PHENOTYPIC MODULATION OF PLURIPOTENT STEM CELLS (PSCs) INDUCED BY MICROFABRICATION MATERIALS

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ABSTRACT

Microstructured Photoresist (PR) has been applied for cell culture substrate, but their effects on cell behaviors are still unclear. Here, we evaluated the effects of PR substrates on human induced pluripotent stem cells (hiPSCs), which have a great potential for clinical applications. By comparing two types of epoxy based PRs with and without antimony salt, both PRs have affected on hiPSC viability after culture for seven days.

KEYWORDS

negative photoresists, polydimethylsiloxane, pluripotent stem cell, cellular phenotype, gene expression

INTRODUCTION

Human induced pluripotent stem cells (hiPSCs) hold a great potential for applications in cell-based therapy, regenerative medicine and drug development/screening. Indeed, hiPSCs can unlimitedly self-renew during culture in vitro without any karyotypical abnormality, and differentiate any kinds of cells in a body. However, there still are concerns of current hiPSC experimental setting consisted with feeder cells, which may release unknown factors resulting low reproducibility for culture and experiments, and cause xenogenic contamination with hPSCs. Thus, it necessitates developing a new hESC/iPSC experimental platform without feeder cells.

Polymer MEMS technology has recently offered a simple three-dimensional (3D) fabrication for microfluidic devices, and is advantageous for creating new cellular microenvironmental cues to control stem cell functions [1]. Especially, there is an on-going trend towards applying microfluidics to stem cell biology. Although nano/micro-fabrications and mechanical properties of photoresist have been well-discussed, chemical effects such as a toxicity of antimony salt (an optical polymerization initiator) in photoresists and its derivatives on cell functions are still unknown.

Therefore, as a first step in utilizing Bio-MEMS and nano/microfluidic devices for applications in stem cell research, we investigated the effects of these photoresist materials on hPSC phenotypes with multiple assays: cell adhesion, pluripotent status, proliferation, cytotoxicity, cellular heterogeneity and gene expression. With this information, we will be able to modify or develop new PRs that are applicable to stem cell research.

EXPERIMENT

hiPSC line (253G1) used in this study were cultured on Mitomycin C (MCC)-treated mouse embryonic fibroblast (MEF) cells in hPS medium, consisting of DMEM/F12 medium supplemented with 15% KnockOut Serum Replacement (KSR), 1% non-essential amino acid (NEAA), 0.1 mM β-mercaptoethanol, and 10 ng/mL basic fibroblast growth factor (bFGF). Media was changed daily, and these cells were passaged with StemPRO EZPassage kit (Invitrogen) weekly.

First, we examined various surface treatments (i.e., corona treatment, gelatin coating, and Matrigel® coating) for commonly used PRs (TMMR-S2000®; called "S2000") to facilitate PSC adhesion (Table 1). To facilitate mESC adhesion on PR substrates, we used these surface treatments to prepare PR substrates and used mouse embryonic stem cells (mESCs) to evaluate them.

Table 1. Summary of treatments used for PR surfaces to improve cell adhesion

Surface treatments	Cell adhesion	Colony formation	Alkaline phosphatase
None	N.D.	N.D.	N.D.
Gelatin	+	N.D.	N.D.
Matrigel	+++	+++	+++
Corona	+	N.D.	N.D.

Briefly, corona treatment is a surface treatment technique that uses a low temperature discharge plasma to prepare a hydrophilic surface. Gelatin and Matrigel coatings on substrates are commonly used for cell cultures, including PSCs. mESCs could not adhere to a non-treated PR surface because of their hydrophobicity. Corona- and gelatin-treated PR surfaces became hydrophilic, although not sufficiently so for mESC adhesion.

Compared with these treatments, Matrigel-coated PR substrates provided for better mESC adhesion. In addition, mESCs grew for seven

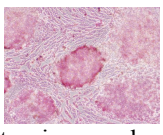

Fig. 1 Photomicrograph of mESCs expressing alkaline phosphatase cultured on S2000 coated with Matrigel.

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days with normal colony morphologies and expression of a pluripotent marker, alkaline phosphatase (AP; Fig. 1). Regarding PDMS, a number of reports including ours [2] have described the use of Matrigel-coated PDMS surfaces for hPSC culture. Thus, we chose the Matrigel-coating method to further evaluate the effects of PRs on human PSCs.

Second, various microfabrication materials, such as a collection of photoresists (S2000, NS3000, S2000 (10%), S2000 (resin) and NS4000, see Table 2), PDMS and glass, were evaluated to investigate their effects on hPSC behaviors (Fig. 2). The efficacy of hPSC adhesion on PR surfaces was evaluated. All PRs were coated with Matrigel to facilitate cell adhesion and 253G1 hiPSCs were inoculated on the Matrigel-coated PR substrates (Fig. 2a). NS4000 showed only small numbers of hPSCs were able to form their colonies. These results indicated that hPSCs could adhere on the surfaces of Matrigel-coated PRs, except for NS4000, and the hPSC colony morphologies were identical to colonies cultured on a conventional plastic dish. Although a few hPSC colonies adhered on the surface of the NS4000 substrate, their colony morphology differed from colonies cultured on a proper substrate and conditions.

We also made cell counts after culture to evaluate the PRs' toxicity on hPSC growth (Fig. 2b). As expected, hPSCs cultured on NS4000 exhibited dramatically lower viability than with the other PRs. hPSCs cultured on both S2000(10%) and S2000(Resin) exhibited higher cell viability after four-day culture compared with S2000 and NS3000. These results clearly indicated that reducing Sb reagents in PRs improved hPSC viability during culture on PR substrates. As PDMS exhibited cell numbers similar to those with glass, it did not affect hPSC growth.

Table 2. Chemical properties of negative PRs

Photoresists	TMMR® S2000	TMMR® S2000(10%)	TMMR® S2000 (Resin)	TMMR® NS3000	TMMR® NS4000
Positive / Negative type	Negative	Negative	–	Negative	Negative
PAG	Sb	Sb	–	Non-Sb	Non-Sb
Polymerization mechanism	Cationic polymerization	Cationic polymerization	–	Cationic polymerization	Radical polymerization

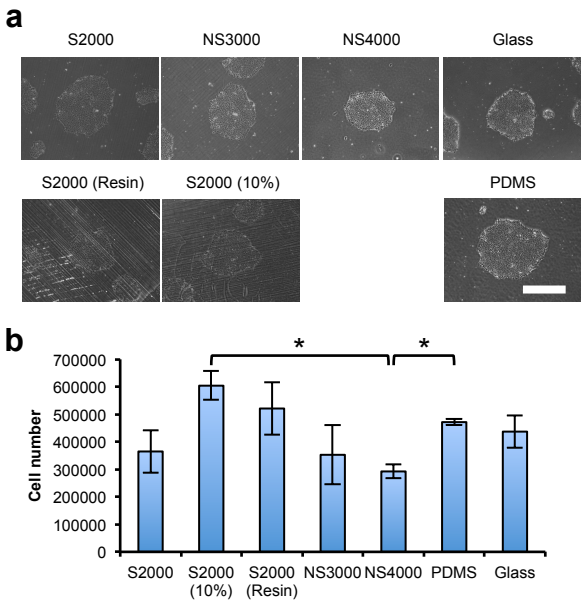

Fig. 2. hPSC adhesion and colony formation on microfabrication materials. (a) Phase-contrast images of hPSCs cultured on PR substrates coated with Matrigel. Scale bar = 200 μm. (b) hPSC cell counts after four-day culture on substrates. *$p < 0.05$. ($n > 4$)

For further evaluation of the effects of microfabrication materials, the pluripotent status of hPSCs cultured on these substrates was examined by AP staining. hPSCs cultured on PRs, including NS4000, retained their AP expression after culture, which was comparable to conventional culture conditions. Flow cytometry analysis was also used to quantitatively determine cell surface markers associated with pluripotency (SSEA4 and TRA-1-60) and differentiation (SSEA1) (Fig. 2d). There were no significant differences in cells' expressions of SSEA4, TRA-1-60, and SSEA1 among the substrates used. As culture on NS4000 resulted in very small numbers of cells, flow cytometry was not applicable for this sample.

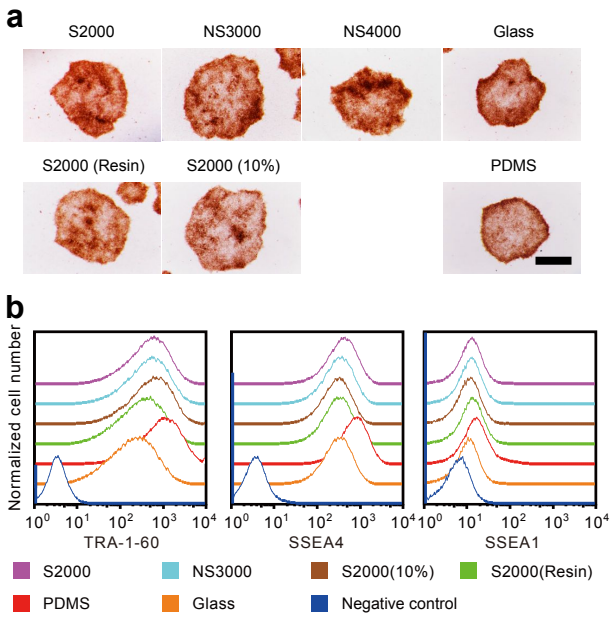

Fig. 3 hPSC pluripotent status on microfabricatin materials. (a) hPSC expression of alkaline phosphatase. Scale bar = 200 µm. (b) Flow cytometry analysis to evaluate PR effects on pluripotency and differentiation status.

To further characterize microfabrication materials on hPSC behavior, quantitative PCR was used to investigate how they altered the expressions of pluripotent stem cell-specific genes and some differentiation genes (Fig. 4). We carefully chose 84 genes, which are related with maintenance and differentiation of hPSCs. We used unsupervised hierarchical clustering to identify those genes specifically induced by PRs or PDMS. Fig. 4a and b show the heat map and the clustered dendrogram of gene expressions obtained by a quantitative PCR array. Negative PRs, except for NS4000, induced similar gene expression patterns. After clustering the gene expression patterns, 84 genes were divided into three categories; these were specifically activated with PRs, PDMS, glass, and a culture dish (Category *i*), NS4000 (Category *ii*), and IMR90 (Category *iii*). According to these clustered data, NS4000 was similar to IMR90, even though the actual gene expression patterns were very different.

In conclusion, we are able to investigate how microfabrication materials affected hPSC behaviors utilizing multiple analyses. We envision that the obtained information will be beneficial to develop the new materials capable for applications in stem cell biology as well as a wide range of biomedical setting.

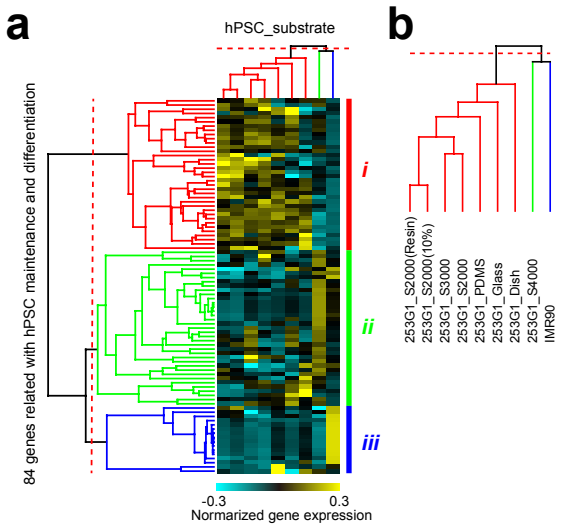

Fig. 4 Hierarchical clustering of 84 mRNAs expressed by hiPSCs (253G1) as determined by quantitative PCR. (a) Two-dimensional heat map and (b) a clustered dendrogram for 253G1 hiPSCs cultured on nano/microfabrication substrates.

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