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PAPER

Continuous Release of bFGF from Multilayer Nanofilm to Maintain Undifferentiated Human iPS Cell Cultures

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In most cases, induced pluripotent stem (iPS) cells, must be maintained by replacing basic fibroblast growth factor (bFGF)-containing medium daily to inhibit spontaneous differentiation. However, complete inhibition of differentiation is difficult to achieve even with frequent replacement of medium because of bFGF instability. Here, we established a novel system for the sustained release of bFGF from a modified surface by using a nanofilm fabrication method.

Induced pluripotent stem (iPS) cells are generated from various types of somatic cells.[1-6] The ultimate goal of iPS cell studies is the generation of patient-specific pluripotent stem cells as a regenerative medicine.[7-8] Human iPS cells are expected to eliminate the classical problems associated with human embryonic stem (ES) cells, such as the various ethical issues relating to the utilization of human eggs.[9-10] Human iPS cells, like ES cells, possess two remarkable properties: self-renewal and the ability to differentiate along any cell lineage.[8,11] To be useful as a regenerative medicine, pluripotent stem cells should be propagated using their self-renewal property, and then subsequently differentiated into specific cell types. Therefore, during their culture it is important to preserve the process of self-renewal while minimizing spontaneous differentiation into other cell types.

It has been demonstrated that basic fibroblast growth factor (bFGF), also called FGF-2, is sufficient to maintain the pluripotency and self-renewing activity of human iPS cells as well as ES cells in culture, although the exact mechanism that maintains the undifferentiated state remains poorly understood.[12-15] However, bFGF has been known to be highly unstable under normal culture conditions.[16-17] Recently, it has been reported that bFGF levels in the culture medium significantly fluctuate even with daily replacement of medium, preventing the complete inhibition of spontaneous differentiation. Indeed, it was also demonstrated that sustained levels of bFGF in culture medium improve the maintenance of human iPS cells as well as ES cells, while preserving their self-renewal property and pluripotency.[18]

In concert with an emerging interest in the application of nanofilms to cell culture platforms, erodible nanosurfaces that deconstruct in aqueous conditions via disassembly and/or breakdown of the constituent building blocks have begun to be explored as potential controlled-release drug delivery films for cell culture as illustrated in fig. 1. Drug-loaded degradable nanofilms have been explored for the programmed release of not only protein therapeutics or plasmid DNA but also active ingredient growth factors.[19-21] By applying bioactive nanofilm surfaces onto cell cultures, the ability to tune cell differentiation and/or proliferation has been demonstrated and used to control the cell culture conditions within these films. In this study, we have generated a novel and biocompatible nanofilm system for the continuous release of bFGF by utilizing Layer-by-Layer (LbL) technique.[22-28] The sustained release of bFGF from nanofilm was observed for over 10 days and the biological activity of the released bFGF was investigated. Based on these results, it was demonstrated that the undifferentiated state of human iPS cells was maintained by the bFGF released from multilayer nanofilm.

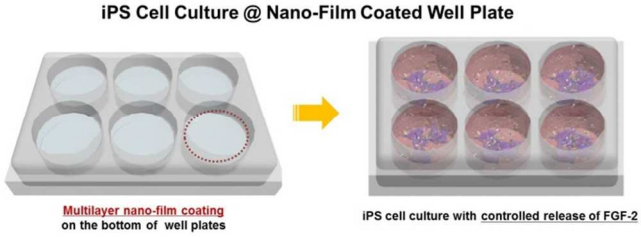

Fig. 1. Schematic illustration of iPS cell cultures with nano-films with effective controlled release of growth factor.

Fig. 2a shows the overall process of Layer-by-Layer (LbL) assembly of Poly-*L*-lysine (PLL), basic fibroblast growth factor (bFGF), and starch (ST) within the designed structure. Multilayer nanofilms were prepared by sequential adsorption of PLL, ST, bFGF, and ST through electrostatic interactions and partial hydrogen bonding. Representative images of $(PLL/ST/bFGF/ST)_n$ *(n = number of tetralayer)* multilayer thin films were obtained; the films exhibited a characteristic color corresponding to each material layer. For LbL assembly, each material solution was adjusted to the appropriate aqueous conditions, as reported previously (described in the Materials and Methods section). The active ingredient bFGF was also successfully incorporated into the multilayer structure. Fig. 2b

shows the chemical structures of PLL and ST used in this study. The polypeptide PLL and the polysaccharide ST were chosen as building blocks for multilayer formation. PLL provides enhanced geometric compatibility and cell adherence with ST as well as increased film stability. In addition, take full advantages of ST gelatinization, nanocrystal layer could incorporate to inner film structure with poor permeability.[29-34] In our approach, controlled b-FGF release mechanisms were prepared by the permeability difference of starch gelatinization based nanofilm inner structures.[35] The thicknesses of the $(PLL/ST/bFGF/ST)_n$ multilayer films were monitored for tetralayer growth, as shown in Fig. 2c. Exponential growth of the thickness profile as a function of tetralayers was observed, which could indicate that the films had a relatively high roughness corresponding with as a function of tetralayer number (Fig. S2).

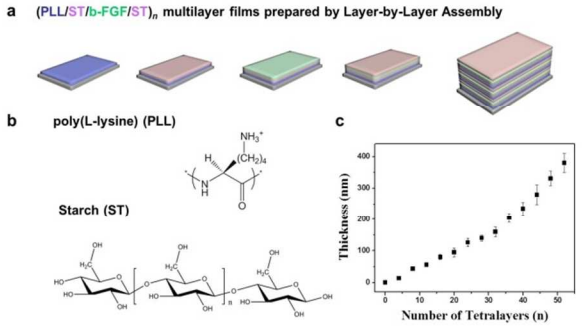

Fig. 2. (a) Schematic depicting the preparation of multilayered structures consisting of PLL, ST, and bFGF. (b) Chemical structures of PLL and ST used in the present study. (c) Growth curve of $(PLL/ST/bFGF/ST)_n$ *(n = number of tetralayer)* multilayer films as a function of tetralayer number.

Fig. 3 shows SEM images of multilayer $(PLL/ST/b\text{-}FGF/ST)_n$ thin nanofilms. These images confirmed the successful preparation of smooth-surfaced multilayer films. Multilayer nanofilms without any notable defects were homogeneously observed over the entire surface. Some unique film structures, such as crystalline structures, were observed as expected. These nanocrystal structures are also shown at high magnification in the FE-SEM image in Fig. 3b. Multilayer films with/without annealing at 40°C for 20 min were comparable. This is because of gelatinization of the nanolayered ST in the multilayer structure. The presence of crystalline structures in ST is well known and has been proven using various experimental tools, including X-ray diffraction, and this crystalline structure serves as a barrier to mass transport.[31-32] Furthermore, the molecular rearrangement of starch molecules in the internal multilayer structures affects growth factor diffusion behavior, including entry and exit of molecules to and from the ST structures.

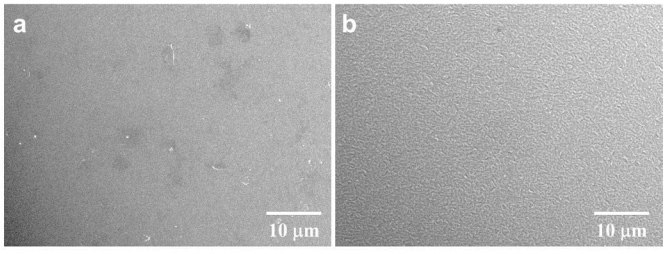

Fig. 3. SEM images of multilayer film at (a) low and (b) high magnification.

The release of bFGF from $(PLL/ST/bFGF/ST)_{20}$ multilayer films after incubation in model physiological conditions (PBS solution, (at 37 °C, 5% CO_2) can be systematically analyzed as a function of incubation time after contact with the PBS solution. The normalized cumulative release profiles show the effect of heat treatment of ST layers on bFGF release kinetics. Heat-treated multilayer films exhibited a linear erosion release profile over a long duration, considering that films are prepared in aqueous solution (Fig. 4). In particular, only about 10% of the bFGF was released from heat-treated multilayer films within the first 24 h of incubation, whereas under the same experimental conditions more than 60% of bFGF was already released from multilayer films that were not heat-treated. The difference in release kinetics of the incorporated bFGF arises from the heat-induced change in the properties of the films, such as porosity, modulus, and crystallinity. As described above, heat treatment of as-prepared ST-containing multilayer films induces the rearrangement of internal film structures, which leads to narrowing of the molecular pathways through which the initially incorporated active agents, such as bFGF, are released. We believe that the molecular release kinetics is affected when the molecules diffuse out of the ST structure in the vertical direction in the present study. In this experiment, the soluble supernatants were collected by centrifugation after the release of bFGF from nanofilm and the concentration of bFGF in the supernatant was measured. Since the immunologically active forms are able to be detected by ELISA, it is believed that the concentration of bFGF shown in Fig. 4 represents a soluble and active form.

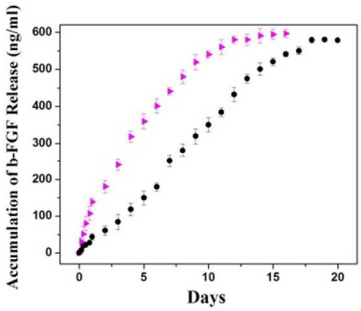

Fig. 4. Normalized cumulative release profile of bFGF from nanofilms before (pink) and after (black) heat treatment as a function of incubation time when rehydrated in PBS with pH 7.4, as measured by ELISA.

At 70% confluency, iPS cell colonies were detached using collagenase and dissociated into small clumps by gentle pipetting. The small iPS cell clusters were seeded onto mitomycin C-treated MEF feeders and cultured in human ES cell culture medium.To evaluate whether pluripotency was maintained by the released bFGF, the release of bFGF from nanofilm was performed in basal culture medium, DMEM. Although several nutrients are contained, it was considered that the cumulative release profile of bFGF is not different in DMEM compared to PBS because the basic physical and chemical properties of DMEM such as conductivity and pH are very similar with PBS. The DMEM containing $(PLL/ST/bFGF/ST)_{20}$ multilayer films was divided into each tubes with same volume and incubated at physiological condition. During the incubation, one tube was collected once a day and the supernatant containing bFGF released from nanofilm (NF-bFGF) was diluted 1:5 in complete ES medium to apply to human iPS cells culture. The NF-bFGF collected on the first day of incubation was applied when the iPS cells were seeded. One day after the start of culture, the medium was replaced with fresh ES medium containing NF-bFGF collected on the second day of incubation. The medium was also replaced with fresh medium

containing NF-bFGF collected on the third day of incubation at the next day and the media were replaced daily in the same way. For comparison, iPS cells were also cultured in medium lacking (negative control) or containing (positive control) 25 ng/mL recombinant bFGF, with daily replacement of the medium. Human iPS cells, like human ES cells, form dome-like colonies, which require a continuous supply of bFGF to maintain the undifferentiated state.[11] In the absence of bFGF, cells begin to differentiate, and the colonies become flat or acquire an epithelium-like morphology. Human iPS cells cultured with NF-bFGF as well as 25 ng/mL recombinant bFGF maintained a tightly packed and endothelial morphology, whereas cultures grown in the absence of bFGF contained mostly differentiated cells after six days of culture (Fig. S1). Furthermore, any inhibitions on cell growth were not observed with NF-bFGF during the culture period (data not shown). According to the release profile, in the case of NF-bFGF, it is considered that the concentration of bFGF in the culture medium was lower than 25 ng/mL by the second day; however, the cells retained their undifferentiated morphology.

The effect of NF-bFGF on the expression of pluripotency markers was further assessed by immunological detection of SSEA-4 and Nanog, which are widely used as pluripotency markers.[36] After six days of culture, three different colonies were examined in each experimental group. In the absence of bFGF, a majority of the cells exhibited a flat, epithelial morphology and barely expressed SSEA-4 and Nanog (Fig. 5a, straight arrows). Although some cells appeared to retain their undifferentiated morphology, the expression levels of SSEA-4 and Nanog were quite low in these cells (Fig. 5a, dashed arrows). However, the cells cultured in the presence of recombinant bFGF and NF-bFGF maintained their spherical shape and strongly expressed SSEA-4 and Nanog (Fig. 5b and 5c). These characteristics were exhibited by most colonies in the presence of recombinant bFGF and NF-bFGF.

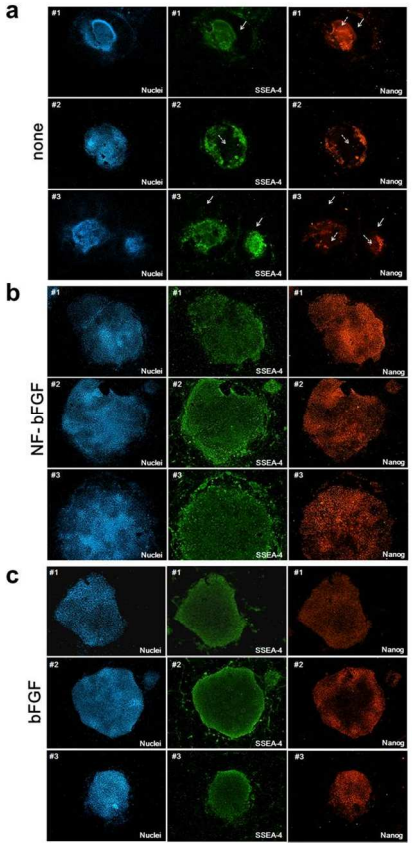

Fig. 5. Effect of NF-bFGF on the expression of pluripotency markers. Expression of SSEA-4 (green) and Nanog (red) in iPS cells cultured with (a) without bFGF, (b) NF-bFGF, and (c) recombinant bFGF. Straight arrows indicate differentiated cells with flat, epithelial morphology. Dashed arrows indicate cells in which SSEA-4 or Nanog expression is suppressed, while their morphologies appear to be maintained. The numbers on the upper left corner of the pictures represent distinct human iPS cell colonies.

The expression level of cell surface AP, which is an early-stage pluripotency marker, was quantitatively analysed.[37] The AP activity of iPS cells was measured after six days of culture and normalized to total protein. The normalized AP activity in the presence of recombinant bFGF is represented as one arbitrary unit. AP activity was reduced to below 40% in the absence of bFGF, whereas it was maintained in the presence of NF-bFGF (Fig. 6a). However, the AP activity was lower than that of cells cultured with recombinant bFGF. This result may be due to the low bFGF concentration at the early stage of culture in the case of NF-bFGF. Next, we investigated the expression level of Oct4, which is a representative transcription factor with a crucial role in pluripotency.[36] Intracellular Oct4 expression was maintained by NF-bFGF supplementation, whereas it significantly decreased in the absence of bFGF (Fig. 6b). These results demonstrate that human iPS cells can grow in an undifferentiated state and maintain their pluripotency with NF-bFGF supplementation.

The main advantage of the LbL nanofilm compared to other sustained release system is believed that the coating onto well-plate is easy to construct a conventional human pluripotent stem cell culture system. Our system could extend the period of medium replacement compared to the established daily change method by coated onto well-plate if other problems such as nutrients depletion and accumulation of toxic by-product are controlled because it is designed to release bFGF over long duration. It has been not investigated in this study whether human iPS cells are able to be cultured directly onto the well-plate coated by $(PLL/ST/bFGF/ST)_{20}$ multilayer films keeping their pluripotency. It was reported that the mechanical properties of multilayer nanofilm influence the physiology of various cells cultured on the nanofilm[38]. In order to culture human iPS cells directly onto LbL nanofilm, the effect on cell responses such as growth, viability and attachment should be addressed in further study. Nevertheless, the human iPS cells not only maintained the undifferentiated state but did not show a significant inhibition on the growth and attachment by the supplementation of NF-bFGF released from LbL structure. Those results indicate that $(PLL/ST/bFGF/ST)_{20}$ multilayer films used in this study is biocompatible with human iPS cell cultures.

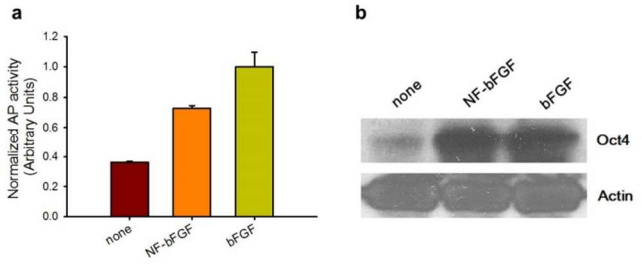

Fig. 6. AP activity and Oct4 expression in iPS cells cultured in the presence of NF-bFGF. Human iPS cells cultured for 6 days were collected and lysed. AP activity was quantitatively analyzed in the lysates and normalized to the total protein. (a) Normalized AP

activity in the iPS cell cultures. (b) Western blot analysis of Oct4 expression.

Conclusion

In summary, we established an LbL technique-based nanofilm system that allows the continuous release of bFGF. The release of bFGF was sustained for over 10 days. Human iPS cells that were grown in the presence of the released bFGF maintained their undifferentiated morphology and expression levels of pluripotency marker proteins such as Oct4, SSEA-4, and Nanog, and AP activity. It is anticipated that these results would provide a beneficial method for maintaining the undifferentiated state of human pluripotent stem cells in culture. Furthermore, it will be expected to be a great tool not only to maintain undifferentiated state of pluripotent stem cells but also in other developmental studies such as the differentiation of iPS cells to neuronal cells, cardiomyocytes, or hepatocytes by conjugating specific differentiation-stimulating factors.

Acknowledgements

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Experimental Section

Experimental details are given in the Supporting Information.

Notes and references

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