ACS APPLIED BIO MATERIALS

Letter

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ACS Appl. Bio Mater., **Just Accepted Manuscript** • DOI: 10.1021/acsabm.8b00686 • Publication Date (Web): 01 Mar 2019

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

A Chemically Well-Defined, Self-Assembling 3D Substrate for Long Term Culture of Human Pluripotent Stem Cells

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KEYWORDS

Xeno-free nanofibrous scaffolds, Ultrashort peptides, Self-assembled peptide hydrogels, Human pluripotent stem cells, Directed 3D differentiation.

ABSTRACT

Clinical applications of human pluripotent stem cells (PSCs) are limited by the lack of chemically well-defined scaffolds for cell expansion, differentiation and implantation. In this study, we systematically screened various self-assembling hexapeptides to identify the best matrix for long-term 3D PSC culture. Lysine-containing Ac-ILVAGK-NH_2 hydrogels maintained best the pluripotency of human embryonic and induced PSCs even after 30 passages. This peptide matrix is also compatible with the use of xeno-free and defined differentiation media. By exploiting its stimuli-responsive sol-gel transition, arrays

of encapsulated PSCs can be bioprinted for large-scale cell expansion and derivation of miniaturized organoid cultures for high-throughput screening.

TEXT

Pluripotent stem cells (PSCs) are steering the development of patient-tailored biomedical applications ranging from cell therapy to tissue models for predicting disease progression and evaluating therapeutic efficacy. Their capacity for self-renewal makes PSCs an important renewable cell source while their expanded cell fate potential facilitates the production of differentiated cells and organoids for cell therapy[1], tissue engineering[2] and drug screening[3]. However, the development of clinical applications is hampered by limitations in substrates for scale-up culture, three-dimensional (3D) differentiation and implantation into human patients.

There is a need for fully-defined and xeno-free cell culture substrates that maintain high proliferative rates while preserving the stemness. To date, the gold-standard for PSC culture is on either inactivated feeder cells or on tissue culture polystyrene coated with recombinant Laminin-511 or Matrigel (solubilized basement membrane preparation

extracted from murine Engelbreth-Holm-Swarm sarcoma). Such 2D cultures are not efficient for scaling-up PSC culture to levels needed for commercial development. While PSCs have been adapted for suspension culture to facilitate efficient expansion in bioreactors[4], not all cells are amenable to the process. The encapsulation of PSCs within a compatible synthetic scaffold or microcarrier would be a viable alternative for supporting the large-scale expansion.

The dynamic complexity of the extracellular microenvironment remains the major challenge in designing ideal 3D PSC culture substrates. The efficacy of Matrigel in both maintaining pluripotency during PSC expansion and supporting differentiation during organoid development implicates the complex interplay of soluble biochemical cues and cell-substrate interactions. In the design of a synthetic matrix, a suitable starting candidate would be a nanofibrous scaffold which mimics the topography of natural extracellular matrix. This criteria is amply fulfilled by a novel class of synthetic ultrashort peptides that self-assemble into biomimetic hydrogels under physiological conditions[5]. The characteristic amphiphilic peptide motif spontaneously organizes into helical fibrils which further aggregate into nanofibers and sheets in aqueous conditions under physiological

conditions. In particular, a subset of hexapeptides containing basic residues demonstrates salt and pH-enhanced self-assembly[6]. This property was harnessed to formulate printable scaffolds that support the growth of stem cells in lieu of Matrigel. Although these synthetic peptides lack ligands for cell attachment, recognition motifs can be easily incorporated using various conjugation strategies. This would also confer better control over the biochemical makeup of the matrix. Peptide synthesis using solid phase chemistry produces a precise, monodisperse product, making the material extremely well-defined chemically. Scaling-up the synthesis to manufacture medical grade product on a commercial scale is relatively trivial due to existing industrial expertise. Thus, when paired with defined media, the peptide hydrogel constitute a completely defined culture environment, free of xenogenic components, for the 3D expansion and differentiation of PSCs.

Identifying candidates that promote stem cell attachment and proliferation. Various self-assembling peptide hydrogel candidates were screened using H1 hESCs to evaluate biocompatibility, cell attachment and proliferation. The candidates chosen were from

three main subclasses – hexamers containing aspartic acid, serine and lysine as the C-terminus polar head group. Members of the lysine subclass were identified to be the most promising substrates (Figure 1A). This suggests that polarity is critical for cell attachment. Cell attachment onto hydrogel-coated tissue-culture polystyrene was observed within 24 hours. By 48 hours, the cells had clustered and begun to proliferate as distinct colonies. At the end of 5 days, dome-shaped colonies were observed on both Ac-LIVAGK-NH_2 and Ac-ILVAGK-NH_2 (Figure 1B). These colonies resemble pluripotent stem cells (PSCs) cultured on feeder layers and on electrospun scaffolds[7], rather than the flat and spread-out morphology observed on Matrigel coatings. It is thus postulated that the nanofibrous microarchitecture of the macromolecular assembly, with its resemblance to native extracellular matrix, influences cell attachment and morphology.

There are several chemically well-defined 2D coatings based on recombinant laminins[8], various polymers[9, 10] and hyaluronic acid[11]. These can be applied as coatings for culturing PSCs on 2D surfaces, as well as to improve attachment to 3D scaffolds for 3D culture. However, this novel class of materials has the advantage of self-assembly into nanofibrous hydrogels, bypassing the need for extraneous 3D supports. The rigidity of

these peptide hydrogels is in the range of 10kPa[6, 12], which supports long term pluripotency maintainence[9].

Encapsulation of human pluripotent stem cells for 3D culture. The lysine subclass of peptidic materials demonstrates stimuli-enhanced gelation, which can be exploited for cell encapsulation and bioprinting. Formulations for encapsulating pluripotent stem cells were designed and optimized. Ac-ILVAGK-NH_2 was selected as the preferred candidate due to its low minimum gelation concentration and rapid gelation kinetics. The optimized peptide concentration for bioprinting was previously determined to be 8mg/mL[6]. The cell suspension in PBS is mixed or co-printed with a 10mg/mL stock peptide solution in a 1:4 ratio. Self-assembly into a clear hydrogel occurs within 5 minutes at 37°C (Supplementary movie), which minimizes the duration of media deprivation. To ensure uniform cell seeding, a single cell suspension was used. However, the viability of PSCs is significantly reduced in both 3D hydrogel and 2D Matrigel cultures. ROCK inhibitor Y-27632 was thus added to the media for the first day to enhance the cell viability. To further improve cell viability, adjustments in formulation can be made. For instance, cells can be re-

suspended in 50% mTeSR or other defined media which do not contain serum components.

Some cell proliferation is observed after one day (Supplementary Figure S1A). Within 48 hours, small distinct colonies are observed (Figure 1C). Over time, the colonies increase in size. On day 5 or 6, some colonies escape from the edge of the hydrogel droplet, as the matrix is no longer able to contain their bulk (Supplementary Figure S1B). Increasing the cell seeding density increases the number of colonies per droplet (Figure 1D). Expanding colonies in close proximity within the droplet also begin to fuse. Discounting the break-away of satellite colonies, the bulk of the hydrogel droplet is stable. It is postulated that the dynamic re-assembly of the peptides around the expanding colony prevents the overall shape from disintegrating under the strong forces exerted by the cells. Transmission electron microscopy (TEM) reveals that the encapsulated cells are very closely packed with significant adhesion interactions between adjacent cells (Supplementary Figure S1C). The dynamic nature of peptide self-assembly also ensures that the expansion of pluripotent stem cell colonies is not confined by the scaffold pore size, like they would be in rigid polymeric scaffolds.

The peptide hydrogel can be bioprinted or moulded into a variety of dimensions following injection into silicon moulds that define the bulk shape. An array of 2.5 μL droplets was selected as the pattern for 3D culture in order to maximise the surface area to volume ratio for diffusion of nutrients and oxygen. Despite so, cell proliferation is slightly slower than in 2D culture, possibly due to reduced diffusion of nutrients and oxygen. The encapsulated cells were passaged at ratios of 1:8 every 5 or 6 days, while 2D Matrigel controls were passaged every 3 days at the same ratio (for H1 cells). This suggests that doubling time is approximately doubled.

The hydrogel adheres well to untreated cover glass surfaces, and the droplets are not easily dislodged during repeated, daily media changes. The optical clarity allows for real-time monitoring of colony growth via microscopy techniques. This bodes well for potential applications in high-throughput screening, as media changes can be performed using liquid handlers. One of the advantages of 3D encapsulation is the entrapment of all the cells in the population, thereby minimizing the loss of poorly-adherent cells, as well as rare phenotypes. There is also better control of the cell density; in 2D culture, the cell density is highly dependent on where the cells attach. On the flip side, dead cells cannot

be automatically separated in 3D cultures, unlike 2D cultures where they detach from the surface and are removed during media changes. Advantageously, it is observed that the cells which are not viable were located at the periphery and did not agglomerate into the growing colonies (Supplementary Figure S1D).

The present class of synthetic peptide matrices addresses the dearth of 3D PSC culture substrates which are easily manipulated. Rigid natural and synthetic polymer scaffolds may replicate the topography of natural extracellular matrix, but their opacity hinders facile observation of cell growth. Furthermore, polymeric scaffolds are very stable, making it difficult to retrieve all the encapsulated cells. Soft hydrogels are ideal candidates as due to the ease of cell retrieval via aspiration. However, many hydrogels, including Matrigel are extremely soft and their structural integrity is weakened by frequent media changes. This results in distortions of the bulk shape over long culture durations. In contrast, self-assembling peptides produces fairly rigid hydrogels [6] which are stable in cell culture conditions while balancing the ease of cell retrieval.

Long term 3D culture of encapsulated pluripotent stem cells. Human embryonic stem cells H1 and H9, as well as induced pluripotent stem cells, were successfully cultured in a 3D format over long durations, with repeated passaging (exceeding 30). To preserve cell viability and minimize the risk of differentiation of cells in the core of the colonies (due to hypoxic and nutrient-poor conditions), passaging is typically done between days 5 and 7. Desired colonies were selected under the microscope and then aspirated with a P1000 pipette. The colonies are washed with half-strength PBS with gentle agitation to soften the surrounding hydrogel. They are subsequently incubated with TrypLE Express for 90 seconds at 37°C. After removing the enzyme solution and replacing with mTeSR containing ROCK inhibitor, the colonies are dissociated by vigorous pipetting for ten times. The cell suspension is diluted with an equivolume of 2X PBS, and then seeded into peptide hydrogel droplets at a 1:8 passage ratio.

Long term encapsulation of the stem cells in the peptide hydrogel did not predispose the cells to differentiate into specific lineages. Cultured in proliferative mTeSR1 media, embryonic and induced pluripotent stem cells maintained their pluripotency, as reflected by the expression of biomarkers via confocal imaging (Figure 2A, Supplementary Figure

S2A). Expression of transcription factors OCT4 and NANOG, as well as surface biomarkers TRA-I-60 and TRA-I-80, are detected in hESC cultured for a single passage and after 30 passages. Analysis of mRNA expression in 3D cultures showed that the genes indicative of pluripotency are comparable to Matrigel cultures (Figure 2B). Karyotype analysis of the cells showed the absence of major chromosomal aberrations, even after 30 passages cultured over 6 months (Figure 2C, Supplementary Figure S2B). When the cells at passage 20 were injected into nude mice, teratomas developed over the course of two months. The tumor tissue consists of cells from all three germ layers (Figure 2D, Supplementary Figure S2C), validating that the cells remain pluripotent after long term culture in the 3D format.

Encapsulation can facilitate scale-up culture of pluripotent stem cells which are not totally compatible with suspension culture in dynamic bioreactors. The hydrogel would serve as a matrix for cell anchorage and shield the expanding colony from the shear forces in rotation culture, which can be detrimental. Besides pluripotent stem cells, the peptide hydrogel may also serve as a potential matrix for other patient-derived cells such as xenografts to support the expansion of difficult-to-culture primary cells. Most

importantly, it would be a viable alternative to Matrigel for cell therapy applications in localising cells to the injection site *in vivo*.

Global gene expression profile of encapsulated cells in the long run. Global gene expression profiling was performed to capture potential changes in the pluripotent cell state (Figure 3). Principle component analysis of the global gene expression profiles shows clustering of the gene expression according to the culture system (Figure 3A). A look at the classes of genes that are differentially regulated shows that genes associated with cell adhesion and cell structure are differentially expressed between the cells that are cultured in 3D versus 2D (Figure 3B). This is expected due to the change in cellular matrix used and the way cells are cultured. Despite differences in cell adhesion, a closer look at the expression profile of pluripotency and differentiation associated genes[13] suggests that the cells cultured in the 3D hydrogel system maintains a pluripotent signature similar to cells cultured on Matrigel (Figure 3C). These results show that the human PSC cultured in our 3D hydrogel system maintain their pluripotent characteristics.

Defined 3D substrate for directed differentiation. The peptide hydrogel does not actively pre-dispose PSCs to either maintain pluripotency or differentiate. The influence of biochemical signals present in the media is integral for pluripotency maintenance and directed differentiation. Incubation of encapsulated cells with different types of induction media drives stem cell differentiation down specific endoderm, mesoderm and ectoderm lineages. In this experiment, the cells were initially incubated with mTeSR containing ROCK inhibitor overnight, before changing to the different induction media for the next 5 days. Cells were stained for representative biomarkers implicated in the early stages of differentiation and were examined under confocal microscopy (Figure 4A). These markers were not detected in the controls cultured in mTESR1 (Supplementary Figure S3). The differentiation into early endoderm, mesoderm and neuronal precursors was further corroborated by mRNA expression analysis using qPCR (Figure 4B). Adaptation and optimization of existing 2D culture protocols would be necessary as the presentation of differentiation factors and chemical gradients would change in 3D culture.

Concurrently, such hydrogels satisfy the niche for 3D substrates that support stem cell differentiation into organoids. Recent developments to mimic various organ stem cell

niches using defined growth factor cocktails have led to rapid advances in developing organoid cultures of brain, retina, lung, stomach, small intestine, liver, kidney and pancreas[3]. Many differentiation protocols for deriving organoids require transition from 2D to 3D culture[14, 15], during which PSC-derived progenitors or adult stem cells are encapsulated within Matrigel[16]. The 3D scaffold, coupled with biochemical cues in the media and cell signaling ligands on the extracellular matrix fibers, stimulate the self-organisation of cells into structures that recapitulate key tissue traits such as the spatial arrangement of heterogeneous cell types, cellular interactions and certain biological processes[17]. These organoid cultures, being more representative of *in vivo* physiology, are promising models to study human organ development and evaluate tissue-specific response to environmental perturbations. In the field of personalized medicine, patient-derived organoids enable the rapid *ex vivo* testing of drug responses on the affected tissue of individual patients[18]. Notably, intestinal organoids prepared from colon biopsies have successfully been used to identify patients who will respond to an experimental cystic fibrosis therapy[19]. While synthetic polymer hydrogels have been used for 3D organoid culture[20], their use for propagating PSCs are unproven. Many such systems

often utilize crosslinking chemistries for sol-gel transition, which can inadvertently alter cell surface chemistries. In contrast, by exploiting self-assembly, peptide scaffolds are foreseeably an easy technology to adopt as a 3D synthetic culture substrate for the large scale culture of pluripotent stem cells for clinical applications.

Conclusion

The development of clinical applications of PSCs relies on concomitant advances in xeno-free 3D substrates for PSC expansion, differentiation and implantation. In this study, we systematically screened various self-assembling peptides and optimized the best candidate for encapsulating H1, H9 and iPSCs for 3D culture. Supported by Ac-ILVAGK-NH_2 peptide scaffolds, all three cell lines maintained their pluripotency over long term culture with repeated passaging. Moreover, the hydrogel matrix does not pre-dispose the cells to differentiate or mutate; stem cell fate is more heavily influenced by chemical factors in the media. As such, the use of self-assembling peptide hydrogels as a culture substrate, when paired with xeno-free media, creates a completely defined culture environment for the expansion and differentiation of PSCs.

Through the design of a chemically well-defined scaffold for 3D PSC culture, this study is a step in the direction of bioprinting PSCs for personalized and regenerative medicine. In the long run, applications in the field of tissue engineering can be expedited by automation and computer-aided design. Moving beyond PSCs, bioprinted peptide hydrogel scaffolds may potentially support the *ex vitro* culture and differentiation of adult organ-derived stem cells and patient-derived cancer cells. Peptide bioinks are thus well-positioned as Matrigel-substitutes to emerging stem-cell based cellular therapies.

FIGURES

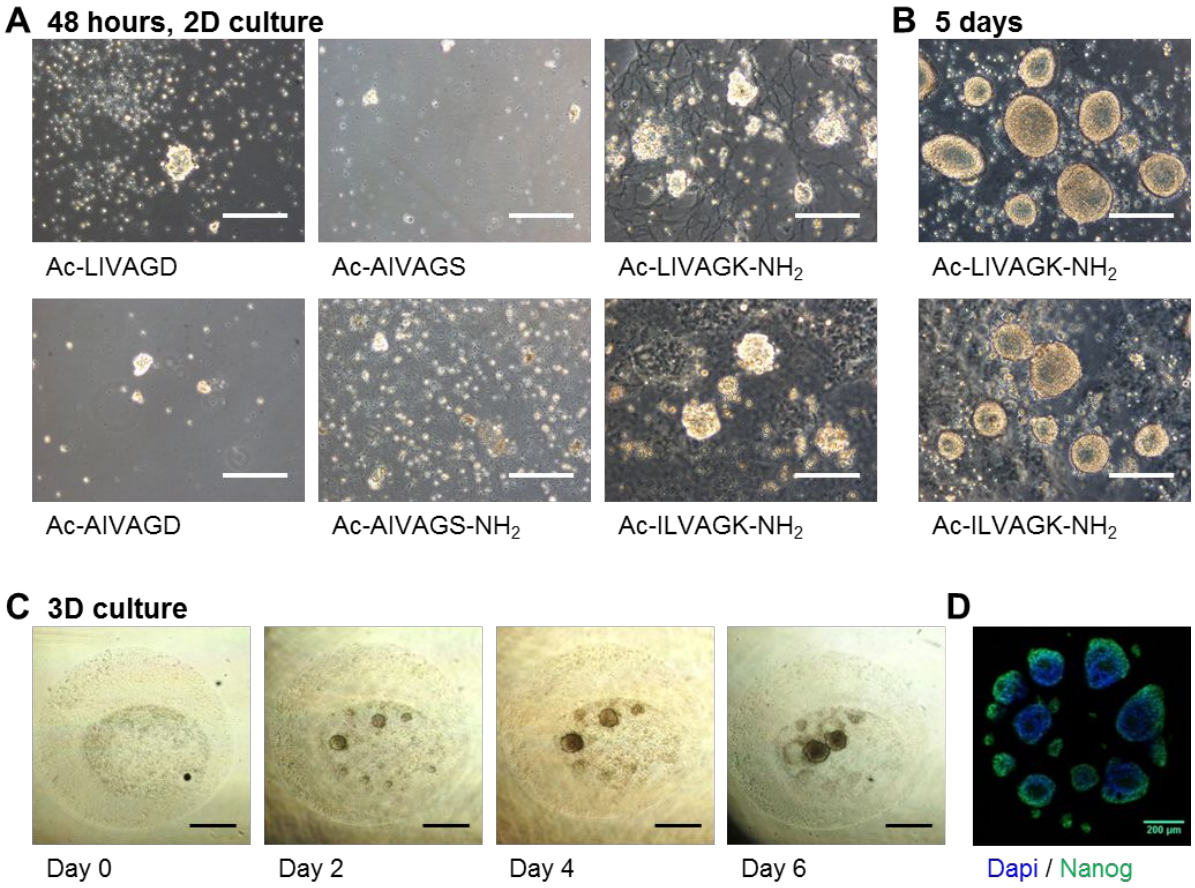

Figure 1. Selection and evaluation of self-assembling peptide hydrogels as culture substrates for pluripotent stem cell culture. (A) Screening of peptide candidates in 2D culture to evaluate cell attachment, proliferation and behaviour. Representative peptides with different C-terminus amino acids were evaluated. Members of the subclass containing lysine were identified to be the most promising substrates that promote attachment and proliferation of H1 human embryonic stem cells within 48 hours. Scale

bar represents 200μm. (B) H1 colony morphology changes over time in 2D culture; after 5 days, the cells stop spreading sideways and grows upwards into a dome-shape reminiscent of cells cultured on feeder layers. (C) Single cell suspension of H1 cells encapsulated in 2.5μL Ac-ILVAGK-NH2 hydrogel droplets. The cells aggregate within the 3D matrix within 48 hours and proliferate to form spherical colonies. Scale bar represents 500μm. (D) The encapsulated H1 embryonic stem cell colonies express the transcription factor Nanog (green), as seen from the Z-projection of a series of confocal images. Scale bar represents 200μm.

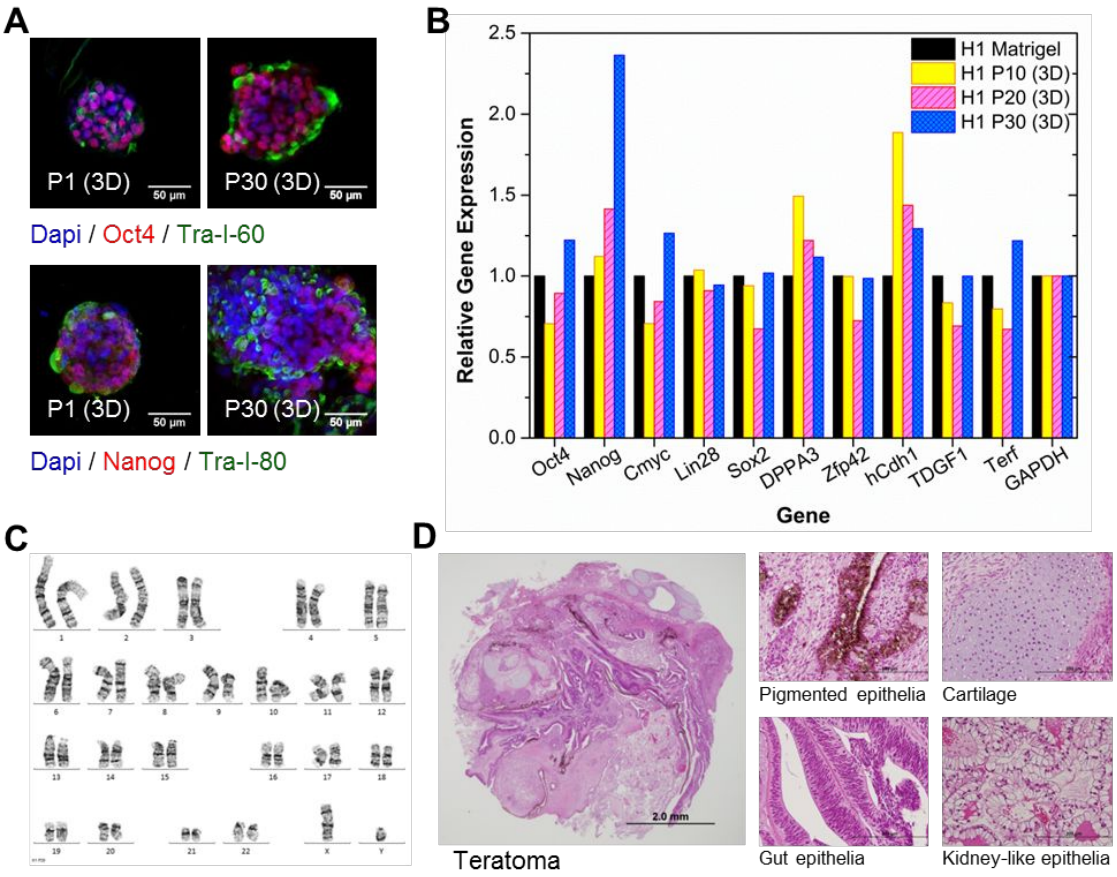

Figure 2. Encapsulated pluripotent stem cells cells maintain their pluripotency, even after long term 3D culture exceeding 30 passages. (A) Expression of pluripotency biomarkers in encapsulated H1 stem cells, verified using confocal imaging at passages 1 and 30. Transcription factors Oct4 and Nanog (in red) are co-localised in the nucleus (blue), while glycans Tra-I-60 and Tra-I-80 (in green) are visualized on the cell surface. (B) The long term expression of genes indicative of pluripotency is comparable to cells cultured on Matrigel, as determined using quantitative PCR. (C) The encapsulated H1 cells which

have been cultured in 3D for 30 passages have normal karyotype, implicating that no major mutations in the form of chromosomal loss, addition or translocation have occurred. (D) Teratoma formation following implantation of H1 cells (after culturing in 3D for 20 passages) into nude mice. Examination of selected areas at higher magnification reveals structures from the three different germ layers. Ectoderm tissue is represented by pigmented epithelia, mesoderm tissue is represented by cartilage, while endoderm tissue is represented by gut and kidney-like epithelia.

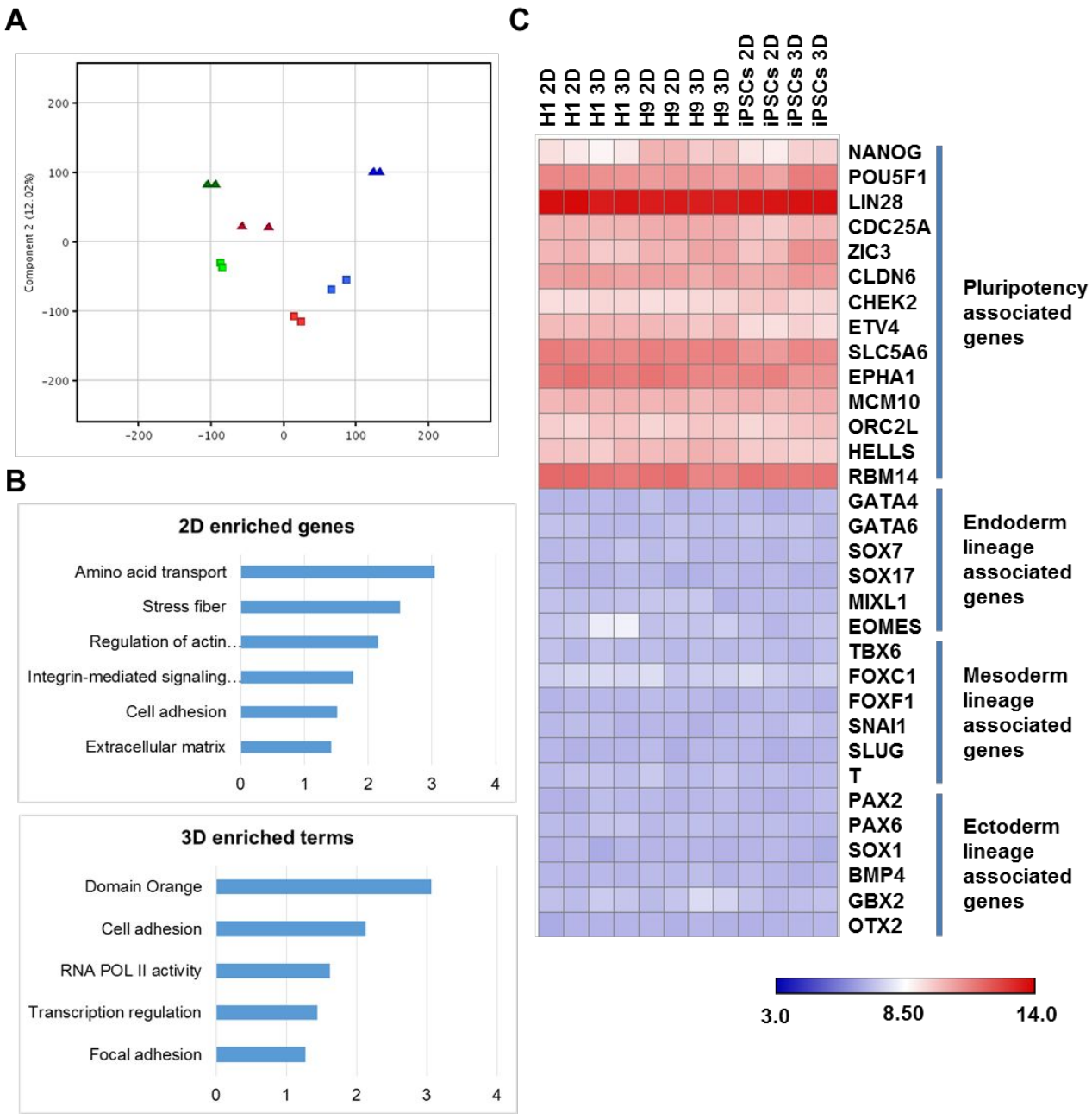

Figure 3. Global expression profiles of human pluripotent stem cells in 2 dimensional and 3 dimensional culture. (A) PCA plot of global gene profiles of two hESC lines and one iPSC line, generated in our previous study[21], were cultured in 2D monolayers on matrigel

or in 3D spheroids in hydrogels over the course of ten passages. PCA analysis shows that the 2D and 3D culture pluripotent stem cells (PSC) display minimal differences in the total gene expression profiles. (B) GO terms of genes enriched in PSC culture in 2D and 3D. Results shows that expression differences between cells culture in the 2 system are largely associated to cell adhesion and cell structure. (C) Expression profiles of genes associated to pluripotency and the 3 germ layers. The PSC culture in both systems shows similar expression patterns for pluripotency and lineage associated genes.

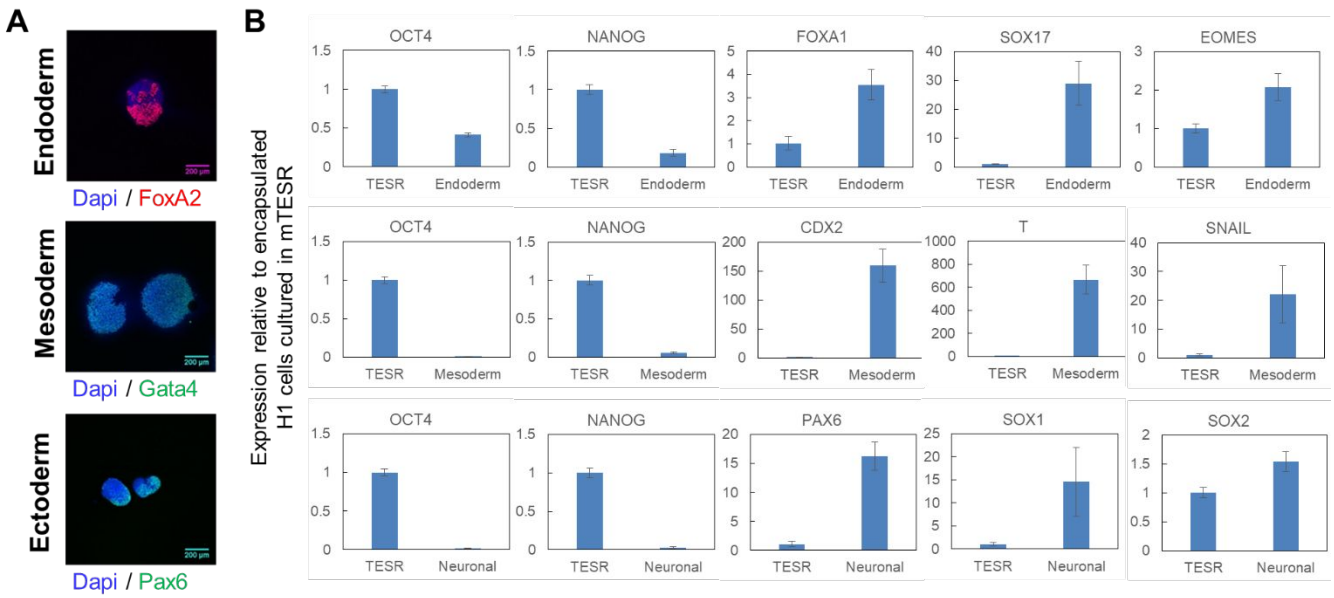

Figure 4. Directed differentiation of encapsulated H1 embryonic stem cells using defined induction media. The matrix is compatible with various types of induction media used. (A) Directed differentiation towards endoderm, mesoderm and ectoderm lineages is respectively reflected by staining of transcription factors FoxA2 (red), Gata4 (green) and Pax6 (green) as visualized under confocal microscopy. These markers are present in the nucleus, co-localising with Dapi (blue) nuclear stain. The scale bar represents 200μm. (B) Quantitative analysis of the mRNA collected from the encapsulated H1 cells corroborated the efficacy of differentiation. Exposure to induction media resulted in down-regulation of pluripotency markers, OCT4 and NANOG in all samples. Colonies cultured in endoderm differentiation media showed concomitant increase in FOXA1, SOX17 and EOMES

expression; mesoderm media induced up-regulation of CDX2, T and SNAIL; ectoderm media induced up-regulation of neural markers PAX6, SOX1 and SOX2.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available on the ACS Publication website (http://pubs.acs.org).

Description of the materials, methods and supplementary figures (PDF file).

Movie demonstrating cell encapsulation by gelation (MOV file).

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Author Contributions

C.A.E.H. conceived the idea of the project. C.A.E.H. together with Y. L. designed the experiments with major inputs from H.H.N. and minor inputs from A.C.A.W. The preliminary screening evaluation and the 3D cell culture were performed by Y. L. and Y.S.C. The confocal microscopy experiments were conducted by Y.L. The teratoma study was done by B.C.P.T. The gene expression and microarray experiments were performed by Y.L., Y.S.C. and I.S. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was supported by the Institute of Bioengineering and Nanotechnology, Genome Institute of Singapore, and Biomedical Research Council (Agency for Science, Technology and Research, Singapore), as well as by grant funding to Charlotte A. E. Hauser from the Agency for Science, Technology and Research (A*STAR) Joint Council Office (JCO) Grant 1231AFG023. Yihua Loo was also supported by the A*STAR BMRC YIG Grant 14/1/07/51/011.

Notes

The animal protocol used was approved by the Institutional Animal Care and Use Committee (IACUC), Biological Resource Centre, (Agency for Science, Technology and Research, Singapore).

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by the Institute of Bioengineering and Nanotechnology, Genome Institute of Singapore, and Biomedical Research Council (Agency for Science, Technology and Research, Singapore), as well as by grant funding to Charlotte A. E. Hauser from the Agency for Science, Technology and Research (A*STAR) Joint Council Office (JCO) Grant 1231AFG023. Yihua Loo was also supported by the A*STAR BMRC YIG Grant 14/1/07/51/011.

The authors would like to thank the Biopolis Shared Facility for the use of their animal facilities, the Institute of Medical Biology Microscopy Unit for the use of their confocal

facilities, as well as Dr. Dharamdajal Kalicharan and Dr Natalia V. Gounko from the IMB-IMCB Joint Electron Microscopy Suite for assistance in preparing and imaging TEM specimens.

ABBREVIATIONS

PSC, pluripotent stem cells; 3D, three dimensional; 2D, two dimensional; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; qPCR, quantitative real-time polymerase chain reaction.

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