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A simple and efficient feeder-free culture system to up-scale iPSCs on polymeric material surface for use in 3D bioprinting

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ABSTRACT

The 3D bioprinting and cell/tissue printing techniques open new possibilities for future applications. To facilitate the 3D bioprinting process, a large amount of living cells are required. Induced pluripotent stem cells (iPSCs) represent a promising cell source for bioprinting. However, the maintenance and expansion of undifferentiated iPSCs are expensive and time consuming. Therefore, in this study a culture method to obtain a sufficient amount of healthy and undifferentiated iPSCs in a short-term period was established. The iPSCs could be passaged for twice on tissue culture polystyrene (TCPS) dish with the conditional medium and could adapt to the feeder-free environment. Feeder-free dishes were further prepared from chitosan, chitosan-hyaluronan, silk fibroin, and polyurethane (PU1 and PU2) two-dimensional substrates. The iPSCs cultured on the chitosan substrates showed a higher proliferation rate without losing the stemness feature. Among the different materials, PU2 could be prepared as a thermoresponsive hydrogel, which was a potential ink for 3D bioprinting. The iPSCs cultured on PU2 substrates well survived when further embedded in PU2 hydrogel. Moreover, PU2 hydrogel printed with iPSCs remained structural integrity. The use of PU2 hydrogel to embed iPSCs reduced the injury to iPSCs by shear stress. These results indicate that iPSCs could be expanded on chitosan or PU2 membranes without the feeder layer and then printed in PU2 hydrogel. The combination of these steps could offer a new possibility for future applications of iPSC-based 3D bioprinting in tissue engineering.

1. Introduction

Induced pluripotent stem cells (iPSCs) are pluripotent cells that can be reprogrammed from autologous somatic cells by introducing four stemness factors while avoiding the controversial step of embryo destruction [1]. Similar to embryonic stem cells, iPSCs can be maintained indefinitely in the undifferentiated state but can differentiate into three germ layers in vitro and in vivo (endoderm, mesoderm or ectoderm). Because of the capacity for unlimited self-renewing and avoidance of ethical/legal issues, iPSCs become the most attractive cell source for regenerative medicine, disease modeling, phenotypic drug screening and in vitro pathophysiology studies [2], as well as for the therapy of various degenerative diseases such as Parkinson's disease [3,4], Alzheimer's disease [5,6], and diabetes mellitus [7]. Notwithstanding, before iPSC can be applied in the transplantation therapy, it is desirable to sustain their original characteristics of stemness. Feeder cells such as mouse embryonic fibroblasts (MEFs), which produce stemness-supporting factors and adhesion molecules, are typically required to prevent spontaneous differentiation and improve iPSC attachment [8]. However, the drawbacks of expensive exogenous leukemia inhibitory factor (LIF) and limited proliferation capacity have significantly hindered the widespread use of MEF feeder cells in regenerative medicine and clinical applications. Besides, the disadvantageous of utilizing animal-derived feeder cells are numerous, including much higher risks of exposure to pathogens, endogenous antigens, as well as concerns over immune rejection. Thus, improvement of iPSC culture systems has mainly focused on feeder-free culture systems using cell-free extracellular matrix (ECM) proteins [9–11], conditioned medium [12] or synthetic biomaterials. Even though various feeder-free culture systems have been developed, relevant studies are still continuously in progress

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to overcome the barriers and challenges associated with low efficiency and high product costs.

SNLP 76/7-4 mouse feeder cell line is an immortalized subclone of the mouse embryonic fibroblast cell line (STO) transformed with neomycin resistance, puromycin resistance, and murine LIF genes [13]. Compared to MEFs, SNLP 76/7-4 abundantly expresses the pleiotropic cytokine LIF to suppress spontaneous differentiation and is much easier to expand in vitro. Despite many features described above, most of the present studies only utilize SNLP 76/7-4 as feeder layers, and few have investigated SNLP 76/7-4 based feeder-free culture system including using the conditioned medium to culture iPSCs, or even further combining with biomaterials that mimic the physiological microenvironment to improve the stemness retaining efficiency.

Natural biomaterials have been extensively studied in the field of tissue engineering and regenerative medicine owing to their intrinsic biological activity and biocompatibility. Several studies have explored the culture of pluripotent stem cells in defined microenvironment offered by natural materials, for example, hyaluronic acid (HA) gel [14], chitosan-alginate scaffolds [15], and cyclic arginine-glycine-aspartic acid (RGD) peptide-modified surface [16]. Despite the greater biocompatibility, natural biomaterials tend to suffer from batch-to-batch variations and lacking proper mechanical strength [17]. Meanwhile, synthetic materials are more stable and amenable to modification. Synthetic polymers for polyurethane (PU) exhibit amphiphilic character with their soft and hard segments. The PU based on polyethylene glycol (PEG) can increase the hydrophilicity and form a gel-like architecture, which provides a biocompatible matrix for chondrogenic differentiation of mesenchymal stem cells (MSCs) [18]. Recently, our research group had successfully synthesized biocompatible and biodegradable polyurethanes by a water-based process. The thermoresponsive polyurethanes could undergo sol-gel phase transition at 37 °C to encapsulate neural stem cells (NSCs) and promote their differentiation [19]. Up to date, only a few research works have employed hydrogel to encapsulate pluripotent stem cells to investigate their ability of differentiation [20,21] or for tissue engineering-related applications [22,23].

In this study, we investigated the feasibility of applying SNLP 76/7-4 conditioned medium to replace feeder cells for murine iPSC culture. A series of biomaterials were combined with feeder-free two-dimensional culture system to obtain iPSCs with higher purity without losing all pluripotency. It was found that iPSCs could be scaled up and continued self-renewal on chitosan or polyurethane membranes. They showed reasonable viability after embedded in polyurethane hydrogel. Finally, iPSCs in polyurethane hydrogel could be printed into a 3D cell-laden hydrogel scaffold. We aimed to establish a biomaterial-based culture system to facilitate the expansion and maintain the pluripotency of iPSCs. A combination of these methods can be employed in the future to build iPSC-based 3D constructs.

2. Materials and methods

2.1. Preparation of MEFs and SNLP 76/7-4 feeder cells

MEFs were established from the embryos of ICR female mice (National Taiwan University Laboratory Animal Center, Taipei, Taiwan). The placentas were removed in phosphate buffered saline (PBS, Invitrogen) after moving embryos out form uterus. Heads, limbs, and internal organs were removed to ensure a pure fibroblastic population. Non-visceral tissues were then trypsinized with 0.25% trypsin-EDTA (Invitrogen) for 5 min and smashed by 1 ml syringe. After the precipitation processes, the supernatant was isolated and centrifuged at 1000 rpm for 5 min. Suspended cell pallets in high glucose Dulbecco's modified Eagle's medium (DMEM-HG, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 2 mM L-glutamine (Gibco, USA), and 100 U/ml penicillin-streptomycin (PS, Caisson Labs, USA) were plated onto culture dishes. SNLP 76/7-4 feeder cells were provided by Dr. Allan Bradley at the Trust Sanger Institute [13]. The SNLP 76/7-4 cells were grown in DMEM-HG with 7% FBS, 100 U/ml PS and 2 mM L-glutamine. The cells were subcultured at 90% confluence. Before being used as feeder layers, both mitotically inactivated MEFs and SNLP 76/7-4 were treated with 10 μg/ml mitomycin C for 2 h (Sigma, USA). After treatment, MEFs and SNLP 76/7-4 were washed extensively with phosphate-buffered saline (PBS) and replated on 0.1% gelatinized dishes to get feeder layers. All cultures were kept at 37 °C in a 5% CO_2 humidified incubator.

2.2. Preparation of conditioned medium

To obtain the conditioned medium from SNLP 76/7-4 cells, M10 medium (DMEM-HG supplemented with 10% FBS, 0.1 μM β-mercaptoethanol, 2 mM L-glutamine, and 50 U/ml PS) and M20 medium (DMEM-HG supplemented with 20% FBS, 0.1 μM β-mercaptoethanol, 2 mM L-glutamine, and 50 U/ml PS) were first prepared. Upon attaining 70–80% confluency, the SNLP 76/7-4 culture medium was aspirated, discarded and replaced with fresh M10 medium. After 8–12 h of culturing, the supernatant of SNLP 76/7-4 culture medium was collected, filtered, and mixed with fresh M20 medium in 1:1 volume ratio. This final mixture was designated as the conditioned medium (CM). It was aliquoted and stored at − 80 °C until further use.

2.3. Culture of iPSCs

iPS-MEF-Ng-20D-17 mouse iPSC lines were generously provided by Dr. Shinya Yamanaka (Center for iPSC Research and Application, Kyoto University, Kyoto, Japan). iPSCs were maintained in the undifferentiated state by propagation on the mitotically inactivated feeder layers. Three types of iPSC culture media were used for the study (Medium 1, Medium 2, and CM), and the full details and ingredients of media are listed in Table 1. Once the iPS colonies had reached the optimum size, the cells were digested with 0.05% Trypsin–Ethylenediaminetetraacetic acid (EDTA, Gibco, USA), cells were then seeded onto freshly prepared feeder layers or feeder-free TCPS dishes.

2.4. Optimization of feeder-free culture system

To optimize the feeder-free culture system for iPSCs, a number of methods (respectively named from FF-1 to FF-7) were employed. Briefly, iPSCs combined with feeder cells were first dissociated enzymatically and replated on the gelatinized dish for 1.5 h. After which, feeder cells had attached to the dish but iPSCs remained in the medium. Isolated iPSCs were then collected by centrifugation and reseeded onto gelatinized dishes or non-coated plates. Seven different feeder-free (FF) culture methods were developed and designated as FF-1 to FF-7, with details shown in Figs. 1 and 2.

2.5. Immunocytochemistry

Cells were rinsed with PBS, fixed in 4% formaldehyde for 15 min at room temperature, and permeabilized for 30 min in 0.1% Triton X-100/PBS (PBST). Blocking was carried out with 2% bovine serum albumin (BSA) in PBST solution for 1 h at ambient temperature to mask non-specific protein binding. To verify the pluripotency, cells were treated with the following antibodies including anti-Oct4, anti-DPPA-2 and anti-SSEA-1 (Merck Millipore, Germany) for 2 h. Nuclei were counterstained with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Merck Millipore, Germany). The fluorescent intensity was analyzed and quantified by an inverted fluorescence microscope (DMIRB, Leica). The numbers of iPSC spheroids were counted when positive stain with antibodies after staining assay was performed.