



WHITEHEAD INSTITUTE

High Throughput Development of Biomaterials for Clonal Growth of Human Embryonic Stem Cells

Krishanu Saha¹, Ying Mei², Said R. Bogatyrev², Jing Yang, Zeynep Ilke Kalcioğlu, Seung-Woo Cho, Maisam Mitalipova¹, Neena Pyzocha¹, Krystyn Van Vliet, Martyn C. Davies, Morgan R. Alexander, Robert Langer², Daniel G. Anderson², Rudolf Jaenisch¹

¹ Whitehead Institute for Biomedical Research, Cambridge, MA USA

² M.I.T. Dept. of Chemical Engineering, Cambridge, MA USA

Abstract

High-throughput combinatorial methods were developed to synthesize and evaluate hundreds of acrylate-based polymeric substrates for their abilities to support the clonal and long-term propagation of human embryonic stem cells (hESCs). Synthetic surfaces were developed that support robust self-renewal of hESCs, preserved a normal karyotype, and maintained full differentiation capacity after prolonged cell culture. The efficiency of clonal hESC growth on the top performing polymers was similar to substrates utilizing mouse embryonic feeders (MEFs), and superior to matrigel, the gold standard of feeder-cell free hESCs culture substrate. Parallel chemical and physical characterization of all members of the library of arrayed polymers allowed us to rapidly map out relationships between cellular responses and polymer properties. Interestingly, propagation of hESCs was associated with an optimal surface energy (water contact angle), but independent of polymer elasticity. Propagating dissociated hES single cells in an undifferentiated state in a xeno-free, feeder-cell free environment has potential to accelerate both basic biological efforts to understand in vitro self-renewal of hESCs. Furthermore, we anticipate that the combinatorial cell and material information described here may facilitate the further development of synthetic surfaces for manipulation of hESCs.

Motivation: Improving human stem cell propagation & clonal growth for gene manipulation

Current methods to clonally expand both hESCs and iPSCs are inefficient and poorly-defined for therapeutic purposes. For instance, hESCs are traditionally maintained on a "feeder" cell layer of mitotically-inactivated mouse embryonic fibroblasts (MEFs), and the production of MEFs is highly laborious. Also, the animal origin of MEFs introduces risks of animal pathogens. Many extracellular matrix (ECM) proteins have been coated on tissue culture polystyrene (TCPS) to develop hESC culture substrates devoid of feeder cells [1-3]. Recent comparison of such "feeder-free" substrates showed that such proteins adsorbed on various biomaterials including TCPS could not support long-term culture of karyotypically normal hESCs [4]. In addition to the strong need to develop feeder-free methods for long-term hESC culture, hESCs, unlike mouse embryonic stem cells, tend to undergo massive cell death after complete dissociation, hence making it challenging to genetically manipulate these cells and direct their differentiation [5].

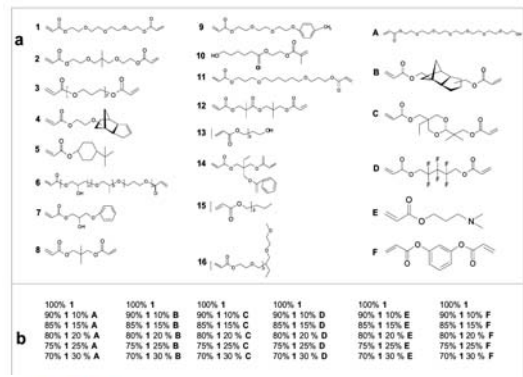


Figure 1. Biomaterial array design. a, monomers used for array synthesis. Monomers were classified into two categories: major monomers that constitute >50% of the reactant mixture and minor monomers that constitute <50% of the mixture. Sixteen major monomers were named numerically, and six minor monomers were named in an alphabetic order. b, 36 different combinations for the major monomer 1 with all 6 different minor monomers. c, Photograph showing one polymer microarray in triplicate with eight polymer spots to show layout.

High-throughput combinatorial methods to synthesize & evaluate biomaterials for clonal growth

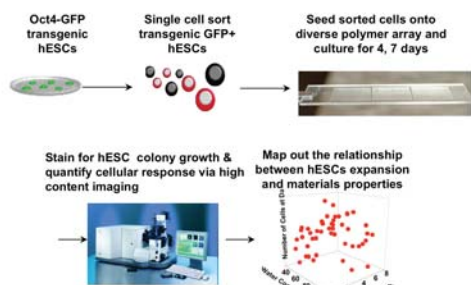


Figure 2: Schematic of initial screening experiments. First transgenic Oct4-GFP hESCs were maintained on MEFs. Then flow cytometry enabled the isolation of highly pure undifferentiated hESCs from the completely dissociated coculture of hESCs and MEFs. Next, sorted cells were seeded onto polymer array. Finally, cellular response on polymer array was quantified by using laser scanning cytometry.

Growth On FBS-Coated Polymer Substrates and Control Surfaces

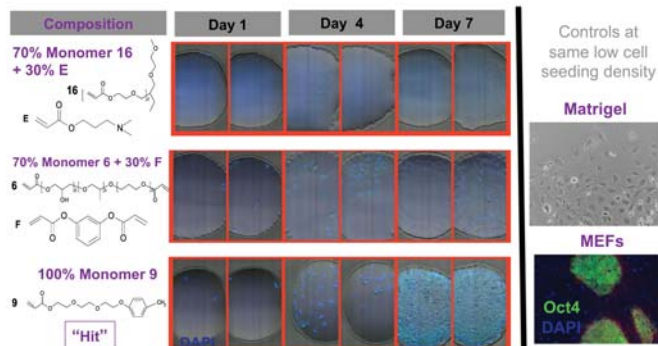
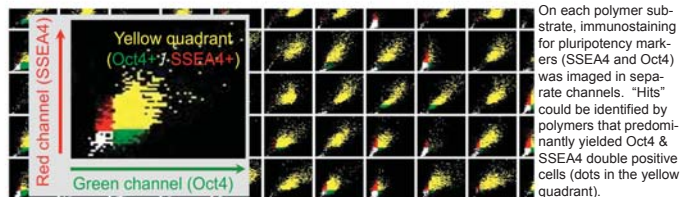


Figure 3. Representative images of cell nuclei (stained by Hoechst in blue) on polymers that do not support either attachment or growth of dissociated hESCs (polymer shown here is 16E-30%F), that support the attachment but not the propagation of dissociated hESCs (shown here is 6F-30%), and on "hit" polymers that support both the attachment and propagation of hESCs (shown here is 9). In standard MEF conditioned media [1], matrigel could not support clonal growth of dissociated hESCs, while MEFs could.

Laser Scanning Cytometry Automated Cell Analysis



Mapping hESC behavior to material properties

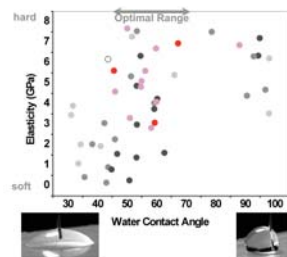


Figure 4. Cell growth on all biomaterial spots on day 7 plotted against both water contact angle and elasticity. Cell number and expression of pluripotency markers correlate well (data not shown). An optimal range of ~45-70 water contact angle supported growth most robustly.

Table 1: Efficiency of various substrates to support the expansion of dissociated hESCs.

	Cell on Day 1*	Cells on Day 7**	Efficiency (%)
MEFs	26	7.1	27.3
Matrigel	32	N/A	N/A
Polymer Hit 9	47	10.0	21.3
Polymer Hit 15A	56	10.3	18.4

*The cell numbers on MEFs and Matrigel on day 1 are quantified per scanned area; the cell number on "hit" polymer spots are quantified per 12 replicates.
**The colony numbers on MEFs and Matrigel on day 7 are quantified per scanned area; the colony number of "hit" polymer spots are quantified per 12 replicates.

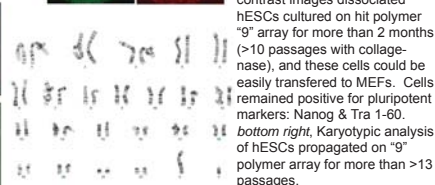
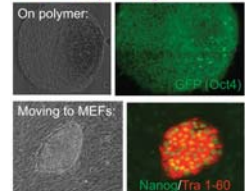
Feeder-Free Long-Term Culture

Pluripotency markers after one passage on hit arrays (~2000 spots):



Figure 5. Long-term feeder-free culture on hit polymer arrays. Top, Immunostaining for pluripotency markers for dissociated hESCs propagated on hit polymer 15A-30% for 7 days: GFP (Oct4), SSEA4, Nanog, & Tra 1-60. bottom left, Phase contrast images dissociated hESCs cultured on hit polymer "9" array for more than 2 months (>10 passages with collagenase), and these cells could be easily transferred to MEFs. Cells remained positive for pluripotency markers: Nanog & Tra 1-60.

After >10 passages:



- Similar results for hit substrates coated with human serum
- In vitro differentiation identical to hESCs grown on MEFs
- Teratoma formation assay in progress

Conclusions

We have developed new synthetic biomaterials for feeder-free culture of hESCs. Two top performing polymers from our material-cell behavior map were selected and used to fabricate "hit" arrays. The hit arrays supported robust self-renewal of hESCs, preserved a normal karyotype, and maintained full differentiation capacity after prolonged cell culture. Importantly, efficiency of clonal expansion on the top performing polymers was similar to substrates utilizing mouse embryonic feeders (MEFs), while matrigel, the gold standard of feeder-cell free hESCs culture substrate, could not efficiently support the clonal expansion of dissociated hES single cells in an undifferentiated state. The current design of polymer array with a 300 μm spots on a cell-resistant poly(HEMA) layer effectively confined the growth of hESCs in the polymer spots, and thus created an artificial colony for the growth of hESCs. Future efforts will be devoted to whether the confined microenvironments created by micrometer spots promote the growth of undifferentiated hESCs. Further it will be of interest to characterize the protein layer adsorbed to the performing polymers and define cell receptor engagement important for self-renewal of hESCs. The biomaterials developed here have strong potential to advance both basic biological efforts to understand in vitro self-renewal of hESCs and applied efforts to propagate therapeutically relevant hESCs in a xeno-free, feeder-free environment.

References

- Xu, C. et al. *Nat Biotechnol* 19, 971-974 (2001).
- Richards, M., Fong, C.Y., Chan, W.K., Wong, P.C. & Bongso, A. *Nat Biotechnol* 20, 933-936 (2002).
- Stojkovic, P. et al. *Stem Cells* 23, 895-902 (2005).
- Hakala, H. et al. *Tissue Eng Part A* (2009).

Acknowledgements

We thank Frank Soltner and Caroline Beard for targeting the BG01-Oct4-GFP cells. We thank all the members of the Jaenisch lab for technical support and helpful discussions. R.J. was supported by NIH grants. D.G.A. and R.J. are advisors to Stemgent.