Engineering Biomaterials for Feeder-Free Maintenance of Human Pluripotent Stem Cells

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Human pluripotent stem cells (hPSCs) are capable of differentiating into any type of somatic cell, a characteristic that imparts significant therapeutic potential. Human embryonic stem cells and induced pluripotent stem cells are types of hPSCs. Although hPSCs have high therapeutic potential, their clinical relevance is limited by the requirement for animal feeder layers, which maintain their pluripotency and self-renewal. hPSCs grown on animal feeder cells are at high risk for pathogen contamination and can be affected by the immunogenicity of the feeder layer. The presence of animal feeder cells also limits the scalability of hPSCs in culture because of the high cost of culturing and batch-to-batch variations. Therefore, development of feeder-free systems is imperative for robust, lower-cost, xeno-free, scalable culture of hPSCs. Biomaterials engineered with bioactive molecules such as adhesion proteins and extracellular matrix proteins, or synthetic materials such as peptides and polymers, may provide alternative substrates to animal feeder cells. This article reviews biomaterial-based, feeder-free systems for hPSC growth and maintenance, which provide clinically relevant alternatives to feeder cell systems.

Keywords: Human pluripotent stem cells, Animal feeder cells, Feeder-free systems, Biomaterials

Introduction

Human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have immense potential for regenerative medicine applications. By definition, hPSCs have the ability to differentiate into cells from all three embryonic germ layers and have high self-renewal capacity (1-3). ESCs are derived from the inner cell mass of the blastocyst during embryogenesis (1, 2), and hiPSCs are reprogrammed to a pluripotent state by overexpression of the transcription factors Oct3/4, Sox2, c-Myc, and Klf-4 (3). Both types of hPSCs are promising cell sources for cell therapy and tissue engineering (1-3).

hPSCs require animal-derived feeder layers for culturing and clonal expansion. Mitotically inactive mouse fibroblasts are typically used as feeder layers to maintain the pluripotency and self-renewal capacity of hPSCs (1-3). Feeder cells contribute to hPSC growth through cell-cell interactions and by providing nutrient support (4). However, culturing hPSCs on mouse feeder cells may be problematic due to feeder cell immunogenicity and microbial or viral transmission (4). Regarding hPSC therapeutics, variability in feeder cells leads to batch-to-batch inconsistencies, which complicate product characterization and quality control. The use of feeder cells increases the cost of hPSC production and is a limitation to the large-scale culture of hPSCs, which is essential for obtaining sufficient cell numbers for clinical applications (5).

Matrigel is composed of growth factors and extracellular matrix (ECM) components secreted by Engelbreth-Holm-Swarm mouse sarcoma cells (6) and is used as an alternative to feeder cells. In combination with mouse embryonic fibroblast-conditioned medium, Matrigel can support the pluripotency of hPSCs. However, because both Matri-

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gel and the conditioned medium originate from mouse tissues, the potential for immunogenic problems and animal pathogen transmission remains (6).

Development of xeno-free, chemically defined hPSC growth conditions is essential for culturing hPSCs for use in a clinical setting. Biomaterial substrates modified with bioactive molecules or synthetic materials provide artificial microenvironments that enhance stem cell growth through cell-matrix interactions (7). Indeed, several studies have demonstrated that biomaterials engineered with synthetic polymers, peptides, and proteins can be used successfully to culture and maintain hPSCs in the undifferentiated state (6). In this review, we discuss biomaterial substrates used for efficient feeder-free maintenance of hPSCs.

Protein-modified substrates

Substrates coated with serum proteins have been tested for feeder-free hPSC culture. Stojkovic et al. reported that hESCs retain their undifferentiated state when cultured on human serum-coated dishes (8). Their work demonstrates that protein components in serum such as growth factors, adhesion molecules, or ECM proteins are critical for maintaining hESC pluripotency (8). However, when hESC-derived medium is removed from the culture or replaced with conditioned medium from human somatic cells, hESCs spontaneously differentiate, indicating that soluble factors secreted by hESCs are also critical for maintenance of pluripotency (8).

ECM proteins have also been investigated as feeder-free hPSC culture substrates. Various ECM components including fibronectin, laminin, collagen, and vitronectin provide conditions that support cell adhesion, proliferation, and differentiation (9). In fact, laminin was found to maintain hESC pluripotency for up to 130 population doublings (9). hESCs cultured on a laminin-coated surface express the pluripotency markers Oct-4 and hTERT and the surface markers SSEA-4, Tra-1-60, and Tra-1-81 and show high telomerase activity (9). More recently, it was reported that recombinant laminin-511 supports long-term maintenance (4 months or 20 passages) of the undifferentiated state in both hESCs and hiPSCs due to the high affinity of laminin for $\alpha_6\beta_1$ integrin on hPSCs (10). Fibronectin has also been used as a culture substrate in combination with medium supplemented with knockout serum replacement, transforming growth factor β_1 , and basic fibroblast growth factor to maintain hESC pluripotency (11). The combination of multiple ECM proteins may also provide a viable alternative to feeder cells (12).

Coating adhesion molecules onto culture dishes is another strategy for development of feeder-free substrates. Cell adhesion molecules that control adhesion, proliferation, differentiation, and morphogenesis of stem cells have the potential for use in substrate engineering. Cadherins are a family of adhesion molecules with these properties. One member, E-cadherin, is a molecule that supports calcium-dependent cell-cell adhesion (13) and is highly expressed in undifferentiated ESCs (6). E-cadherin is especially essential for intercellular adhesion and colony formation of ESCs (14). Thus, E-cadherin-mediated cell-cell adhesion is potentially important for maintaining the pluripotency of ESCs (14).

Substrates coated with E-cadherin were developed for PSC culture without animal feeder cells. Nagaoka et al. constructed an E-cadherin protein fused with an IgG-Fc domain and coated the construct onto tissue culture plates (15). An adhesion protein fused with IgG is easily constructed via conventional genetic engineering techniques, and the Fc fragment mediates adsorption of fusion proteins onto polystyrene surfaces (16). Mouse ESCs retain their pluripotency and demonstrate high proliferation on these chimeric protein-coated surfaces (15). hESCs and hiPSCs also maintain their pluripotency on E-cadherin IgG-Fc constructs under completely defined culture conditions (mTeSR1 medium) (17). Another cadherin family member, N-cadherin, was used to create an IgG-Fc fusion protein construct (16). The N-cadherin-coated surface supports maintenance of an undifferentiated state in mouse embryonic carcinoma cells and enhances neural differentiation of neural stem cells (16). These cadherin-modified surfaces represent promising feeder-free hPSC growth substrates.

Peptide-modified substrates

Proteins used for engineering substrates are usually isolated from animal sources or produced with animal cell culture (18). Although protein-modified substrates are feeder-free systems, they are not xeno-free. Thus, the use of animal-derived proteins may not be free of hazardous pathogens or immunogenic material. In addition, most biological materials, including proteins, are expensive to manufacture and have limited scalability and high batch-to-batch inconsistencies (18). Synthetic materials may circumvent these limitations by providing a fully defined, scalable, reliable alternative to biological materials (19). In particular, synthetic peptides are useful for surface modification because they can be easily incorporated onto a wide variety of surfaces via simple chemical reactions (20).

Synthetic peptides derived from the active domain of ECM proteins can be used to maintain hPSC self-renewal and pluripotency. Melkounian et al. developed a synthetic peptide acrylate surface (PAS) for supporting self-renewal of hESCs in chemically defined xeno-free medium (19). The PAS was prepared by depositing carboxylic acid containing acrylate onto culture dishes and conjugating amine-containing peptides to the surface using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide. Peptides derived from the ECM proteins fibronectin, vitronectin, laminin, and bone sialoprotein were conjugated to PAS and tested for growth support of hESCs (lines H1 and H7). hESCs grown on PAS conjugated to vitronectin and bone sialoprotein-derived peptides retain an undifferentiated phenotype and a normal karyotype for more than ten passages (19). hESCs retrieved after long-term culture on the PAS generate teratomas *in vivo* (19), indicating the pluripotency of hESCs grown on PAS. In addition, PAS-cultured hESCs can be induced to differentiate into functional cardiomyocytes (19). These results demonstrated the utility of PAS for sustaining hESC pluripotency and supporting subsequent differentiation. PAS technology is also scalable, which is a requirement for producing sufficient numbers of cells for cell therapy or tissue engineering applications.

Synthetic surfaces that interact with glycosaminoglycans have also been examined for the ability to support hPSCs under fully defined growth conditions. Peptide sequences with binding affinity to anionic polysaccharides, as observed in heparin, are potential candidates for substrate modification because glycosaminoglycans on the hPSC surface are important for cell adhesion (20). Klim et al. demonstrated that the heparin-binding peptide, GKKQ-RFRHRNRKG, represents the most effective substrate for hPSC adhesion and propagation by supporting hESC and hiPSC growth in mTeSR defined medium for more than 3 months (20). hPSCs grown on peptide-engineered surfaces maintain a normal karyotype and exhibit high levels of pluripotency marker expression. This study demonstrates that synthetic substrates that recognize cell-surface glycosaminoglycans can facilitate long-term culture of hPSCs.

A high-throughput discovery strategy may be useful for screening for novel peptides that bind to the surface of hPSCs. Derda et al. identified several cell-binding peptides with phage display and produced synthetic surfaces capable of supporting the short-term propagation of undifferentiated hESCs (21). Specifically, when hESCs are cultured on self-assembled monolayers of the identified peptide sequences, TVKHPDALHPQ or LTTAPKLP-

KVTR, in mTeSR chemically defined medium, they express markers of pluripotency at levels similar to hESCs cultured on Matrigel (21). This study indicates that a screening strategy may be productive for identifying synthetic materials that can control the growth of hPSCs.

Synthetic polymer-grafted substrates

Synthetic polymers may be used to engineer functional surfaces for long-term, feeder-free maintenance of hPSCs. As repeatedly stressed, naturally derived biomaterials such as Matrigel and ECM proteins have critical limitations to the manufacturing of large batches, long-term storage, and because of safety concerns. Biological proteins are costly and have limited shelf lives. In contrast, polymers can be reproducibly synthesized, handled, and stored with relative ease. Culture dishes grafted with synthetic polymers are chemically defined and have long-term stability. Synthetic polymer substrates are more suitable for mass production and clinical applications.

A recent study demonstrated that synthetic polymer-grafted surfaces support long-term culture of hESCs in different media. Villa-Diaz et al. described a fully defined synthetic polymer coating, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), for hESC growth and culture (22). PMEDSAH was grafted onto ozone-activated surfaces of tissue culture plates. hESCs (line H9) were cultured on PMEDSAH plates in mouse embryonic fibroblast and human conditioned medium and in serum-free defined medium (mTeSR and StemPro). After 25 passages, hESCs cultured on PMEDSAH maintain a normal karyotype and retain pluripotency as demonstrated by teratoma formation in immunosuppressed mice (22).

A high-throughput screening approach demonstrated that new culture substrates can be used to clonally expand hPSCs in a chemically defined, xeno-free, feeder-free manner. Mei et al. manufactured cell-compatible, biomaterial microarrays to facilitate rapid synthesis and analysis of synthetic substrates (23). The polymer microarrays were prepared by combinatorial synthesis of polymers with diverse acrylate monomers (23-25). hESCs and hiPSCs were cultured on the polymer substrates coated with fetal bovine serum or human serum, and then optimal polymer substrate conditions were identified using colony formation efficiency. Material properties including wettability, surface topography, surface chemistry, and elastic modulus of the polymeric substrates were quantified using high-throughput methods to develop correlations between material properties and biological perform-

ance (23, 25). These studies revealed that optimal hPSC growth substrates can be generated from monomers with high acrylate content and with moderate wettability. Optimal polymer substrates adsorb vitronectin from serum and thus promote colony formation through hPSC $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin interactions (23). The integrated high-throughput synthesis and rapid quantification of material/cell interactions may accelerate the combinatorial development of synthetic substrates for hPSC culture.

Another high-throughput screening study identified other types of synthetic polymers that support self-renewal of hPSCs. Brafman et al. identified a synthetic polymer, poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA), which supports long-term attachment, proliferation, and self-renewal of hESCs (lines HUES1 and HUES9) and hiPSCs (26). hPSCs cultured on PMVE-alt-MA maintain their characteristic morphology, express high levels of pluripotency markers, and retain a normal karyotype (26). Such well-defined polymer-based substrates may serve as platforms to produce large numbers of hPSCs for clinical use.

Conclusions

hPSCs have immense potential to treat incurable diseases through cellular therapy. However, current systems of hPSC culture require feeder cells derived from animal sources. The use of animal feeder cells makes it difficult to obtain sufficient numbers of hPSCs that are qualified for clinical therapy due to safety issues, quality control, and high costs. To overcome these limitations, growth substrates have been engineered with biological materials such as ECM proteins and adhesion proteins, or synthetic materials such as peptides and polymers. These substrates in combination with chemically defined medium can be used to successfully replace feeder cells and Matrigel by supporting hPSC pluripotency and self-renewal. These biomaterial substrates provide standardized, controllable, and sustainable culture systems for obtaining clinically relevant hPSCs.

Acknowledgments

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Potential conflict of interest

The authors have no conflicting financial interest.

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