Supplementary Information

Thermoresponsive hydrogel maintains the mouse embryonic stem cell "naïve" pluripotency phenotype

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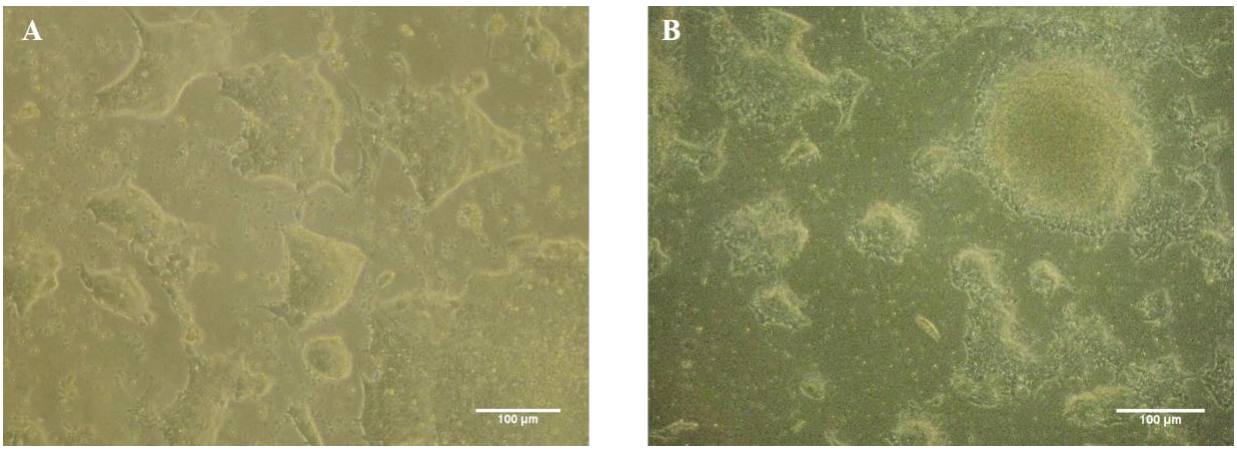

Fig. S1 (A) Brightfield images of HM1 mESCs grown on HG21 in serum-based culture (B) E14TG2a mESCs in defined, serum-free culture. Images were obtained at passage 5 (scale bar = 100 µm).

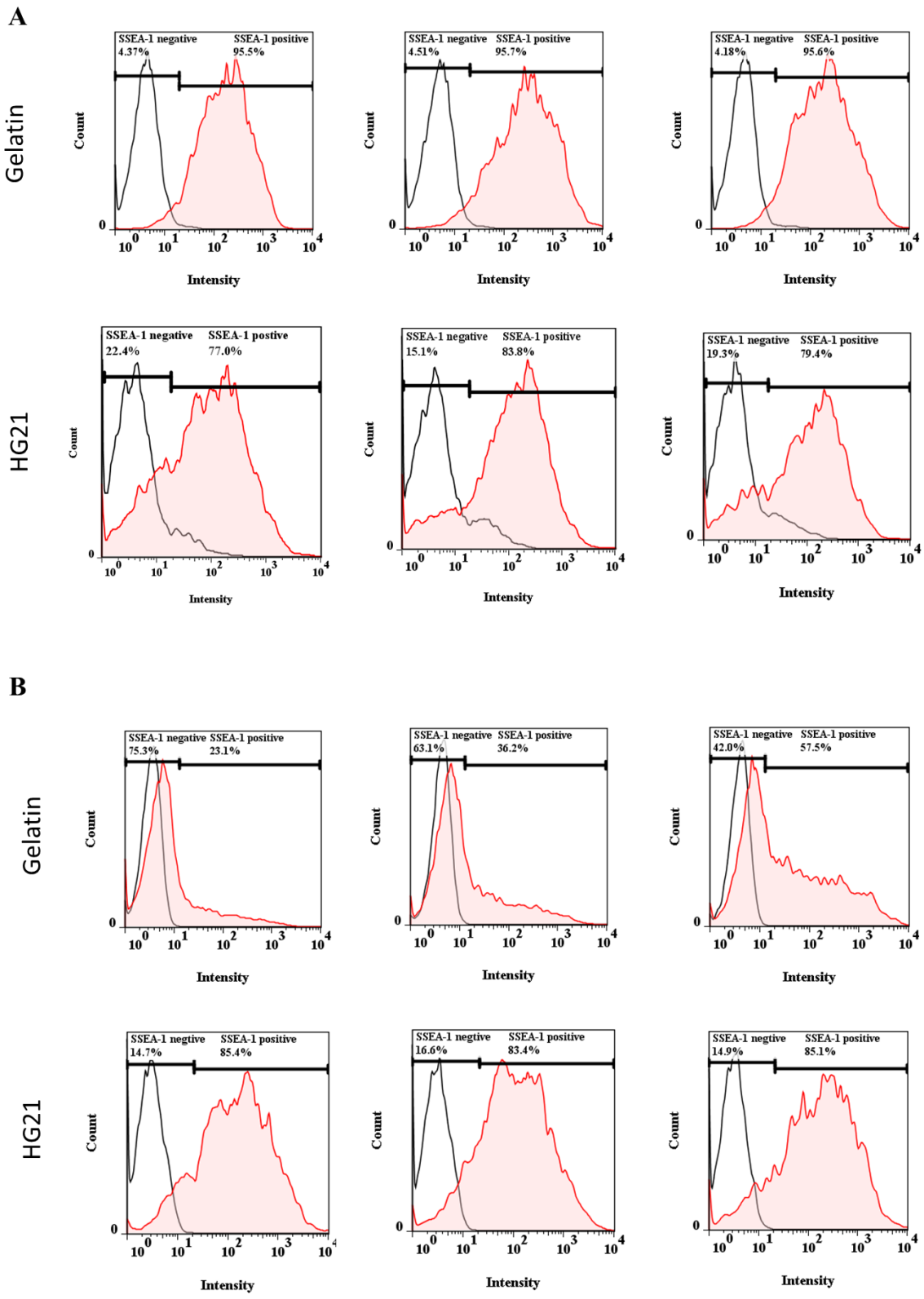

Fig. S2 Flow cytometry histograms (λ_{Ex} 488 nm; λ_{Em} 575 nm) for the distribution of SSEA-1 positive cells (n = 3) at passage 5. (A) Undefined, serum-based culture. (B) Defined, serum free culture.

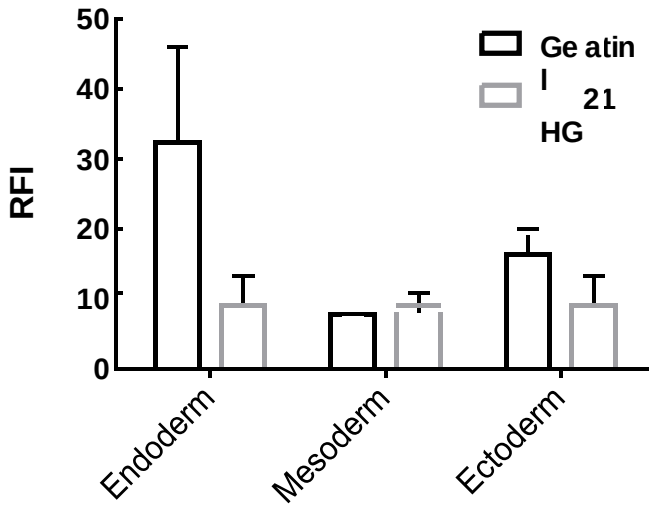

Fig. S3 Relative quantification of fluorescent marker intensity for endoderm (stained for α-fetoprotein), mesoderm (α-smooth muscle) and ectoderm (nestin). For the fluorescent images (n = 2) of the immunostained cells, the fluorescence threshold was manually set (ImageJ, binary contrast enhancement) to identify the area of the marker staining surrounding the nucleus. The mean fluorescence intensity of the area identified was measured and background corrected.

Materials and methods

1. HG21 culture plates

HG21 coated culture dishes were prepared as reported previously.[1] Briefly, 2-(acryloyloxyethyl)trimethylammonium chloride and 2-(diethylamino)ethyl acrylate (3:1 monomer ratio, respectively) with 5% *N*,*N'*-methylene*bis*acrylamide (cross-linker) and 7% 1-hydroxycyclohexyl phenyl ketone (photoinitiator) were added to plasma treated 6-well plates. The plates were covered with polyethylene terephthalate (PET) film and polymerised under UV-light for 20–25 minutes. The PET film was removed and the plates dried at 60 °C for 5 hours, after which the plates were washed with ethanol (× 3), sealed with parafilm, and stored at room temperature.

2. Cell culture

Prior to transfer onto HG21, all cells were cultured on T25 flasks coated with 0.1% gelatin and kept in a humidified incubator at 37 °C with 5% CO_2. 0.05% trypsin/EDTA was used for passaging. HM1 mESCs (acquired from Dr Martin Hooper) were cultured in GMEM supplemented with 10% (v/v) foetal calf serum (Invitrogen), 2 mM glutamine, 1% (v/v) non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), and 10 ng/mL LIF (Millipore). E14TG2a (ECACC #08021401) were cultured in serum free 2i media consisting of N2B27 media with 3 µM CHIR99021 (Calbiochem, Millipore) and 1 µM PD0325901 (Calbiochem, Millipore) with 10 ng/mL LIF (Millipore).[2,3]

3. Long-term mESC culture on HG21

For the long-term culture, both for the HM1 cultured in serum-based media GMEM media and E14TG2a cultured in serum-free 2i media (see above), mESCs were plated at 4×10^5 cells per well on a 6-well plate coated either with HG21 or gelatin, and cultured until confluent (3–7 days). The cells on HG21 were harvested by lowering the temperature to 15 °C for 30 minutes, followed by addition of 2 mL of media/well. The cells were replated in 1:2 splitting ratio. The cells on gelatin were harvested by trypsination (0.05% Trypsin/EDTA). The cells were maintained for 5 passages.

4. Immunostaining

mESCs on HG21 or gelatin were washed with PBS and fixed with 4% paraformaldehyde for 20 minutes. After washing with PBS, the cells were permeabilised with 0.2% (v/v) Igepal (Sigma-Aldrich) for 20 minutes, and subsequently blocked with 10% (v/v) normal rabbit serum (Millipore). Primary antibodies for Oct-4 (Santa-Cruz) at 1:50 and Nanog (R&D) at 1:60 dilution were added, and the plates were incubated overnight at 4 °C. After washing with PBS (× 3), the corresponding secondary antibodies, Alexa Fluor® 488 anti-Oct-4 and Alexa Fluor® 555 anti-Nanog (Invitrogen), were added and incubated for 1 hour at room temperature. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) at 1:1000 dilution for 20 minutes. The cells were visualised using Zeiss Axiovert 200M microscope.

5. Flow cytometry

For the gelatin controls, the cells were harvested with 0.05% Trypsin/EDTA and re-suspended in media. The mESCs grown on HG21 were harvested by lowering the temperature to 15 °C for 30 minutes, and re-suspended in media. The samples were centrifuged at 1100 rpm for 5 minutes. The media was removed and the cells were suspended in 500 uL FACS PBS (0.1% BSA, 0.1% sodium azide in PBS). 100 µL of the cell solution was incubated with 2 µL of PE labelled SSEA-1 (Biolegend) at 4 °C for 20 minutes. The samples were diluted with 2 mL of FACS PBS and centrifuged at 1600 rpm for 5 minutes, the supernatant discarded, and the pellet of cells suspended in 250 µL of FACS PBS. The cells were analysed by FACSCalibur flow cytometer. Flow cytometry histograms were generated using FlowJo.

6. Real time qPCR

RNA was isolated from the mESCs (n = 3) cultured on HG21 and gelatin with Trizol® (Invitrogen). Prior cDNA synthesis, the RNA was treated with RQ1 RNase free DNase kit (Promega). Complementary cDNA was produced using Superscipt III kit (Invitrogen). Amplification of cDNA was done for 40 cycles using Cyber® green polymerase (Thermo Fisher) (n = 3). For the primers and annealing temperatures, see Table S1. cDNA amplification was normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and quantified as $2^{-\Delta Ct}$.

Table S1. qPCR primer sequences and annealing temperatures.[4–13]

Gene	Forward Sequence	Reverse Sequence	Annealing
GAPDH	TCCCACTCTTCCACCTTCGATGC	GGGTCTGGGATGGAAATGTGAGG	58/60 °C
Oct-4	GCAGAAGAGGATCACCTTGG	GATGGTGGTCTGGCTGAACA	60 °C
Nanog	ACCAGTGGTTGAAGACTAGCAAT	CTGCAATGGATGCTGGGATA	60 °C
Sox-2	GCACATGAACGGCTGGAGCAACG	TGCTGCGAGTAGGACATGCTGTAGG	58 °C
KLF4	TTCTCCACGTTCGCGTCCGG	ACGCCAACGGTTAGTCGGGGC	60 °C
Rex1	AGGCCAGTCCAGAATACCAG	GGAACTCGCTTCCAGAACCT	58 °C
Esrrb	TAGGGGTTGAGCAGGACAAG	CTACCAGGCGAGAGTGTTCC	58 °C
Tbx3	CCACCCGTTCCTCAATTTGAACAG	CGGAAGCCATTGATGGTAAAGCTG	58 °C
Dax1	CAGATCCGCTGAACTGAACA	CTACGACCGCTTTCTCCATC	58 °C
FGF4	CGTGGTGAGCATCTTCGGAGTGG	CCTTCTTGGTCCGCCCGTTCTTA	58 °C
Gata6	ATGGCGTAGAAATGCTGAGG	TGAGGTGGTCGCTTGTGTAG	58 °C
Nodal	CGGTTCTCATGCTCTACTCCAACCG	GGCTTCTGTCTGGCAAATGATG	58 °C
FGF5	ACTCCATGCAAGTGCCAAAT	CACTCTCGGCCTGTCTTTTC	58 °C
Brachyury	GAACCTCGGATTCACATCGT	TTCTTTGGCATCAAGGAAGG	58 °C
Sox17	GGCGCAGCAGAATCCAGA	CCACGACTTGCCCAGCAT	58 °C

7. Embryoid body formation and differentiation

HM1 mESCs cultured on HG21 were harvested at passage 5 by thermo-detachment whilst the cells cultured on gelatin were trypsinated. The cells were suspended in GMEM supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 1% (v/v) non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), and 0.1 mM 2-mercaptoethanol (Invitrogen). The cells were plated on 8-well AggreWell™ plates (Stemcell technologies) 1.2×10^6 cells/well. After 4 days of embryoid body (EB) formation, the EBs were collected and grown on 6-well ultra-low adherent well plates (Stemcell technologies) for 7 days (without LIF). The EBs were then transferred to 0.1% gelatin coated 24-well plates and grown in Advanced RPMI (Invitrogen) supplemented with B27 (2% v/v, Invitrogen.) For endoderm formation, 100 ng/mL of Activin A (R&D) was

added to the media and the cells differentiated for 6 days. For mesoderm formation, the cells were kept in media supplemented with 100 ng/mL of Activin A for 1 day followed by 5 day incubation in media containing 10 ng/mL of BMP4 (R&D). For ectoderm formation, the cells were differentiated for 6 days in media supplemented with 5 µM of retinoic acid (Merck).[14]

The differentiated cells were fixed with 4% paraformaldehyde for 20 minutes, washed with PBS, and blocked with 10% (v/v) normal goat serum (Invitrogen). Primary antibodies for α-fetoprotein (R&D) at 1:20 dilution, α-smooth muscle (Abcam) at 1:100 dilution, and nestin at 1:20 dilution (R&D) were added and the plates were incubated overnight at 4 °C. After washing with PBS (×3), the corresponding secondary antibodies, Alexa Fluor® 488 anti-α–fetoprotein, Alexa Fluor® 488 anti-nestin, and Alexa Fluor® 555 anti-α-smooth muscle (Invitrogen) were added and incubated for 1 hour at room temperature. The nuclei were stained with DAPI at 1:1000 dilution for 20 minutes. The cells were visualised using Zeiss Axiovert 200M microscope. The fluorescence intensity of the staining was quantified using Image J.

8. Statistical analysis

Statistical analysis was performed using GraphPad for standard deviation of replicates and student's t-test calculations.

9. References

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