**Polymeric nanofibrous substrates stimulate Pluripotent Stem Cells
to form 3-dimensional multilayered patty-like spheroids in feeder-free culture
and maintain their pluripotency**

Mohammad A. Alamein^{1,2}, Ernst J. Wolvetang³, Dmitry A. Ovchinnikov³, Sebastien
Stephens^{1,2}, Katherine Sanders², Patrick H. Warnke^{1,2 *},

1. The Clem Jones Research Centre for Stem Cells & Tissue Regenerative Therapies,
Bond University, Gold Coast, Queensland, Australia.

2. Facio-Maxillary and Regenerative Surgery, Griffith University, Gold Coast,
Queensland, Australia.

3. Australian Institute for Bioengineering and Nanotechnology, The University of
Queensland, Brisbane, Queensland, Australia.

Corresponding author

Prof. Dr. Patrick H. Warnke

Chair for Facio-Maxillary and Regenerative Surgery

Griffith University Health Centre, Gold Coast, QLD, 4215, Australia

Telephone: +61 756780855

Email: p.warnke@griffith.edu.au

Key words: nanofibers, electrospinning, PMEDSAH, PLGA, Induced Pluripotent Stem
Cells, iPSC, Human Embryonic Stem Cells, HESC, pluripotency, 3-dimensional,
feeder-free, xeno-free, extracellular matrix

Expansion of pluripotent stem cells in defined media devoid of animal-derived feeder-cell to generate multilayered 3-dimensional (3-D) bulk-preparations or spheroids rather than 2-dimensional (2-D) monolayers is advantageous for many regenerative, biological or disease modelling studies. Here we show that electrospun polymer matrices comprising of nanofibers that mimic the architecture of the natural fibrous extracellular matrix allow for feeder-free expansion of pluripotent human induced pluripotent stem cells (IPSC) and human embryonic stem cells (HESC) into multilayered 3-D “patty-like” spheroid structures in defined Xeno-free culture medium. The observation that IPSC and HESC readily revert to 2-D growth in the absence of the synthetic nanofiber membranes suggests that this 3-D expansion behavior is mediated by the physical microenvironment and artificial niche provided by the nanofibers only. Importantly, we could show that such 3-D growth as patties maintained the pluripotency of cells as long as kept on nanofibers. The generation of complex multilayered 3-D structures consisting of only pluripotent cells on biodegradable nanofiber matrices of desired shape and size will enable both industrial scale expansion and intricate organ-tissue -engineering applications with human pluripotent stem cells, where simultaneous coupling of differentiation pathways of all germ layers from one stem cell source may be required for organ formation.

Pluripotent stem cells (PSC) constitute an exceptional tool in developmental biology, disease modeling and future regenerative therapies (Serra et al., 2012) if they can be produced in large numbers under controlled conditions with clinically-feasible protocols (Serra et al., 2012, Zweigerdt et al., 2011). To enable these applications, culture conditions that would allow for xeno-free or feeder-cell-free PSC expansion on

biodegradable scaffolds that could further be clinically manipulated are essential. Furthermore, it might be of higher importance to improve bioreactor-based culture approaches with finely controlled environmental conditions that would reliably propagate 3-D multilayered cell organization or spheroids of PSC in large scales rather than flat expansions in monolayers (Serra et al., 2012, Rodin et al., 2010). In some areas in particular in tumorigenesis and disease modeling for drug evaluation studies there is a call towards utilizing such 3-D cell spheroids for experiments as they provide a more reliable and “natural” response in such culture conditions (Hutmacher et al., 2009, Loessner et al., 2010, Alajati et al., 2009). However, PSC-based tissue engineering may next require these cells to be dissociated and/or differentiated prior to in vivo delivery depending on the method of delivery. Other options in organ engineering could address the controlled seeding of biocompatible scaffolds with PSC aggregates to provide pluripotent cells in the desired spatial organization within the matrix.

In addition, if it comes to bulk production of PSC for future regenerative procedures, it might be necessary to reliably control the pluripotency of PSC or their differentiation stage until full organ tissue development is triggered. In this context, complex organ regeneration probably requires multiplex orchestrated tissue formation (Brady et al., 2009, Warnke, 2010) from cells of different germ layers that could technically be derived from one pluripotent cell source, if it is available in high numbers and could be manipulated by the tissue-engineer. Under this light, it has been shown that the microenvironment or particular niche of a stem cell can significantly determine its fate and behavior (Rodin et al., 2010, Serra et al., 2012). We therefore hypothesized that by controlling the niche, we would be able to expand PSC in 3-D structures on

biodegradable scaffolds that can be manipulated by clinicians and tissue engineers. We envisaged that following differentiation of the PSC into desired lineages, such scaffolds become the starting tool for tissue engineering.

To this end, we manufactured pristine nanofiber (NF) mesh works from synthetic biodegradable polymers via a nanotechnology process called electrospinning that would allow us to recreate the optimal niches and microenvironments of stem cells (Alamein et al., 2013a, Alamein et al., 2013b) (Figure 1). Such NF matrices are designed to mimic the 3-D collagen fiber morphology and architecture of the natural extracellular matrix to a high extend. Importantly, such NF scaffolds can be produced in large scales and of desired sizes and shapes in a highly controlled and uniformed manner under clinical-grade conditions (Alamein et al., 2013a, Alamein et al., 2013b) (Figure 1). Furthermore, our NF matrices have already shown to allow for improved propagation of 3-D organization of mesenchymal stem cells as the NF provide a pro-attachment surface or potentially a sub-cellular 3-D niche-like microenvironment that is not constricted to the conventional flat 2-D surfaces in tissue culture plates (Alamein et al., 2013a, Alamein et al., 2013b). We assumed that such 3-D NF matrices could also influence the behavior of PSC. So far, the common 2-D culturing monolayer methods for PSC even with attempts in advanced bioreactor cultivation have not yielded an effective method of satisfactory xeno-free up-scaled cell expansion with preserved pluripotency for clinical demands.

For this study we initially set out to manufacture clinically compliant biodegradable NF matrices by electrospinning in a clean-room from the biopolymer Poly[lactic-co-glycolic] acid (PLGA), where the resulting nanofibrous substrate would provide

characteristics of human connective tissue extracellular matrix. We then combined PLGA with the recently described novel synthetic polymer Poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH) (Villa-Diaz et al., 2010, Nandivada et al., 2011) for a novel set of NF composite membranes since PMEDSAH was previously shown to be able to replace feeder-cells in 2-D culture systems for PSC (Villa-Diaz et al., 2010, Nandivada et al., 2011).

We next seeded mechanically harvested induced Pluripotent Stem Cells (IPSC) cultured in mTeSR1 on Matrigel® (BD Biosciences) on NF membranes consisting either of electrospun PLGA or the combination of electrospun PMEDSAH and PLGA (Figure 1c-f). To discriminate between the roles of the 3-D microenvironment and the substrate itself, we used flat 2-D non-structured films of both pure PMEDSAH and pure PLGA as controls. Matrigel was further used as a standard control for feeder-free culturing in 2-D cultures. Importantly, no feeder cells were present in any of the cultures at any time.

Our initial thoughts that PMDSA-free NF cultures may require feeder-cells for thriving was incorrect. In fact, cell indiscriminately grew on the PLGA NF and composite PLGA:PMEDSAH NF. We found that both NF matrices with characteristics of the natural extracellular matrix allowed for increased attachment and up-scaled expansion of IPSC when compared to the flat 2-D culture controls of the same polymers. This effect was based on the NF morphology on both NF matrices and therefore independent from the polymer substrate. Both NF supported adhesion and colony formation of over 95 % of the IPSC aggregates (Figure 1d). Throughout 8 weeks, the structure of both, the composite PMEDSAH:PLGA NF and also the pure PLGA NF,

clearly supported attachment and proliferation while also maintaining cell pluripotency (Figure 1d). iPSC cultivated on NF maintained expression of pluripotency markers and displayed a normal karyotype (Supplementary material Figure 1 a-d). They retained pluripotency as shown with their ability to differentiate into three germ-lines after 8 weeks of cultivation (Supplementary material Figure 1 e & f). In contrast, the flat 2-D film controls of the same material allowed for attachment of less than 10 % of iPSC aggregates only, which furthermore spontaneously differentiated throughout the culture period, suggesting that that the 3-D architecture of the electrospun NF membranes itself was critical for maintaining the iPSC in their pluripotency state.

Remarkably, the NF microenvironment evolved to trigger and support the bulk formation of multilayered 3-D "patty-like" iPSC and HESC colonies or dome shaped spheroids (Figure 1 – 3) that comprised of pluripotent stem cells of normal karyotype (Supplementary material Figure 1). As expected the same iPSC and HESC cultured on the flat 2-D surface of the tissue culture plate coated with Matrigel kept expanding in monolayers (Figure 1 – 3).

Confocal imaging revealed that the iPSC patties continued to express pluripotency markers (TRA-1-81, TRA-160 and SSEA-4 and OCT4) throughout the entire 3-D structure (Figure 2). SEM imaging showed that such patties exhibited multiple cell layers. Robust patties with diameters up to 4 mm in height with smooth, elevated edges were identified (Figure 2). This special behavior to form multilayered patties on NF substrates appears to be exclusive for PSC. Such patty formation has not been seen by us with multipotent mesenchymal stem cells or somatic cells cultivated on the same

materials before (Alamein et al., 2013a, Alamein et al., 2013b). Previous studies have shown activation of Rac1 in mouse ESC cultured on synthetic nanofibers (Higuchi et al., 2010, Nur-E-Kamal et al., 2005, Schindler et al., 2005). Rac1 is a Rho GTPase family member protein known to regulate the actin cytoskeleton (Higuchi et al., 2010, Nur-E-Kamal et al., 2005, Schindler et al., 2005). There is evidence suggesting Rac1 activation might have an underlying effect on enhanced proliferation, differentiation, motility and morphological changes associated by culture of mouse ESC on nanofibers (Higuchi et al., 2010, Nur-E-Kamal et al., 2006, Nur-E-Kamal et al., 2005, Schindler et al., 2005).”

Furthermore and in contrast to established protocols, both iPSC and HESC patties did not require constant passage or presence of feeder-cells as they continued to grow without further passaging on NFs in our set up over 5 weeks. Within the first 3 days, PSC cultivated on NFs were seen to attach and expand into small size colonies before they rapidly started to form such patty-like structures (Figure 1). Patty formation followed an outward-to-inward direction, where within about 3 days the iPSC and HESC colonies seemed to form a multilayered structure starting from outer edges of the colony and expanding inwards to the center of the colony (Figure 1 & 2). PSC patties could be easily lifted from the NFs using a surgical spatula (Figure 1c), or alternatively a 1 ml pipette tip. Alternatively the biodegradable nanofiber scaffolds could be picked up to serve as a transfer vehicle of pluripotent cell patties where differentiation into the desired cell types at other sites is required. We believe that this easy manipulation of PSC on scaffolds would allow for straightforward surgical implantation if required and could have high potential to be adopted for mass production of PSC.

We then reached out to confirm the maintained pluripotency of iPSC cell patties in more detail. Flow cytometry analysis to assess for pluripotency markers was performed for iPSC cultured for:

- 1) A total of 5 weeks on PMEDSAH:PLGA NF or pure PLGA NF followed by another 3 weeks back on Matrigel.
- 2) A total of 8 weeks exclusively on Matrigel.
- 3) As well as 8 weeks exclusively on PMEDSAH:PLGA NF or pure PLGA NF.

All groups showed similar expression level of cell marker proteins OCT4 (> 80 %), SSEA-4 (> 85 %) and SOX2 (> 85 %) (Supplementary material Figure 1 b). Further validation using qPCR revealed similar RNA expression levels of OCT4, KLF and SOX2 across all samples (Supplementary material Figure 1 c). Immunostaining revealed expression of OCT4, SSEA-4, TRA-1-60, and TRA-1-81 on iPSCs colonies (Supplementary material Figure 1 a). The pluripotency of iPSCs were confirmed by qPCR (Sox1, T-Brachyury and GATA-4) and immunostaining of the cell markers (Tubulin β -III, CD31, FOXA2) of tri-lineage in spontaneously differentiated embryoid bodies (Supplementary material Figure 1 e & f).

In an additional experiment, we assessed the behavior of iPSC when the underlying surface of the same material was changed during the course of expansion from 3-D to 2-D. We noted that the iPSC patties were re-transferred from NF to flat Matrigel surfaces, cells readily returned to a monolayer format, suggesting this 3-D expansion behavior is solely due to the physical microenvironment. This finding was further supported by our observation that iPSC aggregates cultured on both PMEDSAH:PLGA NF and pure

PLGA NF membranes expanded as 3-D colonies but not on 2-D film of the same material (Figure 1f). In particular, after crossing the margins from structured NFs onto the flat film surfaces of the same polymer material, the patties changed their expansion to a flat monolayer type. Furthermore iPSC that migrated onto flat surfaces increasingly exhibited an adherent fibroblast-like morphology with nucleus-cytoplasm ratios typical of differentiated cells (Figure 1f), while the originating patty on NFs retained shape and pluripotency. This phenomenon highlights the crucial role that surface morphology and roughness can play in growth behavior and maintenance of pluripotency or differentiation of PSC. Indeed, our data suggest that the underlying source of biopolymer was not the crucial factor but that rather its nanofibrous structure determined the fate of the PSC. It will be vital to further investigate the suitability of an NF environment for controlling PSC and such behavior for future clinical applications.

We next set out to investigate whether electrospun NFs could also support the attachment of single cell suspensions of PSC. Usually, single-cell enzymatic expansion is necessary for large-scale expansion of pluripotent stem cells in a bioreactor set up (Hasegawa et al., 2006, Roberts et al., 2012), or for various experimental manipulation or clonal selection (Ellerström et al., 2007). The ongoing survival of PSC usually relies on cell-cell interaction and para/autocrine signals (Pyle et al., 2006). Since the cellular microenvironment provided by the substrate and the constituent of the culture medium have a critical roles in the survival of single human embryonic stem cells (Ellerström et al., 2007) we hypothesized that NFs may also support the attachment and expansion of single-cell preparations of PSC. To test this we seeded single-cell suspensions of two single-cell adapted (Costa et al., 2007, Titmarsh et al., 2013) iPSC lines (iPSC-C11 and

IPSC-C11-EOS-C(3+)-EiP pluripotency reporter) and HES9-EOS-C(3+)-EiP(Hotta et al., 2009) at a cell density of 5.0×10^4 cell/ml² on both the composite PMEDSAH:PLGA NFs and the pure PLGA NFs. These PSC lines contain an EOS-GFP reporter that faithfully reports on pluripotency (Titmarsh et al., 2013). Commercially available Nanofiber-coated plates (Polycaprolactone nanofiber plates, Nanofiber Solutions) and also flat Matrigel-coated plates were used as controls and seeded with the same cells. Remarkably, all NFs matrices tested allowed for efficient attachment of single-cell adapted PSC. Over 90 % of both, the IPSC and HESC seeded on NFs attached to the nanofibers and retained typical morphology and GFP expression for pluripotency (Figure 3). Both, IPSC were circular with large nuclei occupying vast portion of the cells with notable nucleoli (Figure 3). Despite evident loss of a large portion of these single-cell seeded PSC during medium exchanges, the surface of the NFs were partially covered by IPSC and HESC colonies after 3 days of culture again, once more forming the patty like structures (Figure 3). The flow cytometric analysis of reporter IPSC and HESC showed the majority of the population of the cells (> 95%) seeded on NFs were GFP-positive indicating the maintained pluripotency of these cells. This was the same on all tested underlying NF substrates. Confocal microscopy revealed the positive fluorescence of the EOS-GFP reporters within these cells and confirmed their pluripotency once again (Figure 3). In contrast, single PSC seeded on flat Matrigel did not show 3-D patty formation.

Conclusion:

Culture on NF substrates alters the growth pattern of pluripotent stem cells and stimulates the formation of multilayered 3-D patty-like spheroids. Further detailed

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

studies are required to investigate the mechanism by which the extracellular NF microenvironment triggers the 3-D patty formation while preserving the pluripotency of cells. It may not escape the attention of tissue engineers and biologists that such simple feeder-free bulk formation of pluripotent stem cells in multilayered organization from one single PSC source may offer new approaches for organ-like tissue generation or their mass production in clinical-grade bioreactor systems.

Acknowledgements

The initial phase of this study has been funded by the Clem Jones Foundation, Brisbane, Australia. Further funding was received from Griffith University Health Institute grants. We would like to acknowledge Bond University, University of Queensland and Griffith University for providing the facilities to conduct particular elements of this research. Furthermore we thank Mr Stuart Skabo for technical assistance in the initial phase of the project.

Statement

The data of this manuscript has not been published in full or in parts elsewhere. Ethical approval from the relevant boards to generate the iPSC and to apply the embryonic stem cells for research purposes has been gained. The authors declare no conflict of interest.

Figures:

Figure 1 | Culture of IPSC on PMEDSAH:PLGA nanofibers (NFs). a: Clinical-grade electrospinning set up. b: SEM of NFs. c: IPSC patty of 2 mm size (arrow) lifted by a surgical spatula. d: IPSC patties (arrows) as large as 4 mm cultured on NFs in a well plate. e: Close up of an IPSC patty cultured on NFs exhibiting normal morphology of an IPSC colony. Blue arrows pointing at the smooth edges of the colony. f: IPSC patty cultured on the edge of NFs. Once IPSC grow out and extend onto the flat film they start exhibiting differentiated fibroblast-like morphologies. Blue arrows point at the smooth edge of the undifferentiated portion of colony still on NFs. **Two columns of images on right:** Contrast in morphology and the distribution of pluripotency markers (OCT4 (green) and SSEA-4 (red)) between IPSC patties cultured on NFs (left) and IPSC colonies on flat Matrigel (right). IPSC patties on NFs exhibited thicker edges with outwards-to-inwards growth direction. IPSC colonies cultured on flat Matrigel exhibited monolayer-type expansion to outwards

Figure 2 | Morphology of multilayered IPSC patties on PMEDSAH:PLGA NFs compared to flat colonies on Matrigel. SEM images demonstrate morphological contrast between the bulky multilayered patty-like IPSC colonies cultured on NFs (left) compared with the flat monolayer-type colonies propagated on flat Matrigel coated culture plates (right). The 3-D confocal imaging on top left displays distribution of pluripotency-associated surface markers SSEA-4 (green) throughout the IPSC patty on NFs.

Figure 3 | Culture of single cell suspensions of iPSC and HESC. Images on top row show attachment and spreading of single-cell preparations of iPSC cultured either on NFs or Matrigel 16 hours post-seeding. The majority of cells cultured on NFs exhibited typical morphology of an iPSC with large nuclei with distinguishable nucleoli. In contrast, the cells cultured on Matrigel spread flat, exhibiting a fibroblast-like morphology. Second row shows colonies of iPSC derived from single-cell suspension. The PSC cultured on NFs formed patties and were grossly positive for pluripotency marker (SSEA-4; green), whereas a vast number of cells forming flat colonies on Matrigel did not exhibit SSEA-4 marker anymore. SEM images, third row at 3rd day of culture. Patty formations on NF, but the surface of the Matrigel plate appears nearly completely occupied with cells exhibiting morphologies typical for differentiated cells. Fourth row with EOS-GFP positive (green) fluorescence confirming pluripotency throughout multilayered patties grown from single iPSC and HESC preparations on NF. Fifth row with strong positive EOS-GFP fluorescence for pluripotency in multiple patties derived from single iPSC and HESC preparations on NF.

References

Alajati, A., Augustin, H. G., Bartol, A., Korff, T., Laib, A. M. & Weber, H. 2009. Spheroid-based human endothelial cell microvessel formation in vivo. *Nat Protocols*, 4, 1202+.

Alamein, M. A., Liu, Q., Stephens, S., Skabo, S., Warnke, F., Bourke, R., Heiner, P. & Warnke, P. H. 2013a. Nanospiderwebs: artificial 3D extracellular matrix from nanofibers by novel clinical grade electrospinning for stem cell delivery. *Adv Healthc Mater*, 2, 702-17.

Alamein, M. A., Stephens, S., Liu, Q., Skabo, S. & Warnke, P. H. 2013b. Mass production of nanofibrous extracellular matrix with controlled 3D morphology for large-scale soft tissue regeneration. *Tissue Eng Part C Methods*, 19, 458-72.

Brady, M., Sivananthan, S., Warnke, P. & Mudera, V. 2009. Differentiated MSCs seeded in a highly dense collagenous matrix produce novel biphasic Osteochondral constructs. *European Cells and Materials*, 18, 50.

Costa, M., Sourris, K., Hatzistavrou, T., Elefanty, A. G. & Stanley, E. G. 2007. Expansion of Human Embryonic Stem Cells In Vitro. *Current Protocols in Stem Cell Biology*. John Wiley & Sons, Inc.

Ellerstrom, C., Strehl, R., Noaksson, K., Hyllner, J. & Semb, H. 2007. Facilitated Expansion of Human Embryonic Stem Cells by Single-Cell Enzymatic Dissociation. *Stem Cells*, 25, 1690-1696.

Hasegawa, K., Fujioka, T., Nakamura, Y., Nakatsuji, N. & Suemori, H. 2006. A Method for the Selection of Human Embryonic Stem Cell Sublines with High Replating Efficiency After Single-Cell Dissociation. *Stem Cells*, 24, 2649-2660.

Higuchi, Y., Shiraki, N., Yamane, K., Qin, Z., Mochitate, K., Araki, K., Senokuchi, T., Yamagata, K., Hara, M., Kume, K. & Kume, S. 2010. Synthesized basement membranes direct the differentiation of mouse embryonic stem cells into pancreatic lineages. *J Cell Sci*, 123, 2733-42.

Hotta, A., Cheung, A. Y., Farra, N., Vijayagavan, K., Seguin, C. A., Draper, J. S., Pasceri, P., Maksakova, I. A., Mager, D. L., Rossant, J., Bhatia, M. & Ellis, J. 2009. Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency. *Nat Methods*, 6, 370-6.

Hutmacher, D. W., Horch, R. E., Loessner, D., Rizzi, S., Sieh, S., Reichert, J. C., Clements, J. A., Beier, J. P., Arkudas, A., Bleiziffer, O. & Kneser, U. 2009. Translating tissue engineering technology platforms into cancer research. *J Cell Mol Med*, 13, 1417-27.

Loessner, D., Stok, K. S., Lutolf, M. P., Hutmacher, D. W., Clements, J. A. & Rizzi, S. C. 2010. Bioengineered 3D platform to explore cell-ECM interactions and drug resistance of epithelial ovarian cancer cells. *Biomaterials*, 31, 8494-506.

Nandivada, H., Villa-Diaz, L. G., O'Shea, K. S., Smith, G. D., Krebsbach, P. H. & Lahann, J. 2011. Fabrication of synthetic polymer coatings and their use in feeder-free culture of human embryonic stem cells. *Nat Protoc*, 6, 1037-43.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Nur-E-Kamal, A., Ahmed, I., Kamal, J., Schindler, M. & Meiners, S. 2005. Three dimensional nanofibrillar surfaces induce activation of Rac. *Biochemical and Biophysical Research Communications*, 331, 428-434.

Nur-E-Kamal, A., Ahmed, I., Kamal, J., Schindler, M. & Meiners, S. 2006. Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells. *Stem Cells*, 24, 426-33.

Pyle, A. D., Lock, L. F. & Donovan, P. J. 2006. Neurotrophins mediate human embryonic stem cell survival. *Nat Biotechnol*, 24, 344-50.

Roberts, I., Baila, S., Rice, R. B., Janssens, M. E., Nguyen, K., Moens, N., Ruban, L., Hernandez, D., Coffey, P. & Mason, C. 2012. Scale-up of human embryonic stem cell culture using a hollow fibre bioreactor. *Biotechnol Lett*, 34, 2307-15.

Rodin, S., Domogatskaya, A., Strom, S., Hansson, E. M., Chien, K. R., Inzunza, J., Hovatta, O. & Tryggvason, K. 2010. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat Biotechnol*, 28, 611-5.

Serra, M., Brito, C., Correia, C. & Alves, P. M. 2012. Process engineering of human pluripotent stem cells for clinical application. *Trends in Biotechnology*, 30, 350-359.

Schindler, M., Ahmed, I., Kamal, J., Nur-E-Kamal, A., Grafe, T. H., Young Chung, H. & Meiners, S. 2005. A synthetic nanofibrillar matrix promotes in vivo-like organization and morphogenesis for cells in culture. *Biomaterials*, 26, 5624-31.

Titmarch, D. M., Ovchinnikov, D. A., Wolvetang, E. J. & Cooper-White, J. J. 2013. Full factorial screening of human embryonic stem cell maintenance with multiplexed microbioreactor arrays. *Biotechnol J*, 8, 822-34.

Villa-Diaz, L. G., Nandivada, H., Ding, J., Nogueira-De-Souza, N. C., Krebsbach, P. H., O'Shea, K. S., Lahann, J. & Smith, G. D. 2010. Synthetic polymer coatings for long-term growth of human embryonic stem cells. *Nat Biotechnol*, 28, 581-3.

Warnke, P. H. 2010. In-vivo tissue engineering of biological joint replacements. *Lancet*, 376, 394-6.

Zweigerdt, R., Olmer, R., Singh, H., Haverich, A. & Martin, U. 2011. Scalable expansion of human pluripotent stem cells in suspension culture. *Nat Protoc*, 6, 689-700.

Supplementary material

Supplementary material Figure 1 | Characterization of IPSC cultured on PMESAH:PLGA NFs, pure PLGA NFs and Matrigel. **a:** Fluorescence images showing expression of pluripotency-associated surface markers (TRA-1-60, TRA-1-81, SSEA-4) and transcription factor (OCT4) and the presence of alkaline phosphatase (ALP) activity for IPSCs cultured on PLGA NFs for 8 weeks. **b:** Flow cytometry analysis of IPSCs cultured for 8 weeks on PLGA NFs for pluripotency markers OCT4, SSEA-4 and SOX-2. **c:** Expression of pluripotency associated genes (OCT4, KLF, SOX2) of IPSCs cultured for 8 weeks on PMESAH:PLGA NF and PLGA NF and Matrigel (not statistically significant $p>0.05$). **d:** Normal karyotype of IPSCs expanded 8 weeks on PLGA NFs. **e:** Expression of gene specific to three germ-lines on spontaneously differentiated embryoid bodies; ectoderm (SOX1), mesoderm (T-Brachury) and endoderm (GATA-4) for IPSCs cultured on PMESAH:PLGA NF, PLGA NF and Matrigel for 8 weeks (not statistically significant $p>0.05$). **f:** Confocal imaging confirms the presence of cell-marker proteins for three germ-lines; ectoderm (β -tubulin), mesoderm (CD31), endoderm (FOXA), on spontaneously differentiated embryoid bodies derived from IPSCs patties cultured on PLGA NFs for 8 weeks. IPSCs cultured on PMESAH:PLGA and PLGA NFs and the control IPSCs cultured on Matrigel maintained their pluripotency and their ability to differentiate into three germ-lines.

For Peer Review

Figure 1 | Culture of IPSC on PMEDSAH:PLGA nanofibers (NFs). a: Clinical-grade electrospinning set up. b: SEM of NFs. c: IPSC patty of 2 mm size (arrow) lifted by a surgical spatula. d: IPSC patties (arrows) as large as 4 mm cultured on NFs in a well plate. e: Close up of an IPSC patty cultured on NFs exhibiting normal morphology of an IPSC colony. Blue arrows pointing at the smooth edges of the colony. f: IPSC patty cultured on the edge of NFs. Once IPSC grow out and extend onto the flat film they start exhibiting differentiated fibroblast-like morphologies. Blue arrows point at the smooth edge of the undifferentiated portion of colony still on NFs. Two columns of images on right: Contrast in morphology and the distribution of pluripotency markers (OCT4 (green) and SSEA-4 (red)) between IPSC patties cultured on NFs (left) and IPSC colonies on flat Matrigel (right). IPSC patties on NFs exhibited thicker edges with outwards-to-inwards growth direction. IPSC colonies cultured on flat Matrigel exhibited monolayer-type expansion to outwards

Figure 2 | Morphology of multilayered IPSC patties on PMEDSAH:PLGA NFs compared to flat colonies on Matrigel. SEM images demonstrate morphological contrast between the bulky multilayered patty-like IPSC colonies cultured on NFs (left) compared with the flat monolayer-type colonies propagated on flat Matrigel coated culture plates (right). The 3-D confocal imaging on top left displays distribution of pluripotency-associated surface markers SSEA-4 (green) throughout the IPSC patty on NFs.

Figure 3 | Culture of single cell suspensions of IPSC and HESC. Images on top row show attachment and spreading of single-cell preparations of IPSC cultured either on NFs or Matrigel 16 hours post-seeding. The majority of cells cultured on NFs exhibited typical morphology of an IPSC with large nuclei with distinguishable nucleoli. In contrast, the cells cultured on Matrigel spread flat, exhibiting a fibroblast-like morphology. Second row shows colonies of IPSC derived from single-cell suspension. The PSC cultured on NFs formed patties and were grossly positive for pluripotency marker (SSEA-4; green), whereas a vast number of cells forming flat colonies on Matrigel did not exhibit SSEA-4 marker anymore. SEM images, third row at 3rd day of culture. Patty formations on NF, but the surface of the Matrigel plate appears nearly completely occupied with cells exhibiting morphologies typical for differentiated cells. Fourth row with EOS-GFP positive (green) fluorescence confirming pluripotency throughout multilayered patties grown from single IPSC and HESC preparations on NF. Fifth row with strong positive EOS-GFP fluorescence for pluripotency in multiple patties derived from single IPSC and HESC preparations on NF.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

Supplementary material Figure 1 | Characterization of iPSC cultured on PMESAH:PLGA NFs, pure PLGA NFs and Matrigel. a: Fluorescence images showing expression of pluripotency-associated surface markers (TRA-1-60, TRA-1-81, SSEA-4) and transcription factor (OCT4) and the presence of alkaline phosphatase (ALP) activity for iPSCs cultured on PLGA NFs for 8 weeks. b: Flow cytometry analysis of iPSCs cultured for 8 weeks on PLGA NFs for pluripotency markers OCT4, SSEA-4 and SOX-2. c: Expression of pluripotency associated genes (OCT4, KLF, SOX2) of iPSCs cultured for 8 weeks on PMESAH:PLGA NF and PLGA NF and Matrigel (not statistically significant $p>0.05$). d: Normal karyotype of iPSCs expanded 8 weeks on PLGA NFs. e: Expression of gene specific to three germ-lines on spontaneously differentiated embryoid bodies; ectoderm (SOX1), mesoderm (T-Brachury) and endoderm (GATA-4) for iPSCs cultured on PMESAH:PLGA NF, PLGA NF and Matrigel for 8 weeks (not statistically significant $p>0.05$). f: Confocal imaging confirms the presence of cell-marker proteins for three germ-lines; ectoderm (β -tubulin), mesoderm (CD31), endoderm (FOXA), on spontaneously differentiated embryoid bodies derived from iPSCs patties cultured on PLGA NFs for 8 weeks. iPSCs cultured on PMESAH:PLGA and PLGA NFs and the control iPSCs cultured on Matrigel

maintained their pluripotency and their ability to differentiate into three germ-lines.

For Peer Review