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CONTROLLED ASYMMETRICAL DIFFERENTIATION OF MOUSE EMBRYOID BODIES IN MICROWELLS WITH DESIGNED HETEROGENEOUS BIOCHEMICAL FEATURES

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We report a novel engineered microwells to spatially control differentiation of mouse embryoid bodies. With integrating multiple functionally distinct biomaterials by soft-photolithography technology, this method enables simple and reliable manufacture of biochemically heterogeneous microwells that are capable of regulating differentiation of stem cell in a spatial-specific manner. This simple technology offers a new dimension of spatial control over embryoid bodies development and has great potential in tissue engineering and biomedical applications.

Keywords: Stem cell; embryoid bodies (EBs); hydrogel; differentiation.

Stem cell differentiations are highly influenced by environmental cues. It has been demonstrated that in niche both the biophysical features, such as matrix mechanical properties and architecture,[1,2] and biochemical features, such as chemokines, cytokines, and matrix molecules,[3,4] have function in regulation of cellular fate decision-making and lineage development. Especially, spatial regulation is very essential for embryonic development.[5] These foundational findings inspired researchers to develop technology for controlling stem cell fate through programming extracellular microenvironment.

Microwell arrays are clusters of high-density, micron-sized cavities[6] that are topologically patterned on biomaterial surfaces through microfabrication.[7] In comparison with conventional culture substrates, microwell arrays allow up to thousands of samples to be simultaneously captured on a tiny chip within minutes

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and continuously culture and analysis for long period of time. With tremendously reduced cost of mammalian cell culture and significantly enhanced high throughput analysis capability, microwell arrays, particularly those made from hydrogels, have recently emerged as a promising platform for mammalian cell research.[8]

Recent efforts have demonstrated the controllability on stem cell[29] using microwells arrays with designed properties. Microwells can be fabricated with different geometric features to produce stem cell aggregates with well-defined shape.[9] Moreover, specific differentiation of embryo stem cell can be controlled through defining the size of stem cell aggregates formed in microwells with designed dimensions properties. In particular, large size stem cell aggregates formed in microwells with size of 450 um in diameter preferentially underwent cardiac differentiation. By contrast, small size aggregates formed in microwells with size of 150 μm in diameter preferentially underwent endothelial differentiation.[10] In addition to the biophysical properties, biochemical features of microwells were explored to control cell behaivor as well. For instance, by coupling soft-lithography and microcontact printing technique, microwells made from poly(ethylene glycol) diacrylate (PEG) were selectively functionalized at the bottom with specific polypeptide to control cells adhesion. Similarly, using physisorption technology the inner surfaces of microwells can also be functionalized with either fibronectin or lipid bilayers[8,11] to regulate cell behavior.

Controlling stem cell differentiation in engineered microwells has been greatly advanced due to the high fabrication capability of photolithography developed in semi-conductor manufacturing. However, it is still a huge challenge to introduce the desired spatial controllability into stem cell development process mimicking the *in vivo* development process. To achieve this goal, developing microwells with spatially programmable biochemical features may be a potential approach.

In a previous study, it has been demonstrated that the desired spatially controlled differentiation can be achieved through encapsulating embryoid bodies (EBs) in a patterned 3D hybrid-hydrogel.[12] However, besides the complicate fabrication process, directly exposing stem cell to UV which damaged DNA generating long term and unpredictable influence.[13–20] It is a huge hurdle for applying this technology in biomedical applications. For addressing these problems, here we report a simple but yet robust microwells fabrication method to spatially control stem cell differentiation by integrating multiple functionally distinct biomaterials. Specifically, we employed PEG[6] and gelatin methacrylate (GEL)[21] as materials for fabrication, both are photocrosslinkable and biocompatible but are distinct in bioactivities — the former is nondegradable and chemically inert, while the latter is biodegradable and amenable for stem cell differentiation.[12,21–24] Further, we proposed to fabricate biochemically asymmetrical microwells by making one half part of a microwell from PEG and the other half part from GEL through a specifically designed multi-step soft photolithography process. Therefore, a designed spatial heterogeneous biochemical properties was generated in the final fabricated hybrid microwells. After seeded into the hybrid microwells, cells located at different

positions on a cell aggregate will be exposed to distinct microenvironments (PEG or GEL) and consequently undergo various differentiation process accordingly.

To develop a robust but yet feasible photolithography method for biochemically heterogeneous microwells fabrication, we started by examining homogeneous microwell fabrications with PEG and GEL individually. As illustrated in Fig. 1(a), uniform microwell arrays were fabricated by following a protocol with modification from a previous study.[12] Specifically, (i) a glass slide was firstly coated with a 3-(trimethoxysilyl) propylmethacrylate (TMSPMA, Sigma) and a thin layer of PEGDA (MW 1 KDa); (ii) two No. 1 cover glass slides (150 μm in thickness) were stacked at the glass slide two ends as spacers for controlling the thickness, and 10 wt.% PEG (MW 1 KDa) polymer precursor solution with 1 wt.% photoinitiator (IRGACURE 2959 Ciba) was dropped at the center; (iii) another glass slide along with a photomask with design of black circles (300 μm in diameter) were then placed on the top of the PEG solution, polymerization was performed by exposing the whole structure to UV light from top; (iv) in the last step, the photomask, the top glass slide and the spacers were all removed carefully and non-cross-linked PEG solution was washed away by 1× PBS solution thoroughly. The same protocol was used for fabricating microwells from GEL (5 wt.% of gelatin modified with methacrylate group for photocrosslinking[21] and 1 wt.% photoinitiator).

It was clearly observed that both PEG (Fig. 1(b)) and GEL (Fig. 1(c)) arrays have uniform cylinder-like wells with a diameter of $\sim$300 μm, consistent with the dimension of designed black circles on the photomask. However, the surface structure of the two microwells was distinct when imaged using scanning electron microscopy: the PEG well has a smooth surface (Fig. 1(d)) while the GEL well exhibits thin cross-linked fiber network structure (Fig. 1(e)).

To further demonstrate the biochemical difference of PEG and GEL hydrogels and their bioactivity influence on stem cell differentiation, we cultured stem cells in the PEG and GEL microwells, respectively. EBs with an average size around 300 um were made from wild-type mouse embryonic stem cells and then seeded into the PEG and GEL microwells, respectively, by following a protocol of a previous study.[12] Figures 2(a) and 2(c) showed phase contrast images of PEG and GEL microwell arrays with seeded EBs, respectively. The whole microwells with EBs were subsequently cultured in liquid medium with fresh medium changed daily. After six days, a significant morphological difference was observed. The EBs in the PEG microwells remained their spheroid shapes (Fig. 2(b)), in contrast, the EBs in the GEL microwells greatly sprouted (Fig. 2(d)). This is further confirmed by nuclei specific staining using DAPI as shown in Fig. 2(e) (the EBs in the PEG microwells) and Fig. 2(f) (the EBs in the GEL microwells). This result is in agreement with the fact that PEG is non-biodegradable and biochemically inert while GEL has a high biodegradability and capability for cell adhesion and proliferation.[25,26] Consistent with the above results, a higher cell activity of the EBs in the GEL microwells was measured using Alamarblue assay[27] than the EBs cultured in PEG microwells (Fig. 2(g)). These results confirmed the distinct biochemical properties of PEG and

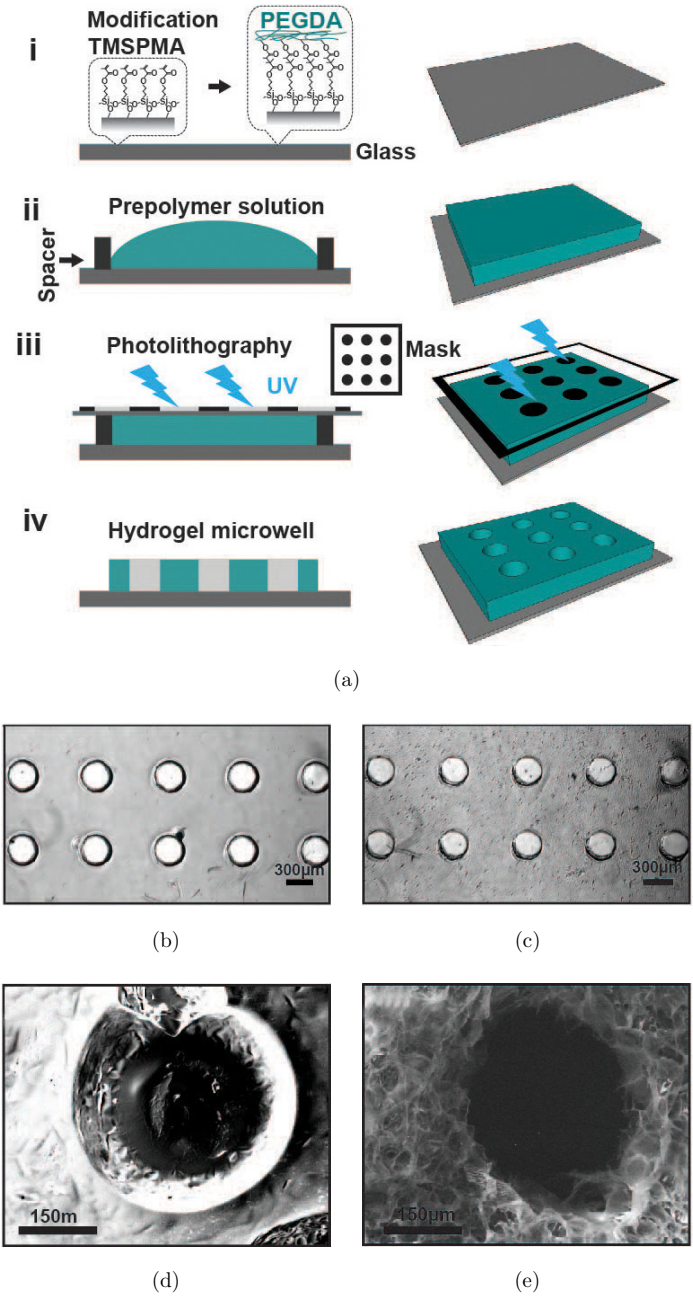

Fig. 1. (Color online) Fabrication of homogeneous microwell arrays. (a) Schematic illustration of microwell array fabrication with a single biomaterial using one-step photolithography. (b) and (c) Phase contrast images of microwell arrays made from PEG (b) and GEL (c) hydrogel polymers. (d) and (e) Scanning electron microscopy images of the PEG (d) and GEL (e) microwells.

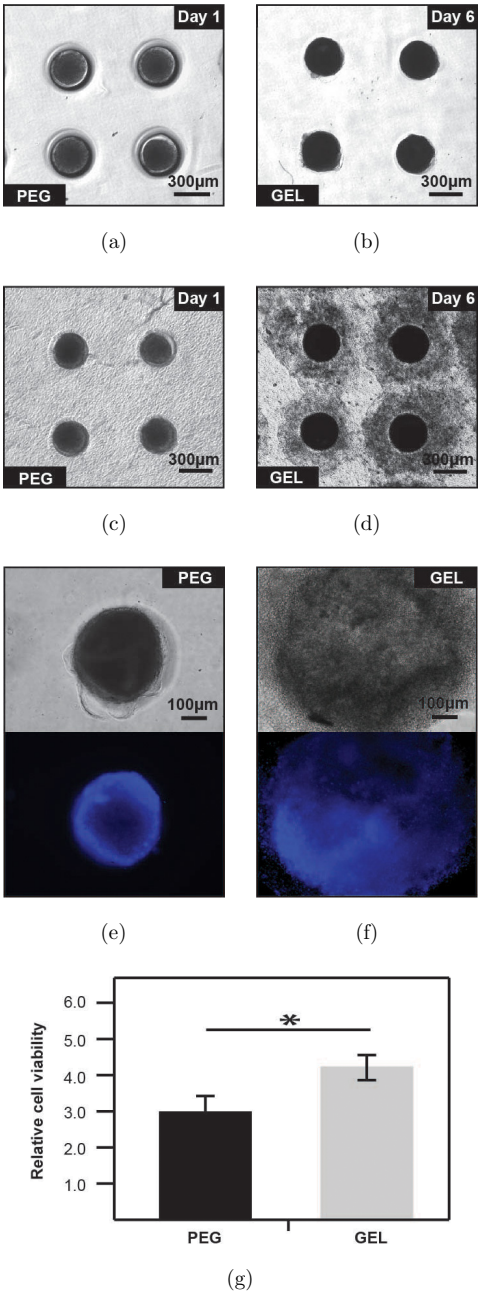

Fig. 2. (Color online) EBs differentiation in homogeneous microwells. (a) and (b) Phase constrast images of EBs at Day 1 (a) and Day 6 (b), when cultured in PEG microwells. (c) and (d) Phase constrast images of EBs at Day 1 (c) and Day 6 (d), when cultured in GEL microwells. (e) and (f) Cellular morphology of EBs at Day 6 in PEG (e) and GEL (f) microwells (top row: phase contrast imaging; bottom row: DAPI cell nuclei staining). (g) Cellular proliferaion of EBs in PEG or GEL microwells at Day 6. The data was measured with Alamarblue assay and normalized by the values at Day 1.

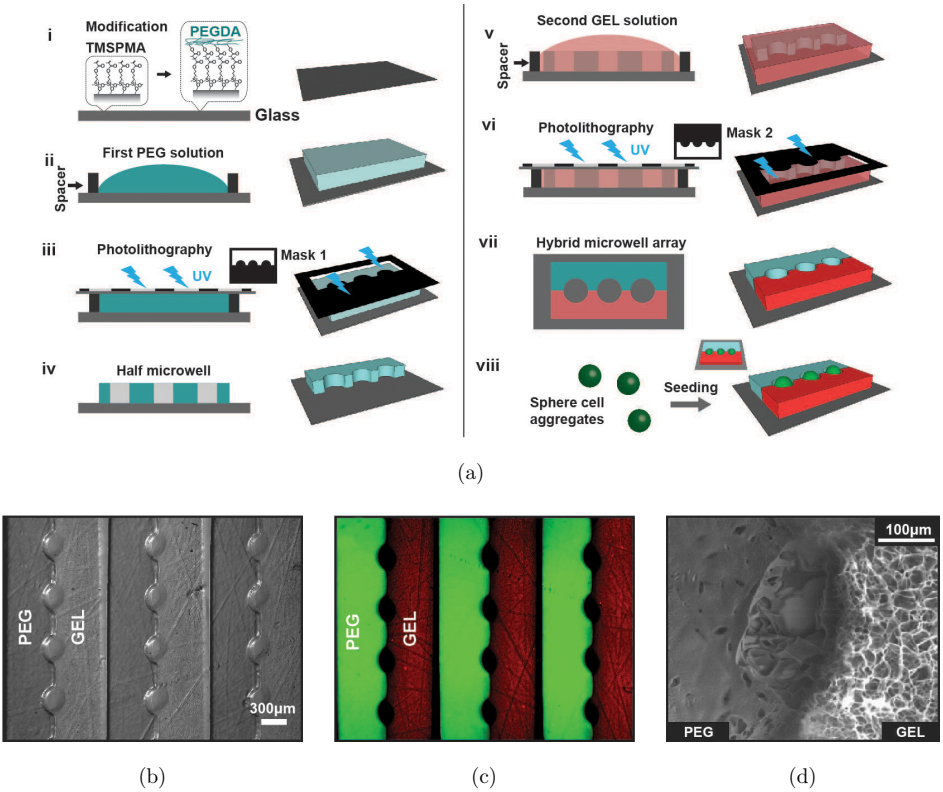

Fig. 3. (Color online) Controlled asymmetrical differention of EBs in the PEG/GEL hybrid microwells. (a) Schematic of individual EBs in hybrid microwells. (b) and (c) Phase constrast images of EBs in the hybrid microwells at Day 1 (b) and Day 6 (c). (d) DAPI cell nuclei staining of the EBs at Day 6. (e) Endothelial cell differentiation of the EBs examined with immunostaining of CD31 of the sample at Day 6.

GEL and also illustrated the possibility of controlling stem cell differentiation in microwells fabricated from designed materials.

Based on the success of the uniform microwell fabrication, we developed a method to fabricate hybrid microwells from two materials of PEG and GEL. As depicted in Fig. 3(a), hybrid microwells fabrication was started by (i) surface modification using TMSPMA and PEGDA (MW 1 KDa), similar to the one-step process in Fig. 1(a). (ii) Subsequently, two No. 1 cover glass slides were stacked at the two ends of the modified substrate glass to serve as spacers for thickness control and PEG hydrogel precursor solution was dropped at the center. (iii) The PEG solution was then covered by another glass slide and then a photomask with specific design of a clear rectangle shape with clear concave half circles along one edge. (iv) PEG polymerization was subsequently achieved by UV exposure from top and those non-cross-linked polymers were washed away by 1× PBS thoroughly. Therefore, a half PEG microwells was fabricated with an open cylinder structure. (v) The other half-microwells was fabricated similarly by adding GEL precursor solution including

5 wt.% of methacrylated gelatin and 1 wt.% photoinitiator to cover the half PEG-microwell structure. (vi) After a horizontal turnover, the same photomask used in step (iii) was used to cover the solution. Here, the concave half-circle on the mask was aligned with the open PEG cylinder structure under microscope. (vii) After polymerization with UV exposure from top, non-cross-linked GEL was washed away by 1× PBS solution thoroughly. (viii) Therefore a hybrid microwells in which two half parts were fabricated from two distinct materials was fabricated and cell aggregates can be seeded in it.

Figure 3(b) showed the final structure of the hybrid microwell arrays in which PEG and GEL half circles were all paired up to form full circle microwells. Furthermore, we labeled the PEG and GEL with red and green fluorescent microbeads, respectively (Fig. 3(c)) and the surface of hybrid microwell was also characterized using scanning electron microcope (Fig. 3(d)). These results demonstrated the success of hybrid microwells fabrication.

Upon succeeding in fabrication of PEG/GEL hybrid microwells, we cultured EBs in the hybrid microwell. Unlike the EBs cultured in the uniform microwells fabricated from single material, EBs in hybrid microwells was exposed simultaneously to two distinct extracellular microenvironments (Fig. 4(a)). As shown in Fig. 4(b), the left half of individual EBs was exposed to PEG while the right half was exposed to GEL (Fig. 4(b)). After six days culture, a single EB showed distinct sprouting patterns on different sides. In comparison with the left half exposed to PEG it remained roughly their initial shape while the right half exposed to GEL had

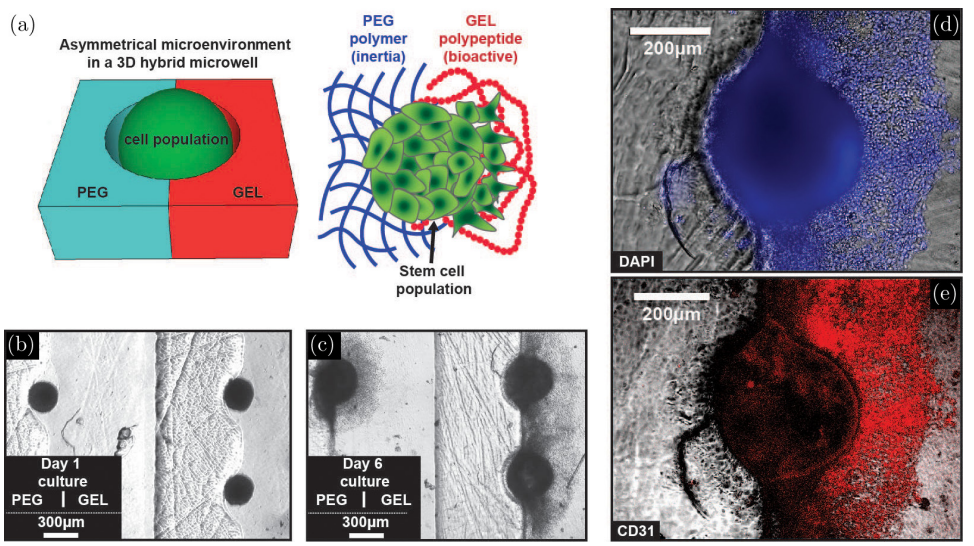

Fig. 4. (Color online) Controlled asymmetrical differention of EBs in the PEG/GEL hybrid microwells. (a) Schematic of individual EBs in hybrid microwells. (b) and (c) Phase constrast images of EBs in the hybrid microwells at Day 1 (b) and Day 6 (c). (d) DAPI Cell nuclei staining of the EBs using DAPI at Day 6. (e) Endothelial cell differentiation of the EBs examined with immunostaining of CD31 of the sample at Day 6.

sprouted significantly (Fig. 4(c)). The morphological difference was further confirmed by DAPI nuclei specific staining (Fig. 4(d)) and immunostaining of the expression pattern of CD31[28] a specific marker for endothelial cell differentiation in vasculogenesis (Fig. 4(e)). It was observed that a significantly higher CD31 expression was detected in the sprouted part of the EB exposed to GEL in the microwells. Taken together, these results demonstrated the spatially controlled differentiation on individual EBs in microwells with designed heterogeneous biochemical properties.

Multipotency and self-renewal of embryonic stem cells are offering exciting opportunities for generating desired cell types and tissues for regenerative medicine, holding a great promise of revolutionizing human healthcare. However, the fine control of cell differentiation has been a longstanding challenge. This study made contribution to addressing the challenge by developing a novel microwell arrays fabrication technology by which functionally distinct biomaterials can be integrated into single wells in a spatially controlled manner. Moreover, in this technology, stem cells are handled using general method without any process e.g., UV radiation exposure, avoiding potential damage and unpredictable influence. The ease of fabrication and the versatility of differentiation control will enable simple and reliable high-throughput studies of stem cells.

Furthermore, this method can be extended to develop more accurate spatial control, creating more complicated geometrical structures for advanced spatiotemporal regulation, and by employing more biomaterials it could enable more flexible control options for cell differentiations, to greatly accelerate *in vitro* stem cell research and its biomedical applications.

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