Hydrogels as feeder-free scaffolds for long-term self-renewal of mouse induced pluripotent stem (miPS) cells

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Abstract

Expanding the undifferentiated induced pluripotent stem (iPS) cells *in vitro* is a basic requirement for application of iPS cell in both fundamental research and clinical regeneration. In this paper, we intended to establish a simple, low cost, and efficient method for the long-term self-renewal of mouse induced pluripotent stem (miPS) cells without using feeder-cells and any adhesive proteins. Three scaffolds, gelatin coated polystyrene (Gelatin-PS) that is the widely used scaffold for self-renewal of mouse embryonic stem (mES) cells, a neutral hydrogel poly(*N,N*-dimethylacrylamide) (PDMAAm), and a negatively charged hydrogel poly(2-acrylamido-2-methyl-propane sulfonic acid sodium salt) (PNaAMPS), were selected for the long-term subculture of miPS cells over 2 months, starting from passage 14 to passage 29. Each passaged miPS cells on these scaffolds were cryopreserved successfully, and the revived cells showed high viability and proliferation. The passaged miPS cells maintained a high level of undifferentiated state on all these 3 scaffolds, and a high level of pluripotency by expressing differentiation markers *in vitro* and forming teratomas in SCID mice with derivatives of all three germ layers. Comparing with Gelatin-PS, the two hydrogels exhibited much better self-renewal performance in terms of their high proliferation rate, high level expression of undifferentiated gene markers, and efficiency in pluripotent teratoma formation. Furthermore, the PNaAMPS hydrogel demonstrated a slightly higher efficiency and a simpler operation of the cell expansion than PDMAAm hydrogel. As a conclusion, the PNaAMPS hydrogel is an excellent feeder-free scaffold for its simplicity, low cost, and high efficiency in expanding a large number of miPS cells *in vitro*.

I. Introduction

Embryonic stem (ES) cells have been considered as an unlimited source of cells for the transplantation therapy in the regenerative medicine. [Evans and Kaufman 1981; Martin 1981; Thomson and Marshall 1998] However, for the clinical application of ES cells, they face immune rejection after transplantation and there are ethical issues regarding the usage of human embryos. [Mclaren 2001; Robertson 2001] These concerns could be overcome by the generation of induced pluripotent stem (iPS) cells, which is a type of pluripotent stem cells derived from somatic cells, by inducing a "forced" expression of certain genes. [Takahashi and Yamanaka 2006; Takahashi et al., 2007; Okita et al., 2007; Takahashi et al., 2007] Like ES cells, iPS cells are able to self-renew indefinitely and to differentiate into cell types of the three germ layers *in vitro* and in teratomas. [Zhang et al., 2009; Karumbayaram et al., 2009; Werning et al., 2008; Iwamuro et al., 2010] Therefore, iPS cells hold great promise for regenerative medicine. However, one of the obstacles of clinical application of iPS cells is that the efficiency of iPS cell derivation is extremely low. [Maherali et al., 2008; Hochemeyer et al., 2008; Hanna et al., 2008] Thence, the self-renewal of iPS cells *in vitro* is a critical issue for iPS cell application not only in fundamental study, but also in regenerative medicine. Self-renewal is the process in which stem cells divide to form more stem cells with maintenance of both the undifferentiated states and pluripotency of stem cells. [He et al., 2009]

The existing method for self-renewal of iPS cells is by culturing them on a feeder layer of mouse embryonic fibroblasts (MEFs) or Mouse Fibroblast STO Cell Line

(SNL) inactivated by treating with mitomycin C (MMC) or γ-irradiation. [Takahashi et al., 2006; Takahashi et al., 2007] The feeder layer provides a suitable substrate for iPS cells attachment, and releases nutrients and signaling factors, conditioning the media for maintenance of iPS cell self-renewal. However, this cell-feeder method has some disadvantages: firstly, the feeder cells are viable for only several passages and for only a few days / passage. Therefore, the method is high cost, very laborious, time consuming, and thus, unsuitable for large scale cell culture; secondly, it is difficult to purify undifferentiated iPS cells from feeder cells because of the strong interaction between iPS cells and feeder cells; lastly, during self-renewal of human iPS (hiPS) cells in clinical application, it is critical not only to maintain undifferentiated hiPS cells, but also to avoid animal-originated conditions. Therefore, the cell-feeder method is not suitable for hiPS in clinical application. [Liu 2008; Amabile and Meissner 2009] Due to the above mentioned disadvantages, recently, some feeder-free culture systems used for hES self-renewal have been attempted for hiPS cell self-renewal, such as the stem cells cultured on surfaces coated with laminnin, fibronectin, and Matrigel^{TM} extracted from Engelbreth-Holm-Swarm mouse sarcoma. [Xu 2001; Richards et al., 2002; Stojkovic et al., 2005] However, the obvious disadvantage of this method is its requirement of the conditioned medium originated from animal sources. Besides this, the high cost also limits the wide usage of this method. That is why the cell-feeder method is still most commonly used for self-renewal of iPS cells, even for the mouse iPS (miPS) cells.

In this paper, we intended to establish a simple, low cost, and efficient method for

the long-term self-renewal of miPS cells without feeder-cells and any surface modification of adhesive proteins. Three scaffolds, gelatin coated polystyrene (Gelatin-PS), the widely used scaffold for self-renewal of mouse embryonic stem (mES) cells, poly(*N*,*N*-dimethylacrylamide) (PDMAAm) hydrogel, and poly(2-acrylamido-2-methyl-propane sulfonic acid sodium salt) (PNaAMPS) hdrogel, were selected for the long term self-renewal experiment. The neutral hydrogel PDMAAm was selected for its good performance as scaffolds for favoring the undifferentiation of miPS cells for short term. [Yang et al.] The negatively charged hydrogel PNaAMPS was selected for its spontaneous cartilage regeneration behavior *in vivo*. [Yasuda et al., 2009] Long-term subculture for 15 passages over 2 months was performed on the 3 scaffolds, starting from the miPS cells of passage 14 (P14) supplied from Riken Cell Bank (RIKEN BRC, Tsukuba, Japan). [Takahashi et al., 2006; Takahashi et al., 2007; Okita et al., 2007; Takahashi et al., 2007] In the case of mES cells, gelatin-coated polystyrene (Gelatin-PS) dish supplemented with Leukemia inhibitory factor (LIF) is widely used for expanding and maintaining the undifferentiated state of mES cells, due to its convenience. [Sirard et al., 1998; Nussbaum et al., 2007; Moeller et al., 2008] iPS cell is considered similar to ES cells, so in this work, the cultivation was also performed with the presence of LIF, a factor that stimulates mouse stem cells self-renewal by binding of the LIF receptorβ/gp130 heterodimer and activation of the JAK/Stat3 signaling pathway. [Niwa et al., 1998; Matsuda et al., 1999; Niwa et al., 2009] The pluripotency of the passaged miPS cells was examined by differentiating them *in vitro* & *in vivo*. The self-renewal on these 3

scaffolds was compared and the most efficient system was discussed.

II. Materials and methods

1. Hydrogels

1-1. Materials

N, N-dimethylacrylamide (DMAAm; Tokyo Kasei Kogyo, Tokyo, Japan) was distilled at a reduced pressure before use. 2-acrylamido-2-methyl-propane sulfonic acid sodium salt monomer (NaAMPS) was obtained by the neutralization of 2-acrylamido-2-methyl-propane sulfonic acid (AMPS), kindly provided by Toagosei (Tokyo, Japan), with sodium hydroxide in ethanol; it was purified by recrystallization from acetone. *N,N′*-methylenebis-(acrylamide) (MBAA; Tokyo Kasei Kogyo, Tokyo, Japan) were purified by recrystallization from ethanol. 2-Oxoglutaric acid (Wako Pure Chemicals, Osaka, Japan), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES; Sigma, St. Louis, MO), phenol red (Sigma, St. Louis, MO), sodium chloride, and sodium hydrogen carboxyl (Junsei chemicals, Tokyo, Japan) were used as purchased. The polystyrene (PS) tissue culture dish (Iwaki & Co., Ltd., Tokyo, Japan) was prepared prior to cell seeding by coating with a solution of 0.1 % porcine gelatin (Sigma-Aldrich) in phosphate buffered saline (PBS, Gibico-BRL), which denoted as Gelatin-PS scaffold in this study.

1-2. Synthesis

The chemical structures of PDMAAm and PNaAMPS are shown in Scheme 1. These hydrogels were synthesized by radical polymerization as previously described.

[Yang et al., 2010; Yang et al., 2010] Sheets of the gels with 1 mm in thickness were synthesized in the reaction cells. A 1 M aqueous solution of monomer, 4 mol% cross-linker (MBAA) and 0.1 mol% initiator (2-oxoglutaric acid) were added to the reaction cells. After purging with nitrogen gas for 30 min, the cells were irradiated with UV light (wavelength, 365 nm) for polymerization (6 h).

After polymerization, the gels were separated from the glass plates and immersed in a large amount of deionized water for 1 week. The water was changed twice daily to remove the unreacted residual chemicals. The gels were then immersed in HEPES buffer solution (HEPES, 5×10^{-3} M; $NaHCO_3$, 1.55×10^{-2} M; NaCl, 0.14 M; pH 7.4). Phenol red (2.5×10^{-3} g/L) was used to visually indicate the gel pH. After reaching equilibrium, the pH and ionic strength of the solution containing the gels were adjusted to 7.4 and approximately 0.15 M, respectively. The gel disks were punched out of the gel plates by a hole-punch with a radius of 7.5 mm. Then the chemically cross-linked hydrogels were sterilized in an autoclave (120 °C, 20 min). After sterilizing, gels were placed in a 24-well polystyrene (PS) tissue culture dish (Iwaki & Co., Ltd., Tokyo, Japan). The gels were incubated in culture media for 6 h before cell culture. Here, it should be noted that, the diameter of the culture dish used in this study was 15.5mm (24-well polystyrene (PS) tissue culture dish). The diameter of gels of 15 mm was selected in this study, which was convenient for our operation. Almost all of the cells adhered and proliferated on the surface of gels although there was a little non-covered part of the plate, which was observed and confirmed by optical micrograph.

The elasticity of the hydrogels was measured by compression test with a tensile compressive tester (Tensilon RTC-1310A, Orientec, Co.). The elastic modulus, *E*, was determined by the average slope of the stress-strain curve over the strain range of 0-0.1. The *E* of PDMAAm and PNaAMPS hydrogels were 162 kPa and 32 kPa, respectively. The degree of swelling *q* was measured as the weight ratio of the gel in its equilibrated state in HEPEES buffer solution to that in its dried state. Each value was averaged over at least three parallel measurements. The *q* of PDMAAm and PNaAMPS hydrogels were 12.5 and 21.6, respectively.

2. Cell culture

The miPS cells of passage 14 (P 14) (Cell No. APS0001, Cell name iPS-MEF-Ng-20D-17, Lot No. 012) were provided by the RIKEN BRC through the Project for Realization of Regenerative Medicine and the National Bio-Resource Project of the MEXT, Japan. [Takahashi et al., 2006; Okita et al., 2007] The cells were cultured according to the protocol. [Takahashi et al., 2007] Cells were cultured in Dulbecco's modified Eagle medium (DMEM: Invitrogen), containing 15 % (v/v) fetal bovine serum (FBS: ES cell Qualified FBS, Invitrogen), 0.1 mM nonessential amino acids (NEAA: Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 50 U/ml penicillin 50 μg/ml streptomycin (Invitrogen), and supplemented with 1000 U/ml of leukemia inhibitory factor (LIF: ESGRO, Millipore). The P 14 miPS cells denoted as input cells were loaded on the surfaces of hydrogels and Gelatin-PS scaffolds with a density of 4.5×10^4 cells/cm^2, and also used as control in this study. Then, the cells were subcultured for 15 passages from P 15 to P 29 over 2 months on these scaffolds

for long-term self-renewal. The cells were passaged every 4 days on these scaffolds. All cells-loaded samples were cultured at 37 °C in a humidified atmosphere of 5% CO_2. The study route was shown in Scheme 2. The medium was changed every 48 h but half of medium was changed for PDMAAm samples to prevent the loss of cells due to the weak adhesion of the cells to the PDMAAm gels.

Cell morphology on these scaffolds was monitored using a phase contrast microscope (OLYMPUS CKX31, Japan) equipped with a digital camera using 10 × objectives lens. Cell density at 4 days (4 d) for every passage was quantified on the basis of the activity of the dehydrogenase in miPS cells using a cell counting kit (Dojindo, Japan), [Yang et al.; Yang et al., 2010] according to the manufacturer's protocol. The cell density was calculated from a standard curve.

3. Real-time polymerase chain reaction (real-time PCR) analysis of undifferentiated and differentiated markers of miPS cells on hydrogels

The miPS cells were cultured on these scaffolds for 4 d, and then, cells were collected from scaffolds respectively for real-time PCR analysis. For the case of cells on PNaAMPS gels, the gels were taken out from the original 24-well culture wells and rinsed with phosphate-buffered saline (PBS). The cells were scraped off the hydrogel surfaces with a cell culture scraper (Iwaki & Co., Ltd., Tokyo, Japan), and collected for RNA extraction. For the case of cells on PDMAAm gels, the cells were directly scraped off the hydrogel surfaces and collected in a centrifuge tube, taking advantage of the weak adhesion of the cell colonies to PDMAAm gels. After

centrifugation, the cells were rinsed with PBS, and then collected for RNA extraction. For the case of cells on Gelatin-PS gels, cells were rinsed with PBS after removing culture medium, and then directly treated for RNA extraction. As positive control, the P 14 miPS cells provided by RIKEN (marked as input cells) were used directly for RNA extraction.

As previously described, [Yang et al.; Yang et al., 2010; Yang et al., 2010] all collected cells were treated according to the RiboPure^{TM} Kit protocol to isolate the total ribonucleic acid (RNA). The RNA was reverse-transcribed into single-strand complementary deoxyribonucleic acid (cDNA) using the PrimeScrit^{TM} RT reagent Kit (TakaraBio, Ohtsu, Japan) according to the protocol. cDNA was stored at –20 °C until required.

All primers used for RT-PCR were designed by TakaraBio. Details of all primer sequences are provided in Table 1. All PCR reactions were performed by SYBR Premix Ex Taq^{TM} II (TakaraBio, Ohtsu, Japan) in standard 25-μL reactions. All PCR data were calculated by using the Delta Delta Ct (ΔΔCt) method. The data were normalized by the appropriate housekeeping Glyceroaldehyde-3-phosphate dehydrogenase (GAPDH) levels for all samples, and further normalized by the levels for the input cells under the same conditions. The data were therefore presented as an increase/decrease in expression of a sequence relative to that of the input cells.

4. Immunocytochemistry

The fluorescence staining was carried out as previously described. [Yang et al.;

Yang et al., 2010] The P 29 miPS cells were washed with sterile phosphate-buffered saline without calcium and magnesium (PBS(-)), fixed with 4% formaldehyde in PBS(-) for 20 min in incubator (37°C, in a humidified atmosphere of 5% CO_2). After washing with PBS(-), the samples were permeabilized with 0.1% Tritox X-100 in PBS(-) at 4°C. The samples were washed with PBS(-) again, followed by pretreatment to block non-specific reaction with 5% BSA in PBS(-) in incubator. Washed it with PBS(-), the immunoreaction was carried out with FITC-conjugated mouse anti-SSEA-1 (BD pharmingen^{TM}) and Alexa488-conjugated anti-Oct-4 (Millipore), respectively. For cell nuclei staining, cells were incubated in Hoechst 33258 (Dojindo, Japan), followed by rinsing with PBS(-). The fluorescence images were recorded with a fluorescence microscope (Eclipse TE2000-E, Nikon, Tokyo, Japan).

5. Quantitative alkaline phosphatase (ALP) activity assay

Quantitative alkaline phosphatase (ALP) activity of miPS cells cultured on these scaffolds was assessed by Quantitative Phosphatase Characterization Kit (Milipore, SCR066), according to the protocol. The P 29 Cells were collected at 4 d on these scaffolds, and approximately 20,000 cells were used for each assay point. Under alkaline condition (pH > 10), ALP can catalyze the hydrolysis of p-nitrophenylphosphate (p-NPP) into phosphate and p-nitrophenol, a yellow colored by-product of the catalytic reaction. The amount of ALP can thus be reliably quantified by reading the amount of p-nitrophenol amassed after the catalytic reaction at 405 nm on a spectrophotometer.

6. Flow cytometry

The P 29 miPS cells passaged on these scaffolds were collected at 4 d, dissociated, and then incubated in FITC-conjugated mouse anti-SSEA-1 (BD pharmingen^{TM}) in a 5 % suspension of BSA (Sigma) in PBS to detect SSEA-1 positive cells. Stained cells were analyzed with a BD FACSCanto flow cytometer (Becton Dickinson Biosciences).

7. In vitro differentiatation of passaged miPS cells

For embryonic body (EB)-induced differentiation, the 3×10^6 P 29 miPS cells were suspended in 10 mL miPS-medium without LIF and cultured in a 10-cm bacterial culture dishes (Iwaki & Co., Ltd., Tokyo, Japan) for 5 days to form EBs. Then the EBs were plated on Gelatin-PS dish and cultured for additional 7 days for differentiation. The differentiated cells were collected and extracted RNA for real-time PCR analysis.

8. In vivo differentiation of passaged miPS cells: teratoma formation

The P 29 Cells were collected at 4 d on these scaffolds, and suspended in culture medium. 2×10^6 miPS cells for each site were injected subcutaneously into the dorsal flank of 8-week-old severe combined immunodeficient (CB-17SCID) mice. 6 injected sites (three mice) for each sample were performed. After 1 month, the teratomas were formed from the miPS cells passaged on hydrogels and retrieved at

the injection sites, but not formed from the iPS cells passaged on Gelatin-PS scaffold. After 2 months, the terotomas were also formed from the miPS cells passaged on Gelatin-PS and retrieved. All teratomas were taken photos, weighted, fixed with 10% formaldehyde in PBS, and embedded in paraffin. Then the tissue sections were prepared and analyzed with Hematoxylin and eosin (HE) staining.

9. Statistical analysis

Data are expressed as mean ± standard deviation at least over three samples. Statistical significance was evaluated using one way analysis of variance (ANOVA). Values of $P < 0.05$ were considered significant.

III. Results

1. Long-term undifferentiation of miPS cells

1-1. Morphology

Fig. 1 shows typical morphology of several passages of miPS cells cultured on PDMAAm, PNaAMPS hydrogels, and Gelatin-PS scaffold for 4 d / passage. The passage 15 (P 15) miPS cells, which was derived from the provided P 14 miPS cells cultured on these hydrogels, formed cell colonies on both PDMAAm and PNaAMPS hydrogels but monolayer cell colonies on Gelatin-PS scaffold. On PDMAAm hydrogels, each colony was from the tightly congregated cells, as revealed by the spherical morphology, and the cells in the colonies cannot be recognized individually. With the increase in the culture time, cell colonies become bigger due to cell

proliferation, and the cell colonies were easy to be detached from hydrogel surfaces to suspend on the surfaces of PDMAAm hydrogels. On PNaAMPS hydrogels, similar cell colonies as that on PDMAAm hydrogels were observed. But the cell colonies on PNaAMPS hydrogels adhered to the hydrogel surfaces during all the proliferative period, and the cell colonies showed oblate morphology. However, monolayer cell colonies were observed on the Gelatin-PS scaffolds, which are completely different from that on hydrogels, indicating strong cell adhesion to Gelatin-PS surface. Furthermore, all cells on these hydrogels and Gelatin-PS scaffold exhibited small, tightly packed morphology with distinct colony borders, which is typical for undifferentiated stem cells.

It can be seen from Fig. 1 that the miPS cells showed similar morphology during subculture for long-term of 15 passages from P 15 to P 29 on these scaffolds. It suggests that the self-renewal behavior of miPS cells on these hydrogels and Gelatin-PS scaffold, regardless of the increase in passages of cells. Meanwhile, it is noteworthy that there were some scattered cells with distinguished cell borders besides of the cell colonies for P 15 miPS cells on these scaffolds, which is the typical morphology of differentiated stem cells. It indicates that the heterogeneity of the initial miPS cells. However, it is interesting to observe that the scattered cells gradually disappeared with the increase in passages of cells, and for P 19 it was very few. The morphology of P 29 miPS cells showed more homogeneous cell phenotype than that of P 15 miPS cells on these scaffolds, especially on the two hydrogels. It means that the heterogeneous miPS cells turned to homogeneous miPS cells on these

scaffolds with increase in passages of cells. Thus, the undifferentiated miPS cells are purified by subcultured on these scaffolds.

1-2. Cell proliferation

As shown in Fig. 2, the cell density of miPS cells initially loaded on these scaffolds, denoted as cell density of input cells, was 4.5×10^4 cells/cm^2. The cell densities of miPS cells proliferated on PDMAAm hydrogels, PNaAMPS hydrogels, and Gelatin-PS scaffold for 4 days (4 d), denoted as cell density of output cells, were 6.1×10^5 cells/cm^2, 7.4×10^5 cells/cm^2, and 5.8×10^5 cells/cm^2, respectively. Compared with the input cells, the output cells increased to approximately 14 folds, 16 folds, and 13 folds on PDMAAm hydrogels, PNaAMPS hydrogels, and Gelatin-PS scaffold, respectively. The high proliferation rate of miPS cells on these hydrogels and Gelatin-PS scaffold indicates the undifferentiated state of miPS cells can be maintained well on these scaffolds, since the undifferentiated stem cells exhibit high proliferation potential, which will decrease as the stem cells mature and then differentiate. [Smith et al., 1988; Williams et al., 1988] It is well known that Gelatin-PS scaffold provides excellent cell adhesion and proliferation for mES cells. [Yang et al., 2010; Kojima et al., 2001] The above results indicate that the two hydrogels have even better performance than Gelatin-PS scaffold, especially the PNaAMPS hydrogels. Furthermore, we found that the proliferation rate of the miPS cells on these scaffolds maintained a constant, which was independent of the change in passages of cells (Data are not shown). This result also indicates the long-term self-renewal of miPS cells on these scaffolds, which is consistent with the

morphology results that the similar morphology of passages of miPS cells was observed on these scaffolds regardless of the change in passages of cells.

1-3. Analysis of the gene expression of undifferentiated markers of miPS cells by real-time PCR

Further to evaluate the intrinsic property of long-term subcultured miPS cells on these scaffolds, the gene expression of undifferentiated markers was examined. During the miPS cells subculture on these scaffolds for long-term of 15 passages from P 15 to P 29, several passages of cells were collected and the gene expression of undifferentiated markers were evaluated: Nanog homeobox (*Nanog*), SRY-box containing gene 2 (*Sox 2*), Octamer-4 (*Oct-4*), and developmental pluripotency associated 5A (*Dppa 5a*) genes, all known to be important for the maintenance of the undifferentiated state of mouse stem cells. [Loh et al., 2006; Palmqvist et al., 2005]

The gene expression levels of undifferentiated markers in the passages of miPS cells subcultured on these scaffolds for 4 d / passage and the input cells were shown in Fig. 3. The gene expression of passages of miPS cells on all the scaffolds shows the same tendency: compared with input cells, all the miPS cells passaged on all the scaffolds show the similar or up-regulated gene expression of undifferentiated markers. Here, the up-regulated expression of undifferentiated markers in passaged cells is attributed to the mixed state of initial miPS cell, which was used as control in the real-time PCR analysis. Additionally, compared with input cells, the gene expression levels of miPS cells cultured on all scaffolds increases with the increase in passage of cells, which is consistent to the morphology results that more

homogeneous cell morphology was found on these scaffolds with increase in passage of cells. The PCR results demonstrated that the undifferentiated state of the miPS cells on these hydrogels and Gelatin-PS scaffold could be maintained well, even subcultured on these scaffolds for long-term of 15 passages. Furthermore, our hypothesis, which is concluded from the morphology results, that the undifferentiated miPS cells were purified by subculturing on these scaffolds is also proved.

1-4. Analysis of undifferentiated protein expression by immunocytochemistry

After the miPS cells subcultured for long-term of 15 passages, the P 29 miPS cells were collected to further verified the undifferentiated state by fluorescence staining of stage-specific embryonic antigen 1 (SSEA-1) and Octamer-4 (Oct-4), which are specific markers for undifferentiated mouse stem cells. [Matsuda et al., 1999] For this immunocytochemistry analysis, the sample preparation for miPS cells on PDMAAm hydrogels should be noted. Before staining, the miPS cells cultured on PDMAAm hydrogels were transfered to the surface of Gelatin-PS dish for further 2 d culture to form cell monolayers. [Bauwens et al., 1999] This is because it is difficult to stain and observe the thick cell colonies formed on PDMAAm hydrogels. The other cells on PNaAMPS hydrogels and Gelatin-PS scaffold were stained directly on the original scaffolds. The expression of SSEA-1 and Oct-4 are shown in Fig. 4a and Fig. 4b, respectively. As shown in Fig. 4a, almost all of the miPS cells on these scaffolds showed positive SSEA-1expression, but parts of the miPS cells on PDMAAm hydrogel and Gelatin-PS scaffold showed negative SSEA-1 expression (area pointed by arrow). As shown in Fig. 4b, almost all of the miPS cells on these scaffolds

showed positive Oct-4 expression, but parts of the miPS cells on PDMAAm hydrogel and Gelatin-PS scaffold showed negative Oct-4 expression (area pointed by arrow). The Oct-4 expression is consistent with SSEA-1 expression. Furthermore, Fig. 4 shows that the miPS cells subcultured on PNaAMPS hydrogel have the most high and homogeneous expression of SSEA-1 and Oct-4, compared with that on PDMAAm hydrogel and Gelatin-PS. In summary, the P 29 miPS cells subcultured on all of the three kinds of scaffolds showed small cell phenotype and large nuclei, which is specific for undifferentiated stem cells. [Yang et al.; Amit et al., 2010] It indicates that the miPS cells can maintain the undifferentiated state well on these scaffolds even subcultured for long-term of 15 passages.

1-5. Quantitative analysis of Alkaline Phosphatase (ALP) activity

The undifferentiated ES/iPS cells usually express high level of membrane ALP. To further confirm the undifferentiated state of the P 29 miPS cells passaged on these scaffolds, the quantitative analysis of ALP activity was performed. After being subcultured for long-term of 15 passages, the P 29 miPS cells were collected to evaluate the ALP expression. Then the results of miPS cells on PNaAMPS hydrogel and Gelatin-PS scaffold were normalized by the result of that on PDMAAm hydrogel, the data were therefore presented as up-regulation or down-regulation of ALP expression in miPS cells in relative to PDMAAm hydrogel.

As shown in Fig. 5a, the miPS cells on PNaAMPS hydrogel and Gelatin-PS scaffold showed up-regulation of ALP expression to that on PDMAAm hydrogel. The miPS cells on PNaAMPS showed the highest ALP expression, which is consistent to

the immune-staining results that the miPS cells on PNaAMPS hydrogel showed excellent SSEA-1 and Oct-4 expression than that on PDMAAm hydrogel and Gelatin-PS scaffold.

1-6. Flow cytometry

As shown by the immune-staining results in Fig. 4a, the P 29 miPS cells had excellent expression of SSEA-1, especially for the miPS cells subcultured on PNaAMPS hydrogels. To further evaluate the positive SSEA-1 level of the miPS cells subcultured on these scaffolds, the staining cells were quantified by flow cytometry. It can be seen from Fig. 5b that the SSEA-1 possitive cells in the P 29 miPS cells harvested from PDMAAm hydrogel was 81.8 %, from PNaAMPS hydrogel was 83.4 %, and from Gelatin-PS was 81.2 %, respectively. That is, more than 80 % miPS cells were positive for SSEA-1 for all scaffolds, and among them, the passaged miPS cells on PNaAMPS hydrogel showed slightly higher expression of SSEA-1 level than that on the other two scaffolds, which is consistent to the immune-staining results shown in Fig. 4 and the ALP expression results shown in Fig. 5a.

2. In vitro & In vivo differentiation of passaged P 29 miPS cells

The results shown in Fig. 3, 4, 5 indicate that the miPS cells can maintain a high level of undifferentiated state for long-term on the 3 scaffolds. As we known, iPS cells are pluripotent cells that have the potential for both indefinite self-renewal and differentiation into all three germ layers of body. Therefore, to verify the pluripotency of the passaged miPS cells subcultured on these scaffolds, the P 29 miPS cells were

collected and differentiated *in vitro* and *in vivo*.

2-1. In vitro differentiation of passaged P 29 miPS cells

To verify the pluripotency of the passaged miPS cells subcultured on these scaffolds *in vitro*, the P 29 miPS cells were collected and differentiated by traditional embryonic bodies (EBs) method. Then the expression levels of genes associated with the three somatic lineage differentiation, ectoderm, mesoderm, and endoderm, of the differentiated miPS cells were examined. [Biswas et al., 2007; Loebela et al., 2003; O'Shea et al., 2004]

The markers associated with ectoderm including nestin (*Nes*), transformation related protein 63 (*Trp 63*), and orthodenticle homolog 2 (*Otx 2*) (Fig. 6a); the markers associated with mesoderm including MyoD family inhibitor domain containing (*mdfic*) and *E-cadherin* (Fig. 6b); and the markers associated with endoderm including alpha-fetoprotein (*AFP*), transthyretin (*Ttr*), and Albumin (*Alb*) (Fig. 6c), were evaluated by real-time PCR analysis. All differentiated gene markers in differentiated miPS cells up- regulated in relative to the input cells, which indicates that the P 29 miPS cells maintain the excellent pluripotency even subcultured on these scaffolds for long-term of 15 passages. Furthermore, among the three lineages, the two mesoderm related markers, *mdfic* and *E-cadherin*, sharply up-regulated in these differentiated miPS cells, which associated with cardiac differentiation. The result is consistent to the morphology results that some beating areas were observed of differentiated miPS cells during the differentiated period.

2-2. In vivo differentiation of passaged P 29 miPS cells

To further verify the pluripotency of the passaged miPS cells subcultured on these scaffolds *in vivo*, the P 29 miPS cells were collected and injected in SCID mice to induce teratoma formation. After 1 month (1 M) implantation, the teratomas were formed derived from the miPS cells passaged on PDMAAm and PNaAMPS hydrogels (Fig. 7a), but no teratoma was observed derived from the miPS cells passaged on Gelatin-PS scaffold. However, after 2 months (2 M) implantation, the teratomas were also formed from Gelatin-PS derived sample (Fig. 7a).

The average weight of retrieved teratomas from 6 balances was shown in Fig. 7b. The teratomas formed for 2 months derived from the miPS cells passaged on Gelatin-PS were marked as Gelatin-PS (2 M). Among these retrieved teratomas, the smallest one was 0.14 g and the largest one was 13.8 g, which is why there are huge error bars in Fig. 7b. The average weight of retrieved teratomas was 1.34 g for PDMAAm-derived sample, 3.55 g for PNaAMPS-derived sample, and 1.32 g for Gelatin-PS-derived sample. Although teratomas could be formed by the miPS cells derived from all these 3 scaffolds, PNaAMPS derived sample is superior to the two other samples, especially, to the Gelatin-PS derived sample that took 2 month to form teratomas.

The histological examination of HE stained sections (Fig. 7c) showed that the formed teratomas contained 3 germ layers. Tissues of ectodermal lineage including epidermis, mesodermal lineage including muscles, and endodermal lineage including epithelium were well recognized (Fig. 7c).

The PCR results in Fig. 3 showed that the similar expression of developmental

pluripotency associated gene was maintained in the miPS on the 3 scaffolds, even subcultured for long-term of 15 passages. Furthermore, the *in vitro* and *in vivo* differentiated results in Fig. 6, 7 also showed that the P 29 miPS cells maintained high level of pluripotency. The results indicate that the long-term self-renewal of miPS cells can be realized by passaging them on these hydrogels and Gelatin-PS scaffold to maintain both excellent undifferentiated state and excellent pluripotency. It provides a simple, low cost culture method to expand miPS cells *in vitro*.

IV. Discussions

1. Long-term self-renewal of miPS cells on hydrogels and Gelatin-PS scaffold

The long-term undifferentiation of miPS cells is realized on the PDMAAm, PNaAMPS hydrogels, and Gelatin-PS scaffold, as shown from Fig. 1-5. Furthermore, the results shown in Fig. 6, 7 indicate that the P 29 miPS cells maintain excellent pluripotency even subcultured on these scaffolds for long-term of 15 passages. It indicates that the long-term self-renewal of miPS cells can be realized on these hydrogels and Gelatin-PS scaffolds. In our previous studies [Yang et al. 2010; Yang et al. 2010], we found that the behaviors of cells on hydrogels were regulated by the adsorbed proteins to hydrogels, which were affected by the charge properties and chemical structures of hydrogels. It indicated that the amount of proteins such as fibronectin adsorbed from the cell culture medium strongly depends on the charge properties and chemical structures of the hydrogels. The behavior of cells cultured on these hydrogels also changed dramatically, consistent with this adhesive protein

adsorption. On neutral hydrogels, cells had a small spreading area and fast migration velocity due to poor protein adsorption, whereas on both negatively charged and hydrophobic structures of hydrogels, cells had a large spreading area and were less mobile due to abundant protein adsorption.

Furthermore, in our recent study, [Yang et al.] we found that there is a collaborative effect of adsorbed proteins on the hydrogel scaffolds and LIF in maintaining short-term self-renewal of miPS cells on hydrogels. However, when there are abundant adsorbed proteins on hydrogels, the collaborative effect disappears and miPS cells were spontaneously differentiated on hydrogels even in the presence of LIF. Therefore, the long-term self-renewal of miPS cells on these scaffolds should be attributed to the appropriate amount of proteins adsorbed onto these scaffolds, which is also verified by the other researches that appropriate amount of protein in extracellular matrix (ECM) favored the self-renewal of stem cells. [Braam et al., 2008; Prowse et al., 2010] In this study, the appropriate amount of adsorbed proteins provided a weak adhesion between cells and scaffolds to enhance cell migration to form cell colonies on all the scaffolds with the undifferentiated phonotype of small cells and big nuclei shown in Fig. 1. In addition, Fig. 1 showed that, the larger 3 dimensional (3D) cell colonies were formed on PDMAAm hydrogels than on PNaAMPS hydrogels, but only the 2D cell colonies were formed on Gelatin-PS scaffolds. This difference is attributed to the different adhesive interaction between miPS cells and these scaffolds, which is regulated by the different amount of proteins adsorbed onto these scaffolds. We have reported that the both the neutral PDMAAm

and the negatively charged PNaAMPS hydrogels have a low protein adsorption, while the protein adsorption on the Gelatin-PS scaffold was over 2 folds than that on the two hydrogels. [Liu et al., 2011] The low protein adsorption on the two hydrogels results in weak adhesion between miPS cells and hydrogels, which favors fast cell migration and collision to form 3D cell colonies. On the contrary, the high protein adsorption on Gelatin-PS scaffolds results in strong adhesion of miPS cells to the scaffolds to form 2D monolayer cell colonies.

Although the long-term self-renewal of miPS cells can be realized on all the three scaffolds, there are obvious differences. Here, we further discuss which scaffold is the most efficient for self-renewal of miPS cells, which is practically important for the application in regenerative medicine. As the results shown in Fig. 2 to Fig. 5 that the highest proliferation was obtained on PNaAMPS hydrogels than the other scaffolds (Fig. 2), and the passaged miPS cells on PNaAMPS hydrogels showed higher efficiency of undifferentiated cells than the other scaffolds (Fig. 3 to Fig. 5). Furthermore, the *in vivo* differentiation results of passaged miPS cells on these scaffolds (Fig. 7) showed that the teratomas were formed more effectively from miPS cells derived from hydrogels than that from the Gelatin-PS scaffold. It indicates that the hydrogels used in this study favor the self-renewal of miPS cells. The results also showed that the miPS cells derived from the PNaAMPS hydrogels had the highest pluripotency among the three scaffolds. However, the teratomas were also formed from the miPS cells derived form Gelatin-PS scaffold. It indicates that the self-renewal of miPS cells were even maintained on Gelatin-PS scaffold, although the

miPS cells strongly adhere on Gelatin-PS scaffold. However, the self-renewal level is not as high as that on the two hydrogels. Another disadvantage on this scaffold is that it is necessary to harvest the undifferentiated cells by enzyme treatment due to the strong adhesion between cells and Gelatin-PS scaffold. In contrast with the Gelatin-PS, it is easy to harvest the undifferentiated cells from the PDMAAm and PNaAMPS hydrogels without enzyme treatment due to the weaker interaction between cells and hydrogels when 3D cell colonies formed on these hydrogels. As mentioned in *1-1*, the largest cell colonies were formed on PDMAAm. However, due to the weak adhesion of the cells to the PDMAAm, they were easily detached from the scaffold to form cell suspension, which makes the cell loss during the culture operation, such as during culture medium change and cell harvest. Furthermore, the results shown in Fig. 4 indicated that the heterogeneous miPS cells containing both undifferentiated cells and differentiated cells were found on PDMAAm hydrogel. The suspension-like culture of miPS cells for cell colonies of large size on PDMAAm hydrogels is similar to the state of EB formation, which should be the reason for the heterogeneous state of miPS cells on the PDMAAm hydrogels. The initial culture of miPS cells on PDMAAm hydrogels favors to maintain the undifferentiated state of miPS cells, but the miPS cells differentiate with increase in culture period due to the suspension-like culture. This is also found by our other study about the dynamic behavior of mouse EBs formed by ES cells on hydrogel scaffolds that the EBs on neutral hydrogels showed high undifferendiated level at initial culture and showed high differentiated level at long period culture. [Liu et al., 2011] As a conclusion,

PNaAMPS hydrogel is the most suitable scaffold among the three substrates investigated in this work in terms of high proliferation rate, easy handling, high expression of undifferentiated markers, and high speed of teratoma formation.

2. Effect of elastic modulus of hydrogels on self-renewal of miPS cells

The long-term self-renewal of miPS can be realized on both PDMAAm and PNaAMPS hydrogels used in this study, which have elasticity of 162 kPa and 32 kPa, respectively. It suggests that the self-renewal behavior of miPS is independent of the elasticity of scaffolds. To confirm this argument, we further investigated the behavior of miPS on PNaAMPS hydrogels of 32, 135, and 656 kPa elastic modulus, and observed the similar self-renewal behavior of miPS on these hydrogels, regardless the change in the elasticity (Data are not shown). These results are in consistent with our recent study [Yang et al.] that demonstrated the self-renewal behavior of miPS cells on hydrogels regardless of the change in elasticity of hydrogels. In our previous studies, we found that the elasticity of hydrogels play different roles on the cell behaviors for the anchorage-dependent cells and anchorage-independent cells. [Yang et al., 2010; Yang et al., 2010; Chen et al., 2011] The behaviors of anchorage-dependent cells, such as endothelial cells, were regulated by elasticity of hydrogels. On the contrary, the anchorage-independent cells, such as chondrocytes, were less sensitive to the change in elasticity of hydrogels. As shown in Fig. 1, the miPS cells could not form confluency on these hydrogels, but form cell colonies on these hydrogels. It indicates that the miPS cells act as anchorage-independent cells on

these hydrogels, which is the reason why the self-renewal of miPS cells is independent of the elasticity of hydrogels.

V. Conclusions

In this study, we have demonstrated a feeder-free method for long-term self-renewal of miPS cells by expanding the cells on synthetic hydrogels without any surface modification by adhesive proteins, and also on Gelatin-PS scaffold. The long-term self-renewal of miPS cells on neutral PDMAAm hydrogels, negatively charged PNaAMPS hydrogels, and Gelatin-PS scaffold was investigated. The undifferentiated state of miPS cells for long-term of 15 passages could be maintained on these synthetic hydrogels and also on the Gelatin-PS scaffold by showing higher undifferentiated gene expression of *Nanog*, *Sox 2*, *Oct-4*, and *Dppa 5a*, and undifferentiated protein expression of SSEA-1 and Oct-4. Meanwhile, the pluripotency of the passaged miPS cells was demonstrated by *in vitro* & *in vivo* differentiation into cells and teratomas of all three germ layers. Furthermore, the PNaAMPS hydrogels is found to be the optimal scaffold among the three scaffolds as demonstrated by its highest proliferation, the highest efficiency of maintaining the undifferentiated state, and the highest pluripotency of passaged miPS cells.

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Captions

Scheme 1 Chemical structures of the polymers used in this study.

Scheme 2 Study route of long-term self-renewal of miPS cells on hydrogels and Gelatin-PS scaffold.

Table 1 Primers used for real-time PCR.

Fig. 1 Phase-contrast micrographs of long-term of miPS cells subcultured on the surfaces of a) PDMAAm hydrogels; b) PNaAMPS hydrogels; and c) Gelatin-PS scaffolds for 4 d from passage 15 (P 15) to passage 29 (P 29). Scale bar: 100 μm

Fig. 2 Cell densities of input cells and output cells on PDMAAm hydrogels, PNaAMPS hydrogels, and Gelatin-PS scaffolds. The error ranges represent the standard deviation over six samples. * denotes $P < 0.05$ with respect to the input cell densities on each hydrogel.

Fig. 3 Real-time PCR analysis of gene expression of undifferentiated markers of input miPS cells and long-term of miPS cells subcultured on a) PDMAAm hydrogels, b) PNaAMPS hydrogels; and c) Gelatin-PS scaffolds for 4 d from P 15 to P 29. The gene expression levels of miPS cells cultured on theses scaffolds were normalized to those of the input cells. The error ranges represent the standard deviation over three samples.

Fig. 4 Immunofluorescence staining images of a) stage-specific embryonic antigen 1 (SSEA-1, green), nuclei (blue), and merged image; b) Octamer-4 (Oct-4, green), nuclei (blue), and merged image of P 29 miPS cells cultured on PDMAAm hydrogels, PNaAMPS hydrogels and Gelatin-PS scaffolds for 4 d. The cells on PDMAAm

hydrogels were subcultured to the surface of Gelatin-PS scaffold for further 2 d for cell adhesion. Scale bar: 100 μm. The arrows indicate the negative expression areas.

Fig. 5 a) Quantitative Alkaline Phosphatase (ALP) analysis of the P 29 miPS cells cultured on PDMAAm hydrogels, PNaAMPS hydrogels, and Gelatin-PS scaffolds for 4 d. The error ranges represent the standard deviation over three samples. The ALP expression of miPS cells cultured on PNaAMPS hydrogels and Gelatin-PS was normalized to those on PDMAAm hydrogels. b) Flow cytometry analysis of P 29 miPS cells subcultured on PDMAAm hydrogels, PNaAMPS hydrogels, and Gelatin-PS scaffolds for 4 d. The values present the positive SSEA-1 level.

Fig. 6 Real-time PCR analysis of expression levels of differentiated markers associated with a) ectoderm, b) mesoderm, and c) endoderm in the input miPS cells and the miPS cells differentiated for 12 d from the P 29 cells subcultured on PDMAAm hydrogels, PNaAMPS hydrogels, and Gelatin-PS scaffolds. The gene expression levels of miPS cells cultured on these scaffolds were normalized to those of the input cells. The error ranges represent the standard deviation over three samples.

Fig. 7 a) photos of teratoma after *in vivo* transplantation, scale bar: 1 cm b) average weight of teratomas after *in vivo* transplantation, c) miPS cells induced *in vivo* teratoma formation including 3 germ layers: epidermal tissues, muscles, epithelial tissues. scale bar: 100 μm.