) of stained H9 hESC colonies cultured on HG21 (left set) and Matrigel ™ (MG; right set). Scale bar equals 100 µm.

Supplementary Figure S11. Blocking of cell adhesion molecules expressed by H9 hESC cultured on HG21 and Matrigel™.



Supplementary Figure S11. Blocking of cell adhesion molecules expressed by H9 hESC cultured on HG21 and Matrigel™.
Blocking of cell adhesion molecules expressed by H9 hESC plated on HG21 and MG in mTesR, or mTesR containing EDTA or a pan-interference cocktail of antibodies against these heterodimers and all of the integrins evaluated by Flow cytometry and ICC. Blocking of integrin heterodimers did not affect colony attachment (black arrowheads) similar to the absence of treatment. Pan-interference impaired adhesion to both HG21 and MG resulting in single cells and aggregates of loosely and densely packed cells (white arrows) in suspension. For both substrates, treatment with EDTA also blocked cell-substrate adhesion and cell-cell interaction in suspension. Scale bar equals 100μm.

Supplementary Table 1. CGH analysis report for RH1 and H9 hESC lines cultured on HG21 or Matrigel

hESCs	Chromosome	Start	End	Size (Kb)	Data Points	Genes	Results	Cytoband	Risk of region showing haploinsufficiency
RH1-p66 MG-p25	chr20	29,341,653	30,961,136	1620	66	35	Duplication	q11.21	High (Exocrine pancreatic insufficiency, dyserythropoie)
	chr13	98,032,054	99,010,744	979	77	7	Deletion	q32.2,q32.3	Low
	chr9	68,496,345	69,942,480	1446	13	8	Deletion		
	chr9	45,284,971	46,702,333	1417	18	4	Deletion		Low
	chr9	44,667,132	45,188,993	522	8	1	Duplication		Low
	chr8	7,240,496	8,164,912	924	42	23	Duplication		Low
RH1-p66 HG21-p10	chr20	29,306,527	30,827,881	1521	63	34	Duplication	q11.21	High (Exocrine pancreatic insufficiency, dyserythropoie)
	chr13	97,910,519	99,010,744	1100	86	8	Deletion	q32.2,q32.3	Low
	chr9	44,595,704	45,188,993	593	9	1	Duplication		Low
	chr8	7,276,315	7,880,533	604	37	17	Duplication		Low
RH1-p66 HG21-p21	chr20	29,306,527	30,280,717	974	48	28	Duplication	q11.21	High (Exocrine pancreatic insufficiency, dyserythropoie)
	chr13	97,910,519	99,010,744	1100	86	8	Deletion	q32.2,q32.3	Low
	chr9	45,284,971	45,997,390	712	7	2	Deletion		Low
	chr8	7,217,188	8,164,912	948	44	24	Duplication		Low
H9-p66 MG-P6	chr20	29,401,318	29,951,666	550	20	16	Duplication	q11.21	High (Exocrine pancreatic insufficiency, dyserythropoie)
	chr14	64,939,983	65,442,211	502	19	2	Duplication	q23.3	Low
H9-p66 HG21-P9	chr20	29,401,318	29,951,666	550	20	16	Duplication	q11.21	High (Exocrine pancreatic insufficiency, dyserythropoie)
	chr16	32,000,280	33,365,421	1365	14	6	Deletion		Low

Supplementary Table S1. CGH analysis report for RH1 and H9 hESC lines cultured on HG21 or Matrigel ™.

CGH analysis showed no major (harmful or lethal) changes in karyotype for HRH1 or H9 hESC cultured on HG21 as comopared to control cells cultured on Matrigel TM (MG). Format of first column: Starting cell line + passage number, followed by substrate + passage number.

Supplementary Table S2. qPCR primers.

	Forward	Reverse	T_{ann}
GAPDH	GGCATGGACTGTGGTCATGAG	TGCACCACCAACTGCTTAGC	59 ºC
Oct3/4	AGTGAGAGGCAACCTGGAGA	GCCTCAAAATCCTCTCGTTG	59 ºC
Nanog	AACTGGCCGAAGAATAGCAA	ACTGGATGTTCTGGGTCTGG	59 ºC
Sox2	AGGGGGAAAGTAGTTTGCTGCCTCT	CTGCCGCCGCCGATGATTGT	58 ºC

Supplementary Table S2; qPCR primers.
List of 5' to 3' sequences and annealing temperature for each primer pair.

Supplementary Methods

Protein capture analysis of HG21. Analysis of protein capture by HG21 was achieved by incubation of coverslips coated with HG21 in mTeSR1 for 24h, followed by three washes and gentle pipetting three times with PBS at 37°C or 15°C, respectively. Coverslips were crushed in eppendorfs and proteins extracted with NuPAGE® LDS sample buffer (Life Technologies) at 100°C for 5 min and separated with electrophoresis using NuPAGE 12% Bis-Tris gels (Life Technologies) and MOPS as gel running buffer at 200v for 50 min. Gels were stained overnight with Gelcode blue, de-stained overnight with distilled water and scanned using a Bio-Rad scanner. Band intensities were analysed with Image Lab software (Bio-Rad) and statistical differences between groups calculated with a student t-test (one-tail), with ***: $p<0.001$ and **: $p<0.01$. Selected bands (3 to 7) were analysed further with Mass Spectrometry (Triple TOF 5600, University of St Andrews, BSRC MS and Proteomics Facility). Before analysis samples were digested with trypsin, while cysteine was alkylated with iodoacetamide. Protein identity was confirmed using the BMSSSO fasta database.

Dielectrophoresis. hESCs were dissociated from Matrigel™ by 2 min incubation in collagenase IV and disaggregated by 3 min incubation in T/E, centrifuged at 100*g* for 5min, resuspended and washed twice in 310 mOsm/kg DEP buffer composed of 8.5 % (w/w) sucrose and 0.5 % (w/w) glucose. The pH of the DEP buffer was adjusted to pH 7.4 using NaOH and its conductivity to 33 mS/m using PBS. Conductivity and osmolarity were measured using an OAKTON-CON-510 conductivity meter and an Advanced Instruments Inc. Model 3300 osmometer, respectively. The transition frequency f_{xo} between negative and positive DEP (DEP crossover frequency) was determined for the embryonic stem cells grown on Matrigel™ and HG21 by applying 3Vpk-pk sinusoidal signals between 10kHz and

200kHz, using interdigitated microelectrodes and the methodology described elsewhere[27]. The cell membrane capacitance C_{mem} was calculated using the following expression[27]:

$$C_{mem=} \frac{\sqrt{2}}{2\pi r f_{xo}} \sigma_m \quad (1)$$

where r is the cell radius and σ_m is the conductivity of the DEP suspending medium.

Rheology and X-ray photoelectron spectroscopy. Rheology analysis was performed with a TA instrument (AR-2000, 40mm 4º steel cone) with an oscillation frequency of 1Hz and oscillation stress of 1Pa. HG21 samples were washed (PBS), refreshed every 24 hours for one week and cut to discs (~3 mm thick and ~2 cm in diameter (while kept hydrated). X-ray photoelectron spectroscopy (XPS) analysis was carried out on a Thermo VG Scientific Sigma Probe, with an Al k_α X-ray source.

Comparative genome hybridization analysis. DNA from approximately 3 million cells per condition was extracted from snap-frozen cell pellets using standard phenol/chloroform precipitation. Comparative genome hybridisation was performed using NimbleGen™ 135K v3.0 whole genome tiling array provided by Western General Hospital Cytogenetics unit, Edinburgh, UK. This array features 135,000 probes, 60mer probe length, and median probe spacing of 12524 bp. Copy number variation analysis was performed using Nimblescan software.

Integrin blocking experiments. Following matrix-appropriate cell detachment cells were washed and plated directly in 12 well plates (coated with Matrigel™, or containing HG21-

coated cover slips) in mTeSR1 in the absence or presence of 2.5mM EDTA, or following a 15 min pre-incubation at 37ºC with a cocktail of 12 integrin blocking antibodies (Chemicon kit ECM435 and Millipore kit ECM430) or a single antibody blocking $\alpha 5\beta 1$ or $\alpha v\beta 3$ (from chemicon kit ECM435); final concentrations 1/1000). After 18h incubation at 37ºC, wells were assessed independently by two researchers (in double blind fashion) and micrographs taken.