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Spider silk for xeno-free long-term self-renewal and differentiation of human pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells (hPSCs) can undergo unlimited self-renewal and have the capacity to differentiate into all somatic cell types, and are therefore an ideal source for the generation of cells and tissues for research and therapy. To realize this potential, defined cell culture systems that allow expansion of hPSCs and subsequent controlled differentiation, ideally in an implantable three-dimensional (3D) matrix, are required. Here we mimic spider silk – Nature's high performance material – for the design of chemically defined 2D and 3D matrices for cell culture. The silk matrices do not only allow xeno-free long-term expansion of hPSCs but also differentiation in both 2D and 3D. These results show that biomimetic spider silk matrices enable hPSC culture in a manner that can be applied for experimental and clinical purposes.

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1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have the unique ability to form any specialized tissue in the human body. These cells therefore represent powerful resources for applications in regenerative medicine and pharmaceutical development. However, several technical challenges must be addressed before hPSCs can be used routinely for clinical therapeutic applications and generation of tissues or organs [1]. First, in order to generate sufficient number of cells, culture systems that are cheap, easy-to handle and chemically defined are needed. Second, mechanically robust 3D matrices that are adaptable, well tolerated by the host and able to regulate stem cell fate commitments have to be developed. Recently, several groups have developed matrices for long-term xeno-free expansion of hPSCs [2–7]. In these reports, hPSCs are maintained on recombinant extracellular matrix (ECM) proteins or synthetic peptides derived from ECM proteins but none of these substrates have been reported to generate 3D scaffolds that support proliferation and differentiation of hPSCs, cf below under 3.4. for further details.

Spider silk is an ideal biomaterial, since it is strong, extendible and is well tolerated and degraded when implanted in living tissues [8,9]. However, spiders are difficult to farm and therefore native spider silk is practically impossible to obtain at large scale. Production in heterologous hosts may be an alternative route to industrial production of spider silk, but this strategy is associated with problems since the spider silk proteins are large and prone to aggregate. Spider silk proteins are composed of an extensive repetitive region flanked by small folded terminal domains that regulate silk assembly [10,11]. The low complexity of the about 3000 amino acid residue long repetitive segment likely contributes both to the impressive mechanical properties and presumed low immunogenicity of spider silk. Despite the technical problems, recent progress has resulted in cost-efficient methods to produce artificial spider silk in heterologous hosts [12]. We have found that a miniature spider silk protein, referred to as 4RepCT, is easily produced in *Escherichia coli*, can be purified to homogeneity and

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assembled into mechanically robust films, foams or up to meter-long fibers under non-denaturing and sterile conditions [13,14]. The 4RepCT matrices are potentially interesting to apply in tissue regeneration since they support self-renewal and differentiation of rat neural stem cells, can be provided with bioactive information by fusion to protein domains and/or peptides and are well tolerated when implanted [15,16]. In the present study, we aimed to develop a biomimetic spider silk cell culture system based on 4RepCT, that enables hPSC long-term self-renewal and subsequent differentiation in a defined xeno-free environment.

2. Materials and methods

2.1. Recombinant spider silk matrices

The recombinant miniature spider silk protein 4RepCT and variants thereof were produced in *E. coli* and purified as described previously [13], including depletion of lipopolysaccharides (LPS) [17].

2.2. hESC cell lines and derivation of human iPS cell lines

hESC lines HS181 [18] and HS360 [19] have been previously described. The hESC cell line H9 [20] was provided by WiCell Research Institute (Madison, Wisconsin). hiPSC lines C5 and C3 were derived using Sendai virus (Invitrogen), a non-integrating method with MOI of 3. Human skin fibroblasts from one female and one male donor were cultured in DMEM supplemented with 10% FBS, 1× Non-essential amino acids (NEAA), and 1× Penicillin–Streptavidin (PEST) (all from Gibco). To generate hiPSCs, approximately 100,000 fibroblasts were transduced and one week later re-plated to irradiated human foreskin fibroblasts (hff, ATCC, CRL-2429). ES-like colonies are visible after 10 days, picked manually and transferred onto irradiated human fibroblasts approximately 3 weeks post-transduction. Both hiPSC lines were cultured in standard hES medium with 8 ng/ml FGF2 during derivation. Medium was changed every day and hiPSC colonies were split manually every five to seven days and extra colonies were frozen in STEM-CELLBANKER solution (ZENOAQ). hiPSCs were split until passage 10 before usage in this study.

2.3. Cell colony forming assay

Around 30,000 hES cells were manually transferred as aggregates to each cell culture plate coated with film made of different functionalized biomimetic spider silk variants or matrigel. After 24 and 48 h incubation the number of colonies was counted under microscope. The fraction of cell aggregates that formed colonies on the coated surface was determined. The mean value and standard deviation of three independent experiments were calculated.

2.4. Long term hPSC cultures

The hESCs and hiPSC lines were cultured on irradiated human foreskin feeder cell layer in Knockout DMEM medium supplemented with 20% knockout serum replacement (KOSR), 1× NEAA, 1× Glutamax, 1× PEST, 0.1 mM β-mecaptoethanol (all from Gibco), and 10 ng/ml FGF2 (R&D), manually dissociated and transferred onto 4RepCT (and variants thereof) films. The cells were then cultured in xeno-free Nutristem medium (Biological Industries) and routinely passaged as aggregates once every 4–5 days using a sterile knife.

For passage as single cells, the hESCs and hiPSCs were treated with Stempro Accutase or 0.05% Trypsin-EDTA (both from Gibco) for 3–5 min at 37 °C, washed in Nutristem medium, and seeded onto freshly prepared VN-4RepCT film or Matrigel in Nutristem medium. 5 μM ROCK inhibitor Y-27632 was added to the medium during the first day after single cell plating.

2.5. RGD competing assay

For the RGD competing assay a cyclic RGD peptide, (cyclo-Arg–Gly–Asp-D-Phe–Lys from Peptides International, Inc. Louisville, KY, USA) was added to the cell culture at a final concentration of 25 μg/ml.

2.6. Cell proliferation assay

For cell proliferation assay, hESCs and hiPSCs cultured on VN-4RepCT film or Matrigel were passaged using Accutase. The cells were briefly washed in D-PBS without Ca and Mg, and then treated with Stempro Accutase solution (Gibco) for 1–2 min at 37 °C. After the Accutase treatment, warm Nutristem medium was added. The cells were gently pipetted 5 times to dissociate the cells, centrifuged at 150× *g* for 3 min, and then resuspended in warm Nutristem medium. One tenth of the total cells were seeded onto freshly prepared VN-4RepCT film or Matrigel in Nutristem medium. To obtain a single cell suspension for counting using an ORFLO Moxi Z automated cell counter, the rest of cells were treated with Accutase for 5 min at 37 °C.

2.7. Immunofluorescence

hPSCs were fixed with 4% (wt/vol) paraformaldehyde for 20 min at room temperature and subsequently washed with PBS for three times. For OCT4 and NANOG immunostaining, cells were blocked with 5% Normal donkey serum (Jackson ImmunoResearch) in PBS containing 0.3% (vol/vol) Triton X-100 for 1 h, and then incubated with primary antibodies diluted in the blocking buffer overnight at 4 °C. Next day, the cells were incubated with secondary antibodies for 1 h at room temperature. After each incubation step the cells were washed with PBS for three times. For SSEA-4 and TRA-1-81 immunostaining, 5% Normal donkey serum in PBS was used as blocking buffer. The primary antibodies used were as follows: rabbit anti-OCT4 (×100, sc-9081, Santa Cruz), goat anti-NANOG (×200, NL-1997G, R&D), mouse anti-SSEA-4 (×20) and mouse anti-TRA-1-81 (×10, both kindly provided by professor Peter Andrews, University of Sheffield, UK), goat anti-human SOX17 (×40, AF1924, R&D), rabbit anti-FOXA2 (×1000, ab40874) and mouse anti-NESTIN (×1000, ab22035, both from abcam), rabbit anti-PAX6 (×200, AB2237, Millipore), mouse anti-cardiac Troponin T (cTnT) (×200, ms-295-p1, Lab Vision), and rabbit anti-NKX2.5 (×100, sc-14033, Santa Cruz).

2.8. Microscopy analysis

Microscopy analysis was performed using a Nikon Eclipse E800 microscope or a Zeiss Axiovert 200M inverted confocal microscope. The software for confocal microscopy was Zeiss Zen 2009. The excitation wavelength and emission filter used were 405 nm and BP420-480IR for DAPI (blue), 488 nm and BP505-530 for green fluorescence, and 543 nm and LP615 for red fluorescence, respectively.

2.9. FACS analysis

hPSCs cultured on VN-4RepCT film were dissociated into single cells by treatment with 0.05% Trypsin-EDTA for 5 min at 37 °C. H9 cells were at passage 20, HS181 and C3 cells at passage 22, HS360 cells at passage 21, and C5 at passage 17 on VN-4RepCT film, respectively. The cells were pelleted by centrifugation at 200 g and then resuspended in FACS buffer (D-PBS without Ca or Mg containing 2% FBS). 1×10^5 cells were incubated with mouse monoclonal antibody against SSEA-1, SSEA-4, TRA-1-60, or TRA-1-81 in 50 μl FACS buffer for 30 min at 4 °C. After subsequent washing with D-PBS without Ca or Mg, the cells were incubated with donkey anti-mouse secondary antibody in 50 μl FACS buffer for 30 min at 4 °C. After two washes in D-PBS without Ca or Mg, the cells were resuspended in 500 μl FACS buffer and analyzed using a BD FACSCalibur flow cytometer. The antibodies used were mouse anti-SSEA4, mouse anti-TRA-1-60, and mouse anti-TRA-1-81 (×10) and Alexa Fluor 488 conjugated donkey anti-mouse IgG antibody (×200, Jackson ImmunoResearch).

2.10. Karyotyping

hPSCs cultured on VN-4RepCT film for 10 passages (H9, C5), 11 passages (HS181), and 13 passages (HS360, C3), respectively, were treated with colcemid KaryoMax (0.025 μg/ml, GIBCO Invitrogen) for 4–5 h or overnight at 37 °C in 5% CO_2. The cells were then treated with 0.05% Trypsin-EDTA for 5 min at 37 °C, pelleted by centrifugation at 150 g for 10 min, resuspended in 0.05 M KCl hypotonic solution and incubated for 40 min at room temperature. An equal volume of fixative (3:1 methanol:acetic acid) was added to the solution. The cells were pelleted by centrifugation at 214× g, washed once and resuspended in fixative solution. The cell solution was dropped onto glass slides and metaphase chromosomes were analyzed under microscope. Totally 25 metaphase spreads were analyzed for each cell line at Labmedicin Skåne, Lund university hospital.

2.11. Teratoma formation

hPSCs cultured on VN-4RepCT film were manually dissociated into small aggregates. We injected H9 cells after 12 passages, HS181 cells after 14 passages, HS360 cells after 13 passages, C5 cells after 14 passages, and C3 after 17 passages on VN-4RepCT. 5×10^5 cells per cell line were mixed with 5×10^5 irradiated human foreskin feeder cells in totally 150 μl Nutristem medium as previously described [21]. 75 μl Matrigel (BD Biosciences) were added to the cell suspension. Cell suspension with only irradiated human foreskin feeder cells was used as control. The whole suspension volume was injected subcutaneously into young SCID mice (4 weeks old, Taconic). Four animals were used per cell line. Teratoma growth was determined by palpation every week and the mice were sacrified when teratoma size reached 1 cm in diameter. The tumor tissue was excised, weighed and fixated for 24 h in 1% formalin and then transferred to 70% ethanol. The tissue was dehydrated, embedded in paraffin and processed into 4 μm thick sections. The sections were stained with hematoxylin and eosin (HE) following standard protocols, and mounted under coverslips.

2.12. In vitro differentiation of hES and iPS cells after long-term culture on VN-4RepCT films

Differentiation towards neuroectoderm was carried out using a previously described protocol [22]. The hPSC lines H9, HS181, HS360, C3, and C5, cultured for over 35 passages on VN-4RepCT film, were seeded as single cells at a density of 18,000 cells/cm^2 on freshly prepared VN-4RepCT film, foam or mesh and cultured for four days. Neuroectoderm differentiation was initiated by treating the cells with

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