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A chemically defined thermoresponsive hydrogel, poly(AEtMA-Cl-*co*-DEAEA) cross-linked with *N,N'*-methylenebisacrylamide, which allows enzyme-free passaging, was used as a substrate to culture mESCs under defined and undefined conditions. Analysis of 14 stem cell markers showed that the mESCs remained in a "naïve" state of pluripotency with differentiation potential to form endoderm, mesoderm, and ectoderm derived lineages. These results validate the use of a chemically defined hydrogel for standardised and inexpensive mESC culture.

Due to their multi-lineage potential, embryonic stem cells (ESCs) have immense therapeutic potential for the regeneration of cells and tissues. ESCs are isolated from the inner cell mass of blastocysts and have the potential to self-renew indefinitely with the ability to differentiate into specific cell lineages provided that suitable culture conditions are provided.[1] For their maintenance and growth, ESCs require a suitable substrate onto which cells can adhere, while cytokines in the culture media provide appropriate signalling cues for self-renewal.

Research has predominately focused onto two types of ESCs, mouse (mESC) and human (hESC), although they display different states of pluripotency. mESCs extracted from the pre-implantation blastocysts exist in the so-called "naïve" state,[2] whereas hESCs isolated from similarly staged embryos are described as existing in "primed" state, which differs in growth factor dependence and signal transduction pathway activity necessary to maintain an undifferentiated state.[3] A primed state of pluripotency in humans is comparable to that exhibited by mouse post-implantation embryo epiblast derived stem cell populations (mEpiSC).[2a,4] Both the "naïve" and "primed" state phenotypes can be classified as pluripotent as they are Oct-4, Nanog, and Sox-2 positive.[2a] mESCs are further characterised by the additional "naïve" state markers KLF4, Rex-1, FGF4, Essrb, Dax1, and Tbx3, whereas mEpiSCs express FGF5, nodal, Gata6, Sox-17 and Brachyury.[2d,4,5] mESCs respond to LIF/STAT3 signalling and are typically grown on gelatin in serum with leukaemia inhibitory factor (LIF) or in serum-free media with LIF and GSK-3β and MAPK/ERK inhibitors (so called 2i media).[2c,3,6] mEpiSCs, which are also cultured on gelatin, respond to FGF/Activin A/nodal signalling pathway.[3] hESCs, also respond to FGF/Activin A/nodal signalling pathway but are typically grown on Matrigel™ in serum free media with growth factors, basic fibroblast growth factor (BFGF) and transforming growth factor beta (TGFβ).[4,7] hESCs can be converted to the naïve state of pluripotency responding to LIF/STAT3 signalling by the use of LIF, GSK-3β and MAPK/ERK inhibitors, and Forskolin (adenyl cyclase activator).[8,9] In the "naïve" state, hESCs have a higher capacity for differentiation into endoderm.[9]

Their availability, "naïve" state phenotype, and the ease of producing genetically manipulated phenotypes make mESCs good models in embryology, in research for genetic disorders, and cancer.[4,10] For example, both mESCs and hESCs can produce teratomas, and during differentiation they undergo chromatin changes altering accessibility to *e.g.* developmental genes like HoxB locus, a process that also occurs during cancer development.[11] Although mESCs cannot be applied in regenerative medicine, they can be used as a readily available cell line for developing protocols and applications for hESCs. For example, dopamine-producing neurons have been generated from mESCs and subsequently used to produce neurons in dopamine deficient rodents.[12]

The maintenance and expansion of mESCs and hESCs *in vitro* remains a challenge. To replace animal derived gelatin or Matrigel™, research has focused on the identification of synthetic substrates, such as polymer and peptide-based materials, to allow more defined culture systems.[13] The self-renewal and differentiation potential of ESCs is determined by

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both the physical (*e.g.* stiffness, roughness) and chemical properties (*e.g.* charge, hydrophilicity) of these materials.[14] Polymer-based materials also have potential as 3D scaffolds and they can be modified to release or immobilise bioactive molecules such as peptides and cytokines, which can control stem cell fate.[14b] Many examples of polymers as substrates for ESCs exist; however, they often rely on the addition of adhesion proteins, such as fibronectin and vitronectin, and require enzymatic or mechanical treatment for passaging.[15]

Hydrogels are attractive substrates for ESCs as their structure can be tuned to be biocompatible with thermo-responsive properties.[16] Mouse embryonic fibroblasts have been cultured on 3D poly(*N*-isopropylacrylamide) microbeams, which upon temperature reduction undergo swelling thus releasing the cells.[17] Recently, we reported a hydrogel HG21 (Fig. 1), a copolymer 2-(acryloyloxyethyl) trimethylammonium chloride (AEtMA-Cl) and 2-(diethylamino)ethyl acrylate (DEAEA) (with the cross-linker *N,N'*-methylenebisacrylamide), which supported long-term culture (>20 passages) of hESCs in serum-free media without the addition of adhesion proteins.[13a] Furthermore, this hydrogel allowed gentle, enzyme free passaging via thermo-modulation. Here, the hydrogel HG21 was investigated as a substrate for mESCs with the cells evaluated for "naïve" and "primed" state markers.

The first objective of the study was to verify whether HG21 had the ability to support growth of mESCs in their naïve state of pluripotency. mESC cell lines HM1 and E14TG2a were grown on HG21 in undefined (serum-supplemented GMEM) and defined (serum-free 2i media) conditions, respectively. For the cells on HG21, thermo-detachment (15 °C for 30 minutes) was used for passaging, while the control cells on gelatin required enzymatic passaging (trypsin/EDTA). The cells were maintained for 5 passages during which the mESCs cultured on HG21 grew with "dome shaped" morphology (a characteristic of mESCs) similar to the cells on gelatin (ESI Fig. S1).

After continuous culture for 5 passages on HG21 in undefined or defined media, the pluripotency of the mESCs was investigated by immunostaining for the markers Oct-4 and Nanog (the key regulators for pluripotency in all ESCs), and for SSEA-1 (stage-specific embryonic antigen 1, also known as CD15 or Lewis^X), a specific surface pluripotency marker for mESCs. SSEA-1 is a cell surface carbohydrate antigen, which is involved in cell–cell interactions during development and is strongly expressed in mouse embryos at the pre-implantation stage and in undifferentiated mESCs in which it is involved in the formation of compact colonies.[18] Immunostaining and fluorescent imaging of the mESCs, both in undefined and in defined culture, showed the clear presence of Oct-4 and Nanog on cells grown on both HG21 and gelatin, proving that HG21 maintained mESCs in a pluripotent state (Fig. 2). Flow cytometry analysis of the mESCs maintained in undefined

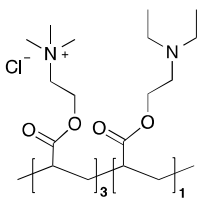

Fig. 1 HG21 is a random copolymer of 2-(acryloyloxyethyl) trimethylammonium chloride (AEtMA-Cl) and 2-(diethylamino)ethyl acrylate (DEAEA) polymerised in a 3:1 monomer ratio, respectively, with 5% of *N,N'*-methylene*bis*acrylamide as a crosslinker.

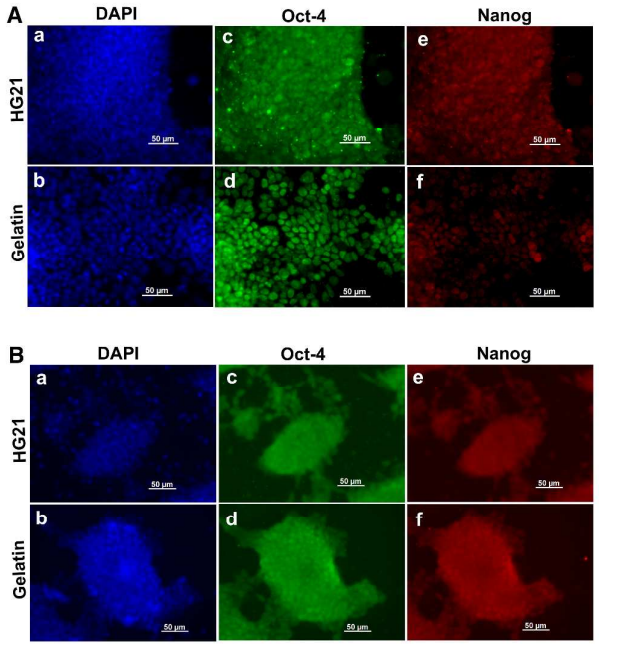

Fig. 2 Immunostaining of mESCs cultured on HG21 or gelatin for pluripotency markers Oct-4 and Nanog (Scale bar = 50 µm). (A) HM1 mESCs in undefined, serum-based culture stained for DAPI (blue, $\lambda_{Ex/Em}$ 364/454 nm), (c–d) Oct-4 (green, $\lambda_{Ex/Em}$ 490/525 nm), (e–f) Nanog (red, $\lambda_{Ex/Em}$ 555/580 nm). (B) Immunostaining of E14TG2a mESCs in defined, serum-free culture stained for (a–b) DAPI (blue), (c–d) Oct-4 (green), (e–f) Nanog (red).

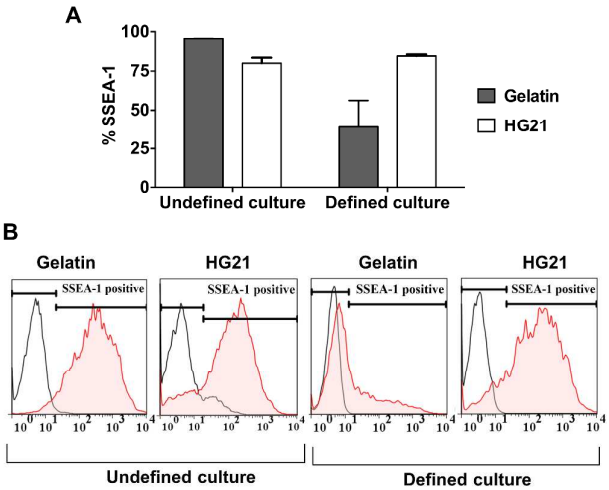

Fig. 3 Flow cytometry analysis (λEx/λEm 488/575 nm, n = 3) of SSEA-1 expression on mESCs (passage 5) cultured on HG21 (thermo-detachment) or gelatin (trypsination). (A) The percentage of SSEA-1 positive cells in undefined, serum-based culture (HM1) and in defined, serum-free culture (E14TG2a). (B) Representative flow cytometry histograms (x-axis = fluorescence intensity). The population stained for SSEA-1 is shown in red and the grey line represents unstained population.

media showed high levels of SSEA-1 (80% on HG21, 96% on gelatin, n = 3) (Fig. 3, ESI Fig. S2A). mESCs cultured on HG21 in defined media showed high expression of SSEA-1 (85%); however,the cells cultured on gelatin showed a notably lower marker expression (39%) (Fig. 3, ESI Fig. S2B). The apparent low expression of SSEA-1 on mESCs on gelatin may be partly attributed to the use of trypsin passaging, which is likely to remove/damage cell surface markers particularly in defined culture, which lacks serum protecting factors (protease inhibitors, α1-antitrypsin) against trypsin. This highlights the potential advantage of the thermo-detachment protocol, where the mESCs are passaged with minimum interference allowing better recovery after passaging.

To further explore the pluripotency of mESCs and to determine if the cells had remained in the naïve state during culture on HG21, the cells were analysed for pluripotency and naïve state gene markers by qPCR. At passage 5, RNA was isolated from the mESCs and the corresponding cDNA amplified for pluripotency markers Oct-4, Nanog, and Sox-2, and naïve state markers KLF4, Rex1, Tbx3, Essrb, Dax1, and FGF4. In addition, the expression of differentiation/mEpiSC state markers Nodal, FGF5, Gata6, Sox17, and Brachyury was investigated. qPCR analysis showed that the pluripotency and naïve markers were present on the cells grown both on gelatin and HG21, both in undefined and defined conditions, as shown by the expression of pluripotency markers Oct-4, Sox-2 and Nanog, and naïve markers KLF4, Rex1, Esrrb, Dax1, Tbx3, and FGF4 (Fig. 4). Nanog and Oct-4 are known to actively target transcribing genes promoting the naïve state,[19] especially Nanog, which is central in defining mESC pluripotency, is known to target Rex1 and Esrrb for activation,[20] which are further augmented by Oct-4 and Sox-2.[20b] These naïve markers along with the pluripotency markers were in fact higher in mESCs cultured on defined, serum-free media (Fig. 4B).

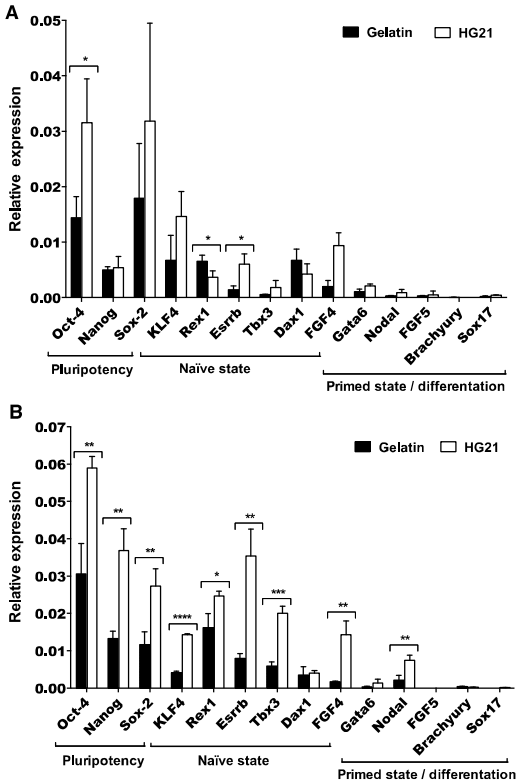

Fig. 4 qPCR analysis of pluripotency mRNA in mESCs cultured on HG21 and gelatin normalised to GAPDH (quantified as $2^{-\Delta Ct}$) (biological samples n = 3, PCR replicates n = 3). (A) Undefined, serum-based culture. (B) Defined, serum-free culture (****p ≤ 0.0001; ***p ≤ 0.001; **p ≤ 0.01; *p ≤ 0.05).

The cells showed low expression of differentiation/mEpiSC markers FGF5, Gata6, Sox17, and Brachyury, especially under defined culture. Nodal, although at a low expression levels, was present on cells cultured both under undefined and defined conditions, as autocrine endogenous activity of Nodal may promote mESC propagation.[21] The gene expression profile of mESCs in defined culture showed a significantly higher expression of Oct-4, and Nanog on HG21 compared to cells grown on gelatin (Figure 4B). Similarly in defined culture, expression of naïve markers KLF4, Rex1, Essrb, Tbx3, and FGF4 was significantly higher on HG21 compared to the mESCs grown on gelatin. The high levels of these genes in mESCs grown on HG21, implies that the substrate is better than gelatin in maintaining pluripotency and the cells are more actively transcribing genes involved in the naïve state of pluripotency than genes involved in differentiation. Under defined conditions, as there is no interference from the serum component, the differences in marker expressions were more evident between the two substrates compared to the mESCs grown in undefined conditions. The relative expression of the pluripotency and naïve state genes is higher in defined serum-free culture, which may have interesting implications for future work with induced naïve hESC.

After 5 passages on HG21 in undefined media, the mESCs were selectively differentiated into endoderm, mesoderm, and ectoderm. After embryoid body formation, treatment with Activin A, Activin A with BMP4, or retinoic acid induced endoderm, mesoderm, and ectoderm formation, respectively. Immunostaining for α-fetoprotein (marker for endoderm), α-smooth muscle (mesoderm), and early neural marker nestin[22] (ectoderm) confirmed that the mESCs cultured on HG21 had maintained their pluripotent potential to differentiate into all the three lineages (Fig. 5). Overall, the degree of differentiation was comparable to cells grown on gelatin; however, relative quantification of the differentiation suggested better endoderm formation with the cells grown on gelatin (ESI Fig. S3).

Conclusions

HG21 was able to sustain mESCs culture while allowing gentle passaging without the use enzymatic treatment, whilst keeping mESCs in the naïve state of pluripotency – in effect, HG21 is a replacement for gelatin in mESC culture. The maintenance of the naïve state phenotype on HG21 was proven by qPCR analysis of 14 genes involved in pluripotency and naïve and

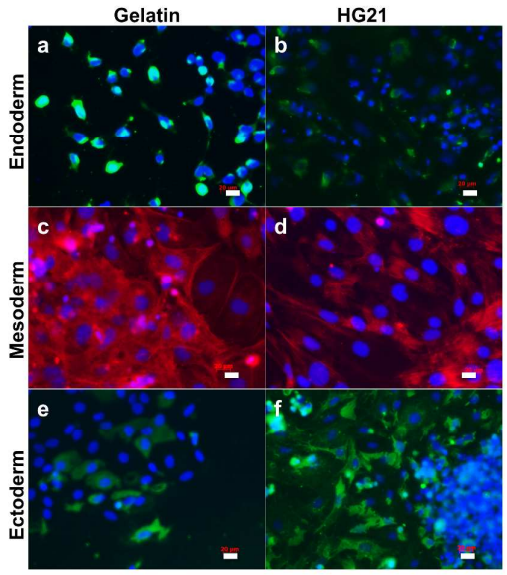

Fig. 5 Immunostaining of HM1 mECSs cultured on HG21 and gelatin after selective differentiation into endoderm, mesoderm and ectoderm. (a–b) Endoderm stained for DAPI (blue, $\lambda_{Ex/Em}$ 364/454 nm) and α-fetoprotein (green, $\lambda_{Ex/Em}$ 490/525 nm), (c–d) Mesoderm stained for DAPI (blue) and α-smooth muscle (red, $\lambda_{Ex/Em}$ 555/580 nm), (e–f) Ectoderm stained for DAPI (blue) and Nestin (green, $\lambda_{Ex/Em}$ 490/525 nm). Scale bar = 20 μm.

primed states. In undefined culture on HG21, the appropriate markers were expressed in levels comparable to mESCs grown on gelatin, whereas in defined serum-free culture, the expression levels were higher on HG21 for pluripotency and primed state markers compared to gelatin. To fully determine the naïve state of these mESCs, future characterisation should include teratoma or chimera formation and epigenetic testing (X inactivation chromosome profile).

HG21 was originally developed as a substrate for long-term hESC culture. The substrate inarguably plays an important role in the maintanence of both the "primed" hESCs and "naïve mESCs"; however, the naïve and primed states also rely on specific cytokines that are required in maintaining these phenotypes. Depending what culture conditions are used, specific signalling pathways are activated and the cells can be driven either towards the naïve or primed state. Since HG21 supported the naïve phenotype of mESCs, it would be interesting to investigate whether HG21 supports hESCs in their naïve like state under defined conditions, since it is in this state that hESCs undergo more robust differentiation.

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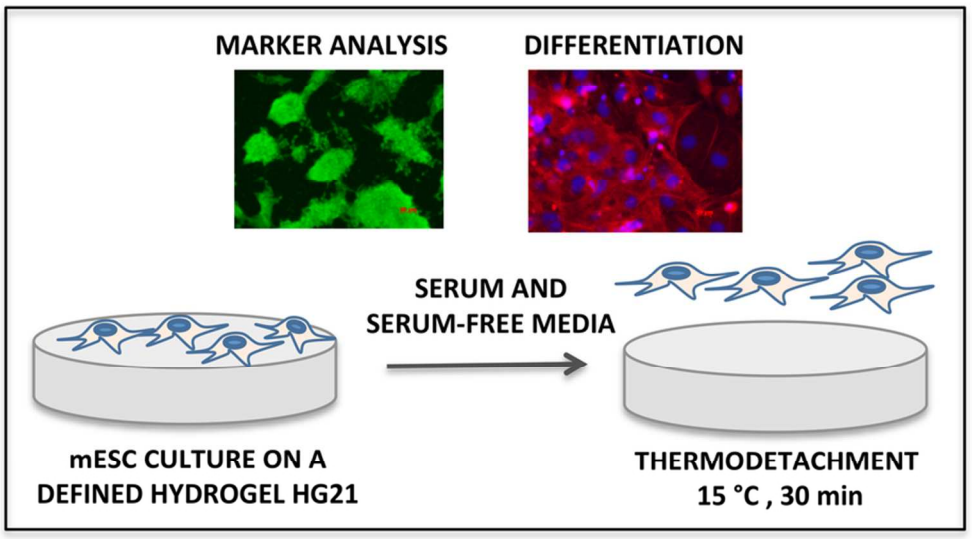
A chemically defined hydrogel HG21, which allows enzyme-free passaging, is a substitute for gelatin allowing standardised and inexpensive mESC culture.



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