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# Biomaterials for *In Vitro* Expansion of Embryonic Stem Cells

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## 1. Introduction

*In vitro* expansion of stem cells is very important for achievement of regenerative medicine. For stem cell expansion methods, several demands are needed to be fulfilled, such as, efficient support of cell proliferation, uniform maintenance of pluripotency, appropriate purity of the stem cells which means less contamination of other cells or potent immunogen, efficient viability after the procedures and so on. However, it is very difficult to efficiently and safely culture some stem cells, such as embryonic stem cells or iPS cells. For example, human embryonic stem (hES) cells cultured on mouse feeder cells expressed an immunogenic nonhuman sialic acid [Martin, 2005]. Human iPS cell developed by Yamanaka's group is cultured on Matrigel™ which is a solubilized basement membrane extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins [Takahashi, 2007]. To eliminate non-human cells and materials, some substitutional methods are devised. Human derived cells [Miyamoto, 2004], human derived materials [Ueno, 2006; Nagase, 2009; Furue, 2008], recombinant proteins [Nagaoka, 2010; Rodin, 2010] and culture medium which enable feeder free culture are investigated by many researchers. Here our strategies for development of biomaterials for expansion of ES cells are discussed.

## 2. Culture of ES cells on chemically fixed feeder cells

ES cells were first derived from the inner cell mass of mouse blastocysts in the early 1980s [Evans, 1981]. ES cells have the unique ability to give rise to any type of somatic cell lineage. Even after the establishment of iPS cells, ES cell features are the standards of pluripotent stem cells, such as, molecular marker expressions and potency to respond to differentiation signals. Therefore, ES cells are still important for analyzing the nature of pluripotent stem cells such as, molecular mechanisms of self-renewal and *in vitro* model of embryogenesis. In addition, the implementation of cell-based or regenerative therapies requires pluripotent stem cells as a renewable source of cells. Efficient *in vitro* expansion of stem cells is therefore important for the development of ES cell technologies and other pluripotent stem cell technologies.

When ES cells are cultured, the environment in which they are propagated has the potential to influence their capacity to act as therapeutic agents in tissue engineering. Animal derived materials may change the potent immunogenic features. Artificial materials may alter the

pluripotency or differentiation state. It is therefore important to understand the response of ES cells to synthetic or biological matrices to ascertain their usefulness as implant materials. Previous studies demonstrate that mouse embryonic fibroblasts (MEFs) support the continued propagation of ES cells in the primitive undifferentiated state while retaining their pluripotency. In the absence of MEFs, gelatin can similarly support the growth and propagation of mouse ES cells in the presence of cytokine leukemia inhibitory factor (LIF), as described by Hamazaki et al. [Hamazaki, 2004]

In the case of human ES cells, bFGF is reported to be useful for growth of human ES cells with keeping the undifferentiated state [Xu, 2005]. However, these replacements cannot be generalized for all lines of ES cells. For culture of primate ES cells including human ES cells, some types of human feeder cells were reported [Cheng, 2003; Richard, 2002]. Among them, human amniotic endothelial (HAE) cell, which was reported by Miyamoto et al. [Miyamoto, 2004], are useful by taking into consideration the easiness of acquisition. However, there are two disadvantages of usage of these feeder cells. One is troublesome for preparation before each ES cell culture. In addition, the feeder cells cannot be completely removed for the next culture after trypsinization.

As we reported, chemically fixed feeder cells supported the growth of hematopoietic stem cells [Ito, 2006]. Therefore, the feeder cells were chemically fixed for supporting the growth of ES cells with keeping the undifferentiated state (Figure 1) [Ito, 2007]. Glutaraldehyde (GA)- or paraformaldehyde (PFA)-fixed MEF cells and HAE cells were prepared for culture of mouse and primate (monkey) ES cells, respectively. HAE was immortalized by infection with hTert cDNA.

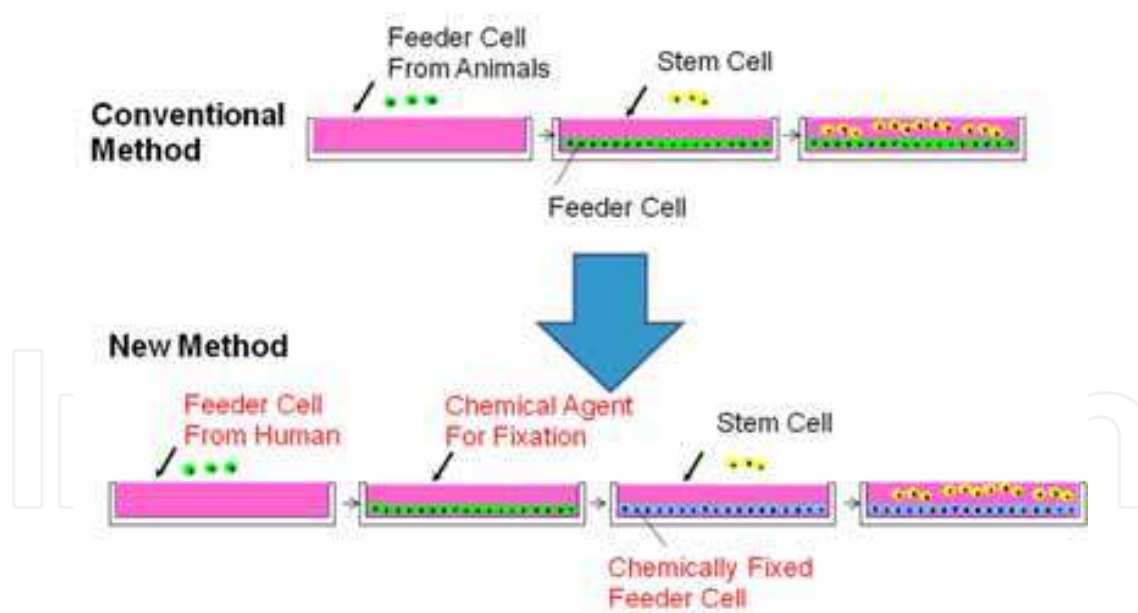


Fig. 1. Chemically fixed feeder cells were prepared as a new technology for culture of stem cells

Figure 2 shows the micrographs of mouse ES cells cultured on several materials. Although the colony size of mouse ES cells cultured on chemically fixed MEF cells was a little smaller than that on MEF which was treated with mitomycin C, they have clear outlines. This result indicated that the mouse ES cells grew with keeping the undifferentiated state. Freeze-dried chemically fixed MEF cells also supported colony formation of mouse ES cells.

On the other hand, on gelatin-coated surface, colony formation was not sufficient and ES cells spread out from the colonies. This indicates that the differentiation began on the gelatin-coated surface. On freeze-dried non-fixed MEF cells ES cells formed colonies but spread out a little.

To confirm the undifferentiation state, the activity of alkaline phosphatase of mouse ES cells was measured by staining (Figure 3). The cells on chemically-fixed cells were stained stronger, even with and without freeze-drying, than that cultured on MEF treated with mitomycin C. On the other hand, the cells cultured only on the gelatin-coated dish were not stained so much.

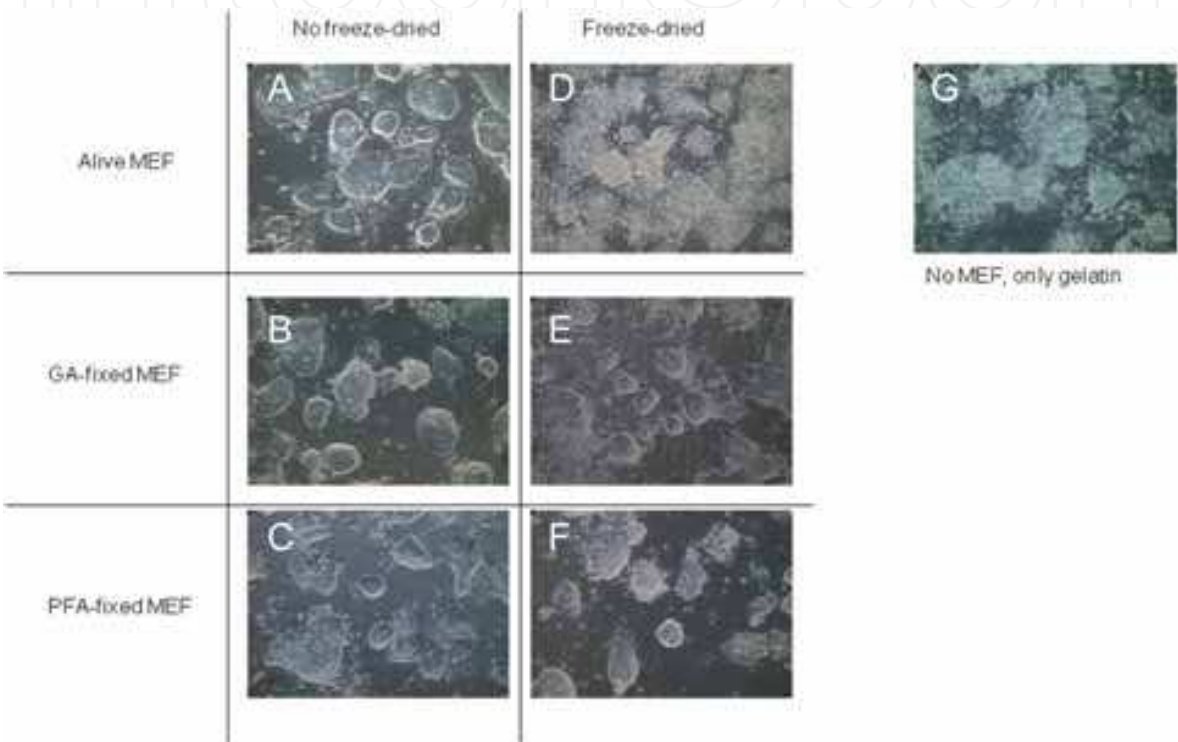


Fig. 2. Phase contrast micrographs of mouse ES cells cultured on different materials for 4 days. A: mitomycin-C-treated MEF, B: GA-fixed MEF, C: PFA-fixed MEF, D: freeze-dried MEF, E: freeze-dried GA-fixed MEF, F: freeze-dried PFA-fixed MEF, G: Gelatin. Reproduced with permission [Ito, 2007]

Immunostaining of SSEA-1 and SSEA-4, which are known to be the markers of undifferentiation and differentiation of mouse ES cells, respectively, were performed and the undifferentiation of mouse ES cells on chemically fixed cells was checked. Expression of transcription factor Oct-3/4 of mouse ES cells was investigated by RT-PCR. On all materials, mouse ES cells expressed SSEA-1 and Oct-3/4, but not SSEA-4. Considering that LIF was added into all culture media, the expression was considered to be natural.

Growth rates of mouse ES cells on different materials were investigated and no significant difference among mouse ES cells on the surfaces was observed. These results indicate that the chemically-fixed MEF cells supported the growth of mouse ES cells with keeping undifferentiated state as well as native MEF cells even with freeze-drying.

Monkey ES cells were also investigated if they can retain the undifferentiation state on fixed feeder cells. Although the monkey ES cells spread out on gelatin-coated dish, the cells on MEF treated with mitomycin C or on chemically fixed HAE cells grew with forming

colonies (Figure 4). On the MEF cells, the shape of colonies was round, but on the fixed and immortalized HAE, the shape of colonies were spindle. It seemed that the monkey ES cells interacted with HAE more than MEF. In this investigation, because the immortalized HAE did not survive in the presence of mitomycin C, the cells were not prepared as a control. Therefore, MEF treated with mitomycin C was employed as the control in the present study.

When the cells were stained by using the activity of alkaline phosphatase, all of the colonies were stained except for the cells on gelatin-coated dish as shown in Figure 5. Staining of SSEA-1 and SSEA-4 was performed and the result indicates the cells kept the undifferentiated state on these materials. This was also supported from the data on Oct-4 expression. Freeze-drying did not reduce the supporting activity. These results confirm the difference between mouse ES cells and primate ES cells in an aspect of self undifferentiation state retaining mechanism.

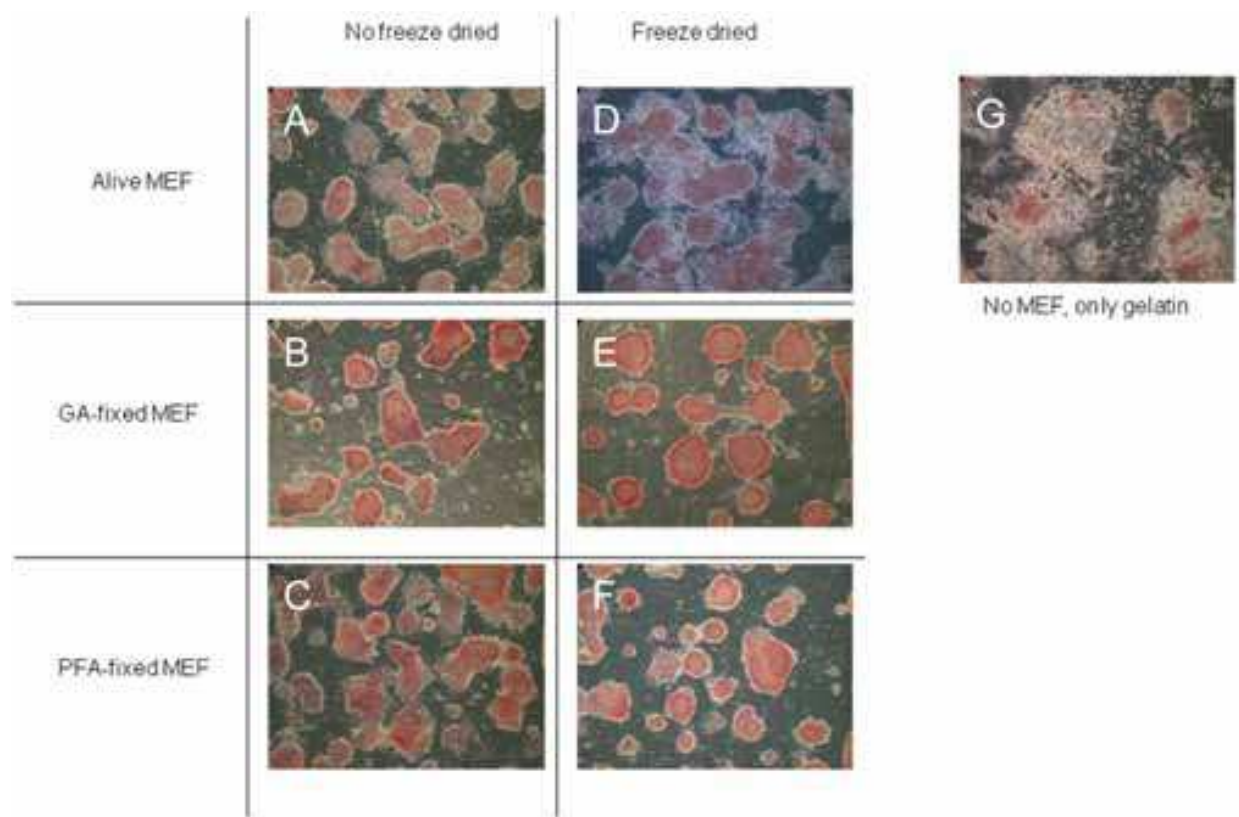


Fig. 3. Staining for Alkaline phosphatase of mouse ES cells cultured on different materials for 6 days. A: mitomycin-C-treated MEF, B: GA-fixed MEF, C: PFA-fixed MEF, D: freeze-dried MEF, E: freeze-dried GA-fixed MEF, F: freeze-dried PFA-fixed. Reproduced with permission [Ito, 2007]

Although fixed HAE cells have incompleteness supporting ability of monkey ES cells, they support the growth and colony formation. Neither chemically fixation nor freeze drying significantly affected the supporting activity, although the growth rates were different from each other. Considering fixation reagent, growth supporting ability of PFA-fixed HAE was a little higher than that of GA-fixed one even before and after freeze-drying. On gelatin-coated surface monkey ES cells grew with differentiation.

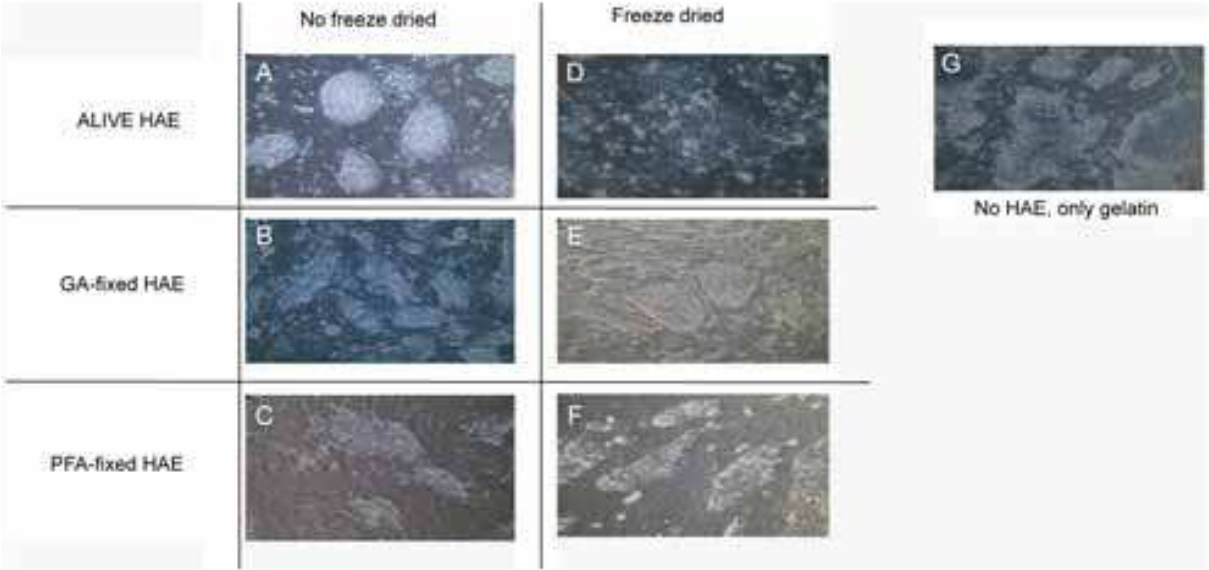


Fig. 4. Phase contrast micrographs of monkey ES cells cultured on mitomycin-C-treated MEF (A), GA-treated HAE (B), PFA-treated HAE (C), freeze-dried HAE (D), freeze-dried GA-fixed HAE (E), freeze-dried PFA-fixed HAE (F), and gelatin (G) for 4 days. Reproduced with permission [Ito, 2007]

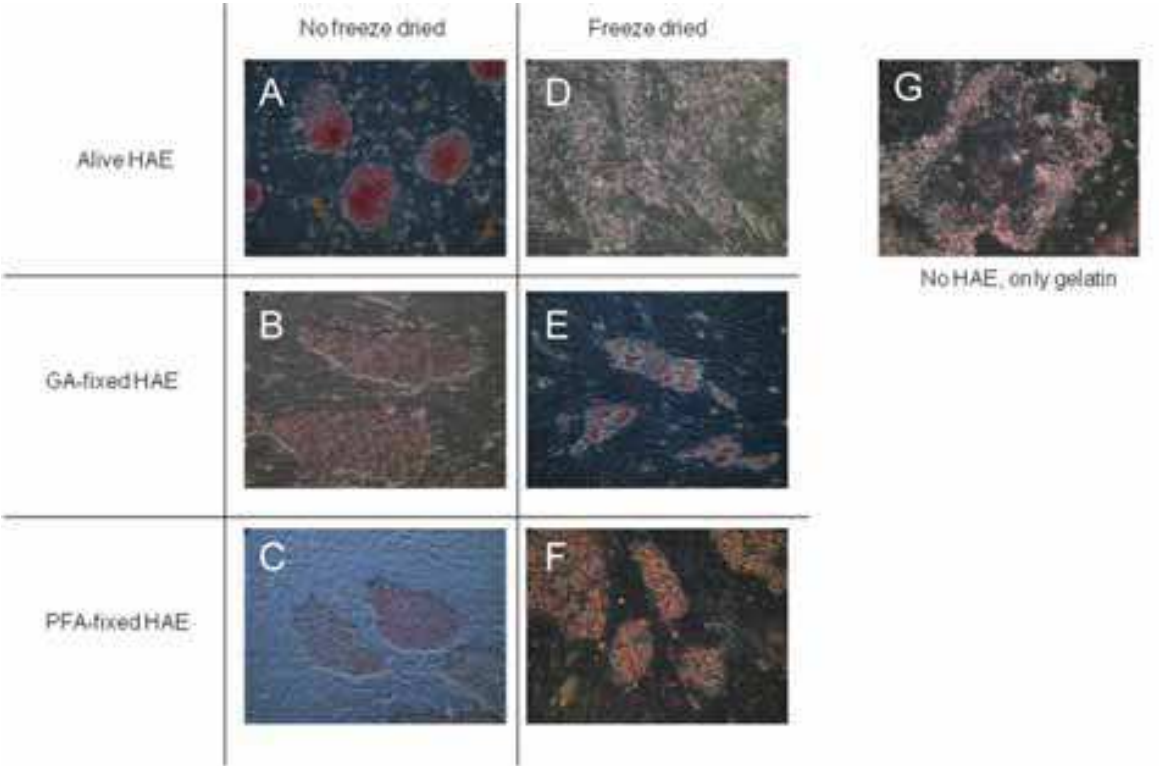


Fig. 5. Staining for Alkaline phosphatase of monkey ES cells cultured on mitomycin-C-treated MEF (A), GA-treated HAE (B), PFA-treated HAE (C), and freeze-dried HAE (D) for 3 days, and freeze-dried GA-fixed HAE (E), freeze-dried PFA-fixed HAE (F), and gelatin (G) for 8 days. Reproduced with permission [Ito, 2007]

### 3. Synthetic biomaterials for ES cells maintenance and EB formation

Synthetic culture substrates for ES cells were also reported. Harrison et al. [Harrison, 2004] reported that fluoride-containing hydroxyapatite supported mouse ES cell growth and the biodegradable substrate affected the pluripotency. Horak et al. [Horak, 2004] cultured mouse ES cells on poly(2-hydroxyethyl methacrylate)-based slabs. Langer's group [Levenberg, 2003; Anderson, 2004] cultured human ES cells in the presence of various materials. A Germany group [Neuss, 2008] also investigated the combination of biomaterials with stem cells. Considering the importance of immobilized biosignal molecules [Ito, 2008], Makino et al. [Makino, 2004] and Nagaoka et al. [Nagaoka, 2008] reported that immobilized LIF and cadherin support the growth of undifferentiated mouse ES cells, respectively. On the other hand, Kurosawa et al. [Kurosawa, 2003] and Konno et al. [Konno, 2005] employed polypropylene tubes and phospholipid polymers, respectively, for the formation of embryoid bodies.

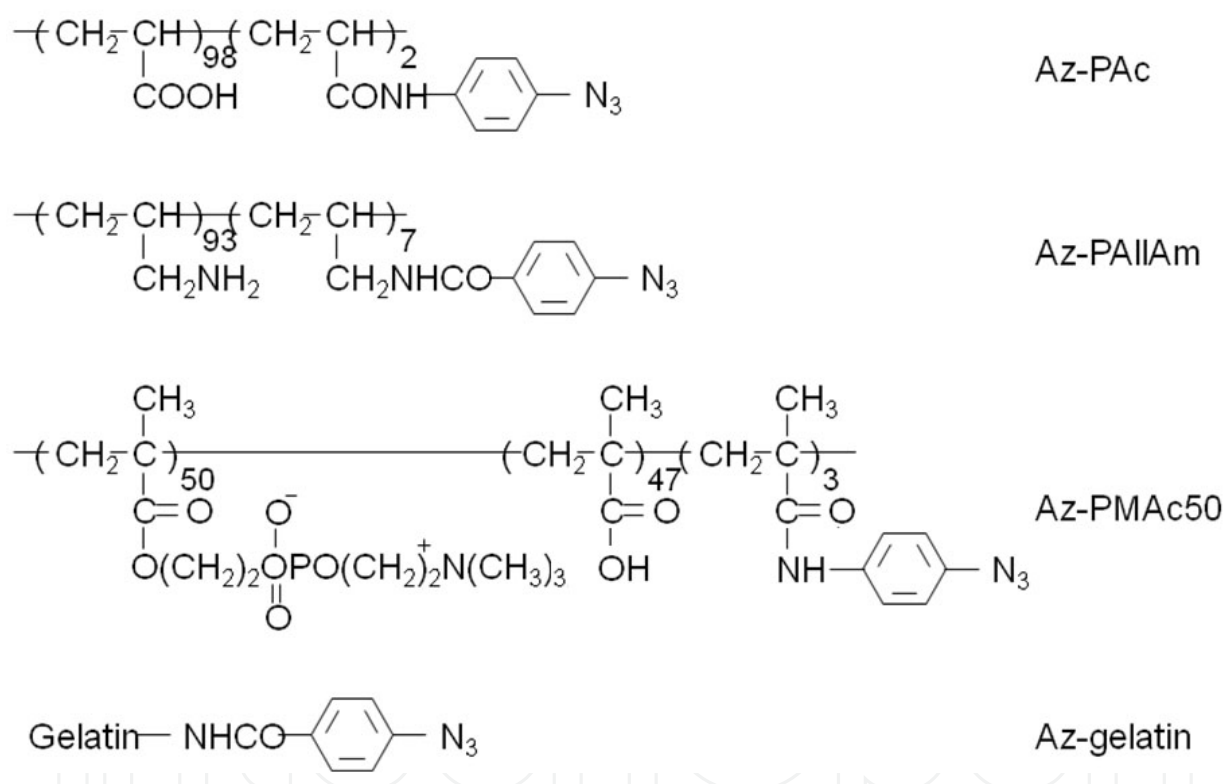


Fig. 6. Chemical structures of biomaterials used for ES cells in this investigation.

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We prepared different types of polymers and covalently immobilized them on conventional polystyrene tissue culture dishes to investigate the effects of polymer surface properties on the culture of mouse ES cells (Figure 6) [Konno, 2006]. Four types of photoreactive polymer were prepared and photoimmobilized: azidophenyl-derivatized poly(acrylic acid) (Az-PAC) as an anionic polymer, azidophenyl-derivatized polyallylamine (Az-PAllAm) as a cationic polymer, azidophenyl-derivatized poly(2-methacryloyloxyethyl phosphorylcholine-co-methacrylic acid) (Az-PMAC50) as a zwitterionic polymer, and azidophenyl-derivatized gelatin (Az-gelatin) as a biological polymer.

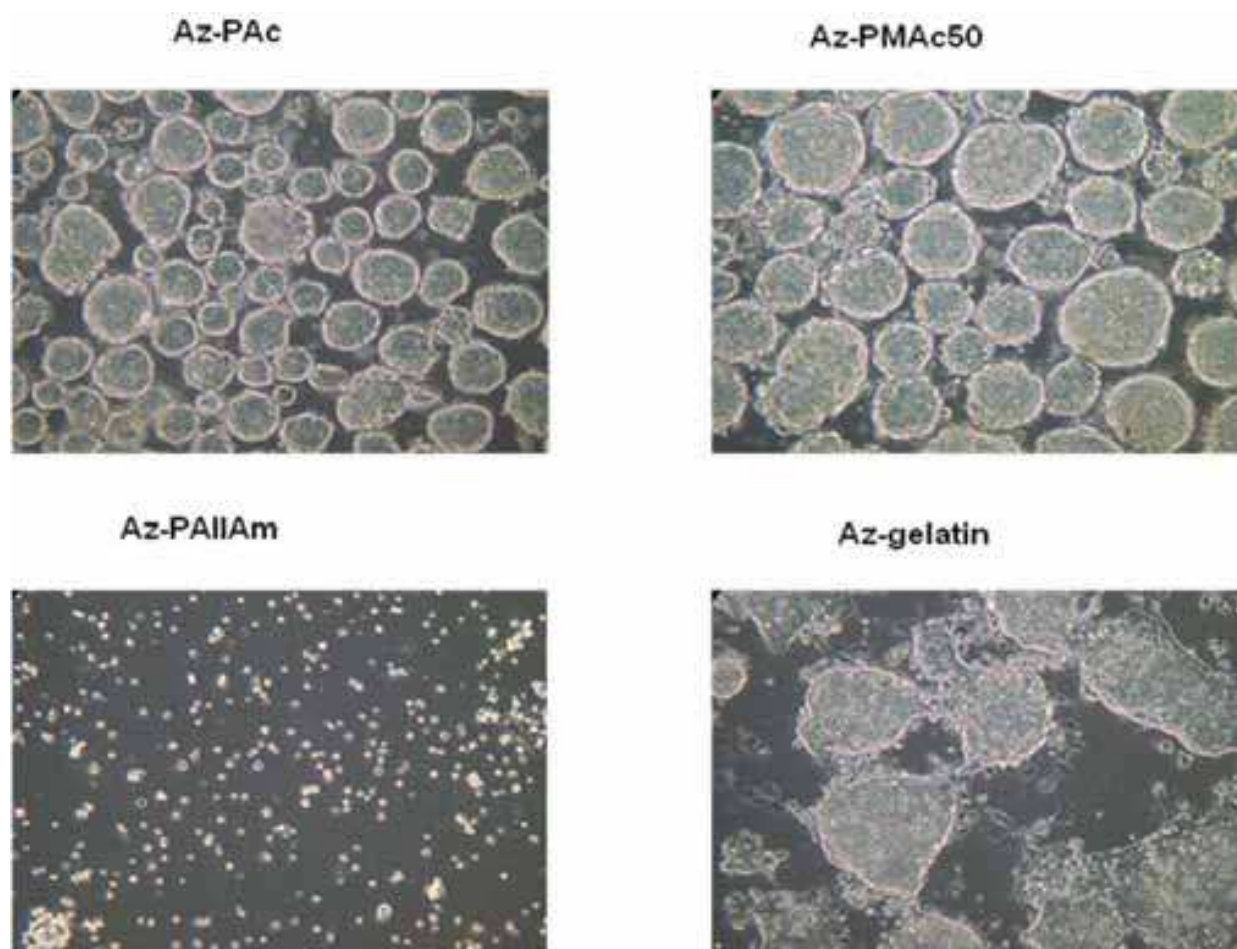


Fig. 7. Mouse ES cells cultured for 3 days on synthetic biomaterials. Reproduced with permission [Konno, 2006]

Phase contrast microscopic images of ES cells after three days of culture on the photoimmobilized polymers are shown in Figure 7. The ES cells immediately and spontaneously aggregated on the Az-PMac50 surface, which comprised concentrated phospholipid polar groups. The Az-PAC surface, which was anionically charged, also induced ES cell aggregation. The ES cells appeared to form embryoid body (EB)-like cell aggregations that did not adhere to the Az-PMac50 surface or the Az-PAC surface. The cell aggregates moved randomly when the Az-PMac50 and the Az-PAC culture plates were shaken. The ES cell aggregates that formed on the Az-PMac50 surface were larger than the cell aggregates formed on the Az-PAC surface. In contrast, the ES cells strongly adhered to the Az-PAllAm surface, which was positively charged, but did not form aggregates. On the Az-gelatin surface, the ES cells adhered and formed colonies. The cells did not move when the Az-PAllAm and Az-gelatin culture plates were shaken.

Figure 8 shows the results of staining for ALP, a marker of the undifferentiated state of the cells, after four days of culture. On the Az-PAC and Az-PMac50 surfaces, the cell aggregates that formed readily stained for ALP, although on the Az-PAllAm it was difficult to detect the staining of the colonies of cells, because the cells did not form colonies. On the Az-gelatin surface, ES cell colonies were not densely stained for ALP, which indicates partial differentiation. It is considered that this result related to the cell spreading from colonies or the deformation of colonies, as shown in Figure 7.

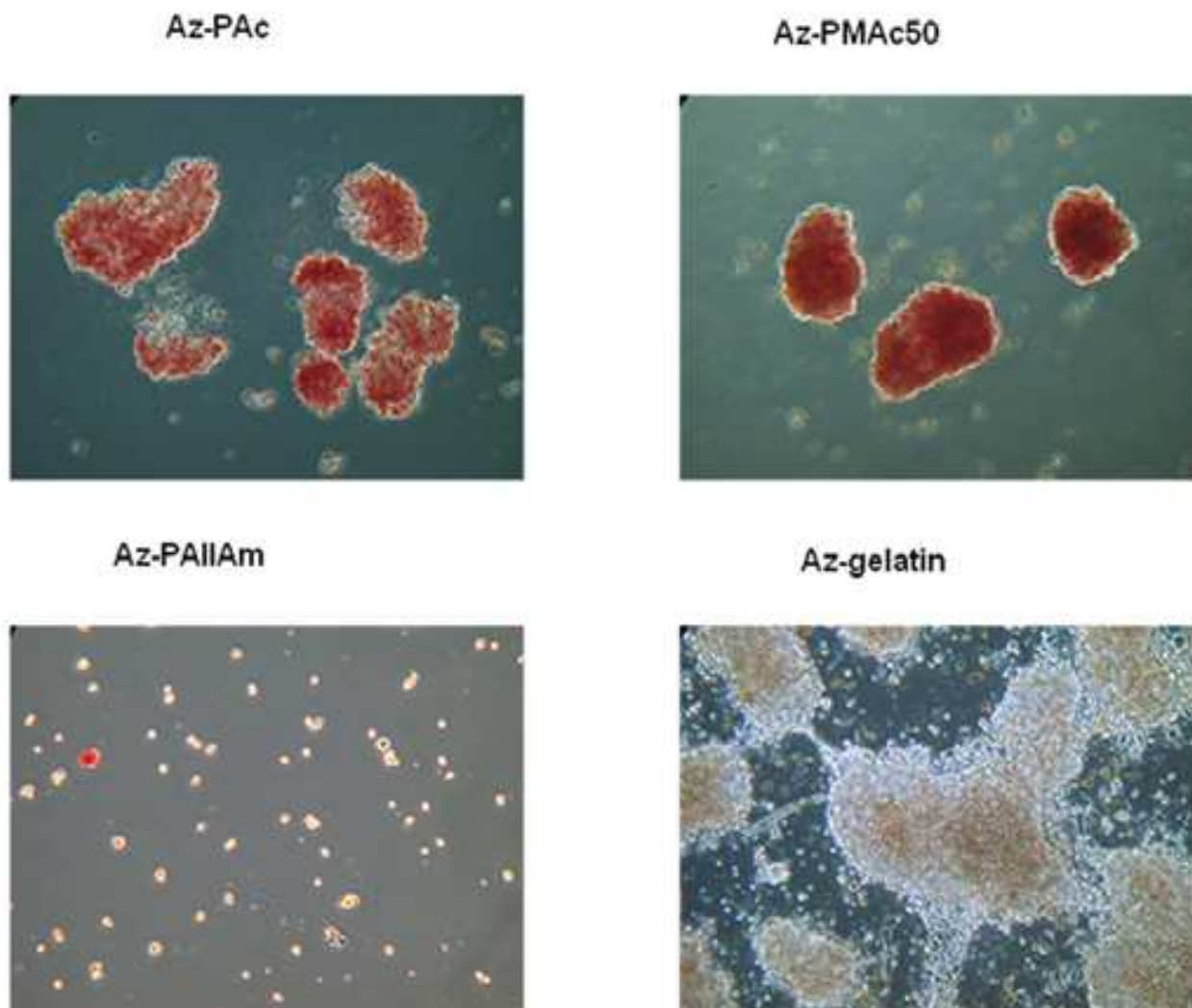


Fig. 8. ALP staining of mouse ES cells cultured on polymers for 4 days. Reproduced with permission [Konno, 2006]

Figure 9 shows the increase in cell numbers on the photoimmobilized polymer surfaces after five days. The highest number of cells occurred on the Az-gelatin surface. The growth of cells on the Az-PMAC50 and Az-PAC surfaces, to which the ES cells did not adhere, was lower than the growth of cells on the Az-gelatin surface. The lowest growth rate was observed on the Az-PAllAm surface, although the cells adhered to this surface. These results indicate that the biological interaction of gelatin is important for cell growth, rather than the simple adhesive interaction.

The LIF/stat3 signaling cascade is involved in maintenance mechanism of mouse ES cell undifferentiation state [Matsuda, 1999; Sekkai, 2005]. The cytoplasmic protein stat3 is activated by the binding of LIF to the LIF and gp130 receptors, which both reside on the mouse ES cell membrane. Activated stat3 (phosphorylated stat3) induces the expression of the transcription factor Oct3/4. In this study, phosphorylated stat3 (p-stat3) and total stat3 (t-stat3) in cells on the immobilized photoreactive polymer surfaces were detected using western blotting. The relative intensity of p-stat3 to t-stat3 (p-stat3/t-stat3), which indicates the activation of signal transduction, was investigated. As a result, phosphorylation of stat3 was observed on all polymer surfaces, and no significant difference between the surfaces

was found. Although ALP staining was not observed in the cells on the Az-PAllAm surface by microscopy, at the molecular level of detection, the undifferentiated state of the cells was revealed.

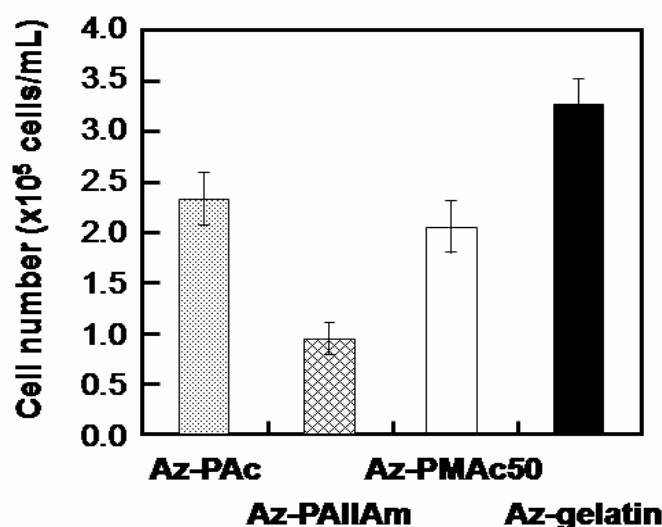


Fig. 9. Growth of mouse ES cells on polymers after 5 days culture ( $n = 5$ ). Reproduced with permission [Konno, 2006]

Expression of the transcription factor Oct3/4 is necessary for ES cells to maintain an undifferentiated state [Niwa, 2005]. Phosphorylation of stat3 induces the expression of Oct3/4. We confirmed the expression of Oct3/4 by RT-PCR. When the relative expression of Oct3/4 to G3PDH (HKG) was calculated using image analysis, the same level of expression was observed in cells on all polymer surfaces (Figure 10).

The expression of GATA4, which is a molecular marker for the early endodermal state or differentiation to the EB [Kelly, 1993; Grepin, 1997], was also measured. Using the data in Figure 10, the relative intensity of GATA4 to G3PDH was calculated. The values were 0.4, 0.2, 0.18, and 0.02 on Az-PMAC, Az-PAC, Az-gelatin, and Az-PAllAm, respectively. High expression of GATA4 occurred in cells on the Az-PMAC50 surface. This indicates that the Az-PMAC50 surface effectively induced EB formation and differentiation of ES cells. Strong expression of GATA4 was not observed in the cells on the Az-PAC surface, although the cells aggregated on this surface. Similar expression was observed in the cells on the Az-gelatin, but, in this case, the colonies were spread out. In contrast, cells grown on the Az-PAllAm surface did not express GATA4, which we considered was due to the lack of cell aggregation.

Many researchers have investigated the behavior of ES cells cultured on various biomaterials. Properties such as hydrophilicity / hydrophobicity, electrostatic charge, and topographical roughness affect cell adhesion, growth, and differentiation. In this study, the effect of electrostatic charge on mouse ES cells was investigated in the presence of LIF. Considering that it is known that LIF activates stat 3 [Matsuda, 1999; Sekkai, 2005], and expression of Oct3/4 [Niwa, 2005], and ALP [Singla, 2006], it is reasonable that the undifferentiated state of cells was maintained on all surfaces in the presence of LIF.

However, the morphology, growth, and differentiation of ES cells to EBs depended on polymer surface properties. Neither Az-PAc nor Az-PMc50 surfaces induced adhesion of ES cells, which led to EB formation. When an EB did form, the growth of cells was reduced. Negative charges or zwitterions reduced the interaction of ES cells with the polymer surface and the cells aggregated to form an EB. EB size and expression of GATA4 (an indicator of EB formation) in cells grown on the Az-PMAc50 surface were greater compared with cells grown on the Az-PAc surface. Recently, we reported that EB formation was efficiently induced on a phosphorylcholine-derived polymer-coated surface [Konno, 2005]. Therefore, the reduction of cell-surface interactions with this polymer would enhance the cell-cell interactions. The lower interaction leads formation of EBs, and thus that explain the larger size of the EBs and stronger expression of GATA4 on the Az-PMAc50 surface.

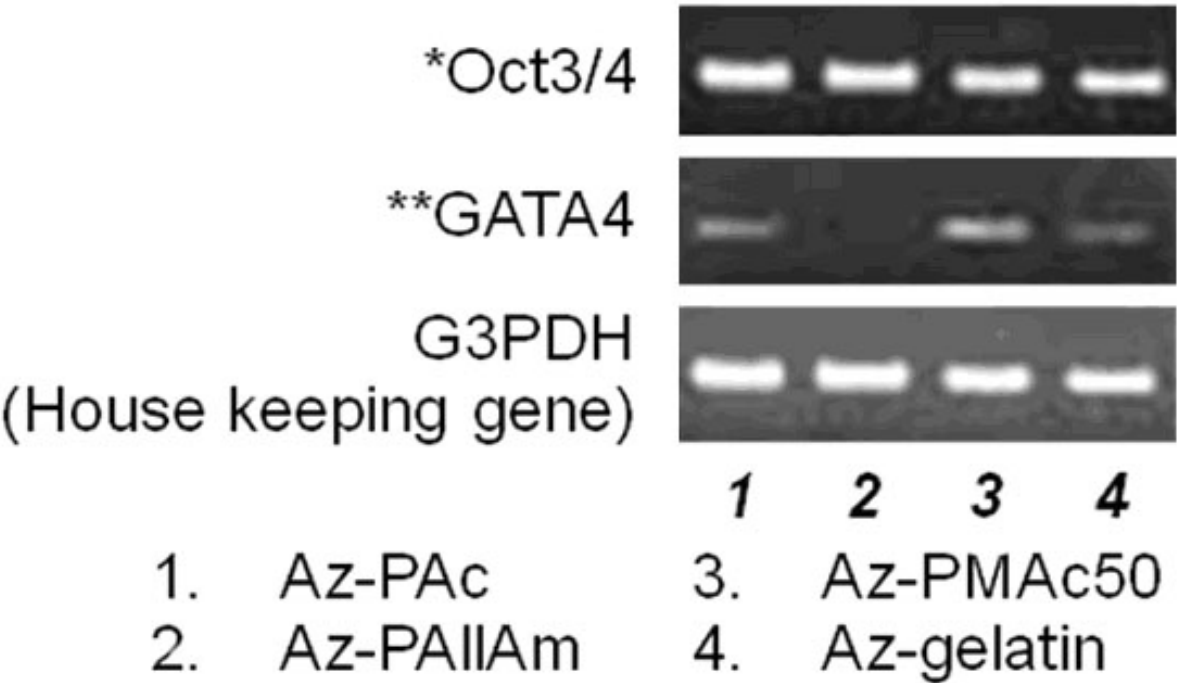


Fig. 10. RT-PCR detection of G3PDH, Oct3/4, and GATA4 from mouse ES cells cultured on polymers for 6 days. Reproduced with permission [Konno, 2006]

ES cells adhere to cationic or gelatin surfaces. However, mouse ES cells formed colonies on the gelatin surface, but not on the Az-PAllAm surface. Some biological interactions are required to induce ES cells to aggregate and adhere to a surface. The growth of ES cells was significantly enhanced on the Az-gelatin surface, and GATA4 expression was slightly induced. These phenomena are the same as those for normal gelatin-coated surfaces.

4. Thermoresponsive polymer for ES cells detachment

Among the procedures of in vitro expansion of ES cell, enzymatic detachment of the cells from the culture plate is one of the most deleterious procedures to the cells. It is known that enzymatic separation of the ES cell colonies into single cells causes massive cell death for human ES cells, and chemical inhibitor of the massive cell death was investigated [Watanabe, 2007; Ohgushi, 2010].

An approach known as cell sheet engineering has been proposed for culturing and passaging cells without the use of trypsin [Okano, 1993]. Using a coating of a thermally responsive polymer, the surface properties of the substrate can be changed by changing the temperature of the environment. Poly(N-isopropylacrylamide) (PNIPAAm) is a popular polymer of this type. It exhibits a lower critical solution temperature (LCST) of 32–33 °C, being hydrophilic at low temperatures and precipitating above the critical phase transition temperature [Xia, 2005]. This unique physical property has been exploited in the fabrication of thermally responsive surfaces for cell sheet engineering [Okano, 1993; Yamato, 2001; Liu, 2002]. At cell culture temperatures above the LCST, the surface is hydrophobic, and cells or tissues attach to the substrate. When the temperature is lowered below the LCST, the surface becomes hydrophilic, and they detach. This mild technique of cell detachment preserves cell-cell and cell-extracellular matrix (ECM) interactions, unlike trypsinization [Kushida, 1999; Shimizu, 2003].

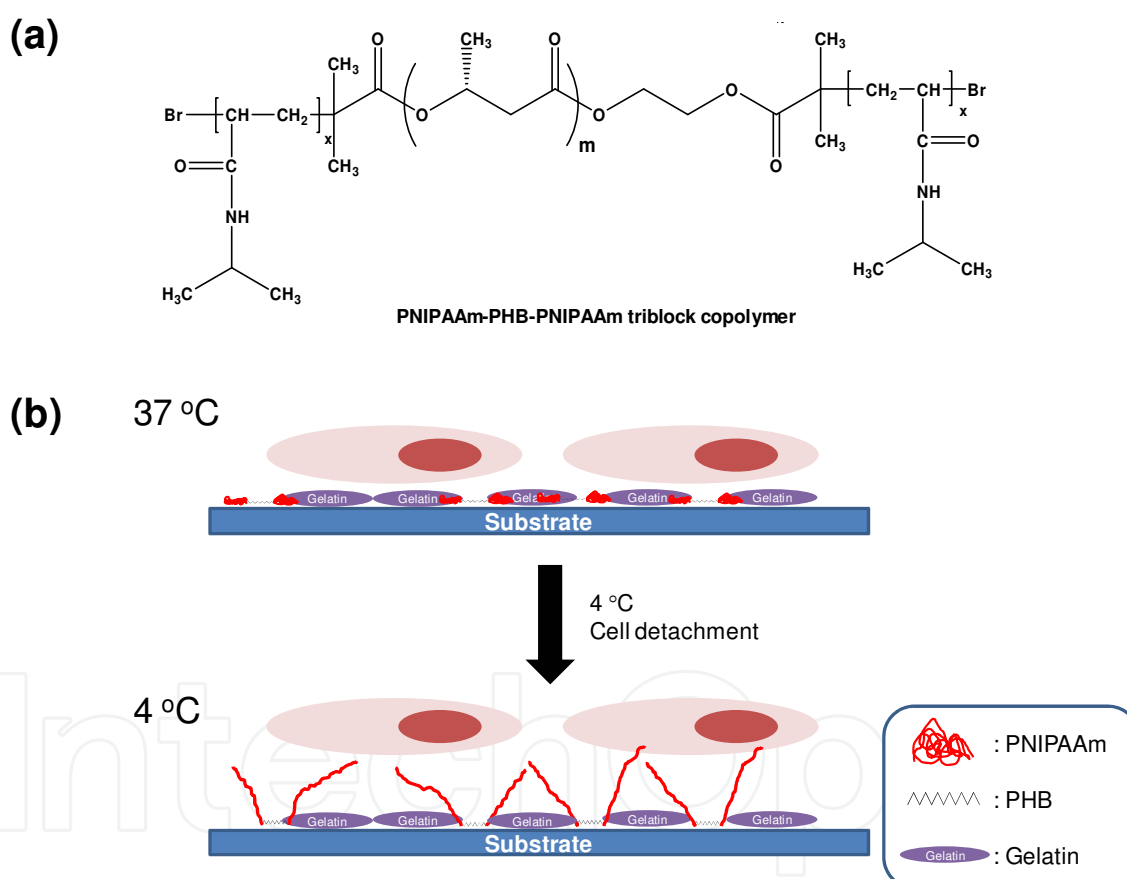


Fig. 11. Chemically fixed feeder cells were prepared as a new technology for culture of stem cells. Reproduced with permission [Loh, 2009]

Several papers have reported that the formation of ES cell aggregates is essential prior to the differentiation of these cells [Lee, 2000; Dang, 2004; Konno, 2005]. The formation of ES cell aggregates takes a minimum of 3 days, thus cells which are freshly trypsinized cannot be used for immediate differentiation. So, there are some advantages to detach the cells without trypsinization, and not to separate the colony into single cells prior to the differentiation.

We synthesized thermoresponsive polymer to recovering ES cells without enzymatic detachment or separation into single cells. We used a triblock copolymer of poly(N-isopropylacrylamide)-poly[(R)-3-hydroxybutyrate]-poly(N-isopropylacrylamide) (PNIPAAm-PHB-PNIPAAm) [Loh, 2009] and prepared a mixture of the thermoresponsive polymer [Loh, 2009] and gelatin with simple drop-casting technique (Figure 11). As the thermoresponsive polymer PHB belongs to a class of naturally derived biologically synthesized polyesters known as poly[(R)-3-hydroxyalkanoate]s [Loh, 2007]. PHB has also been extracted from genetically modified plants [Petrasovits, 2007; Purnell, 2007]. Based on its advantageous properties, PHB might be suitable for a variety of biomedical applications such as tissue engineering scaffolds, and these have been reported to be suitable for enabling cell adhesion [Chen, 2003; Wang, 2005].

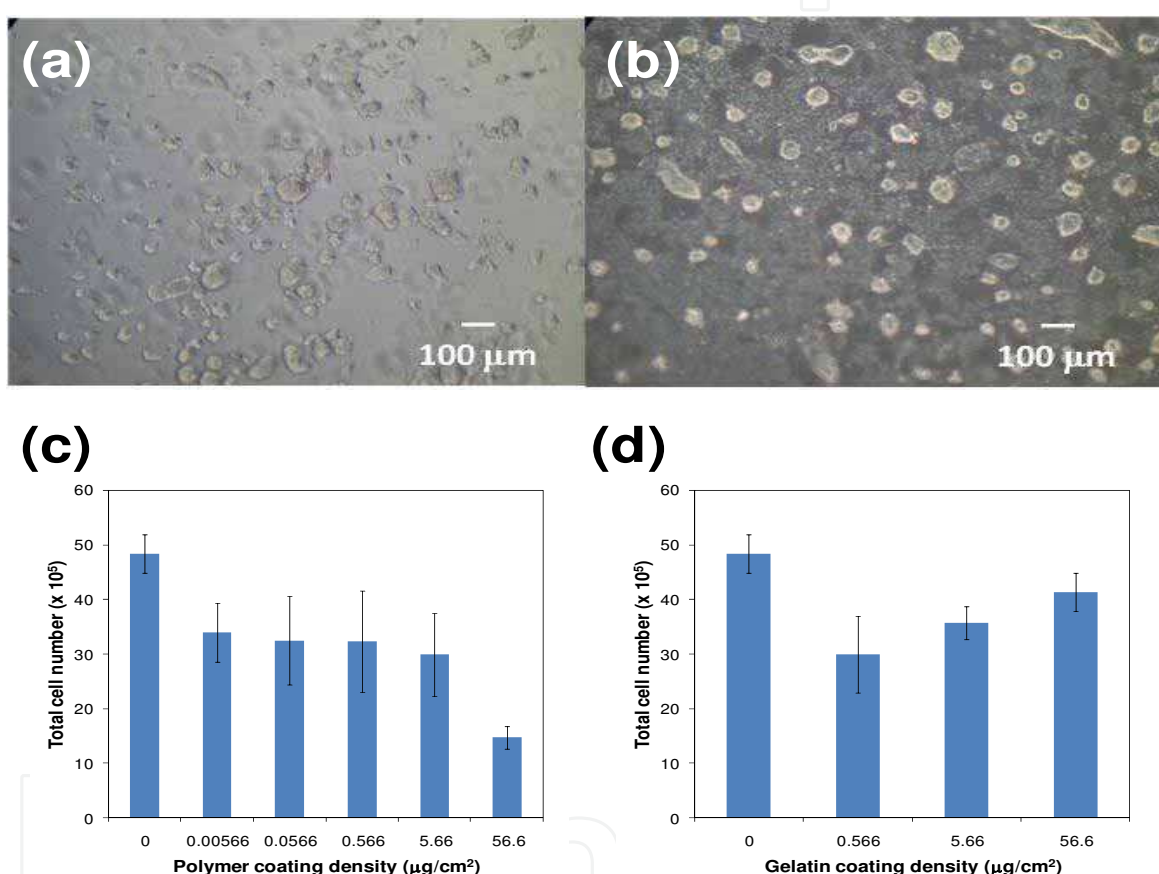


Fig. 12. Morphology of mouse ES cells cultured on gelatin/PNIPAAm-PHB-PNIPAAm surfaces of different thicknesses: (a) 5.66 µg/cm² and (b) 56.6 µg/cm². (c) Cell growth on different copolymer coating densities of gelatin/PNIPAAm-PHB-PNIPAAm after 3 days. (Gelatin coating density = 0.566 µg/cm²). (d) Cell growth on different gelatin coating densities of gelatin/PNIPAAm-PHB-PNIPAAm after 3 days (Copolymer coating density = 5.66 µg/cm²). Reproduced with permission [Loh, 2009]

As coating method, we used a simple drop-casting technique for the preparation of a homogeneous thermoresponsive surface, instead of using spin coating.

Figure 12 shows the micrographs of mouse ES cells cultured on thermoresponsive polymer at different densities. On the surfaces at 56.6 µg/cm², colonies had clear outlines. While, the colonies on the surface at 5.66 µg/cm² had less (compare Figure 12a and Figure 12b), these

result suggested that the mouse ES cells grew with keeping the undifferentiated state on the surfaces at  $56.6 \mu\text{g}/\text{cm}^2$ .

Cell numbers were counted for the layers with different surface densities and are shown in Figure 12c. The cell numbers were generally similar up to  $5.66 \mu\text{g}/\text{cm}^2$ . Cells grew on the layers at  $56.6 \mu\text{g}/\text{cm}^2$ , but the cell proliferation rate was much lower than on layers of lower coating density (Figure 12c). The amount of gelatin incorporated into the coating also affected cell proliferation (Figure 12d).

Considering that our aim was to achieve a high growth rate of ES cells as well as a maximal thermal response, we decided to use substrates with gelatin and polymer coating densities of  $0.566$  and  $5.66 \mu\text{g}/\text{cm}^2$ , respectively. Mouse ES cells were cultured on five different coated surfaces. The cell growth was monitored over 4 days. The growth rate was found to be in the following order: gelatin > gelatin/PNIPAAm-PHB-PNIPAAm > gelatin/PNIPAAm homopolymer > PNIPAAm-PHB-PNIPAAm > PNIPAAm homopolymer. It is known that gelatin is essential for culture of ES cells. Gelatin immobilized on acrylic acid grafted poly(L-lactide-co- $\epsilon$ -caprolactone) aided in the growth and adhesion of human mesenchymal stem cells, compared with the nonimmobilized polymer [Shin, 2008]. Our results showed that although cells could be cultured on the plain polymer surfaces, incorporation of the gelatin coating significantly improved both cell growth and adhesion. On the other hand, it appeared that incorporation of the polymers stunted the growth of the cells compared with the substrate coated with gelatin.

The highest growth rate of ES cell was obtained with the gelatin/PNIPAAm-PHB-PNIPAAm coating. This was more effective than the PNIPAAm coating, probably because of the presence of the PHB segment. As PHB-based scaffolds are suitable for cell adhesion [Chen, 2003; Wang; 2005; Wu, 2008], the PHB segment might contribute to the growth enhancement of anchorage-dependent cells although the effect was less than that of gelatin. Cell detachment was tested by incubating the culture dish at  $4^\circ\text{C}$  for 20 min. As a control, cells growing on the gelatin-coated dish were also incubated at  $4^\circ\text{C}$  for the same period. Figure 13a shows the thermoresponsive cell detachment from the cooled gelatin/PNIPAAm-PHB-PNIPAAm surface. The cell colonies eventually detached after 20 min. On the other hand, as shown in Figure 13b, cells grown on the control gelatin surface did not detach.

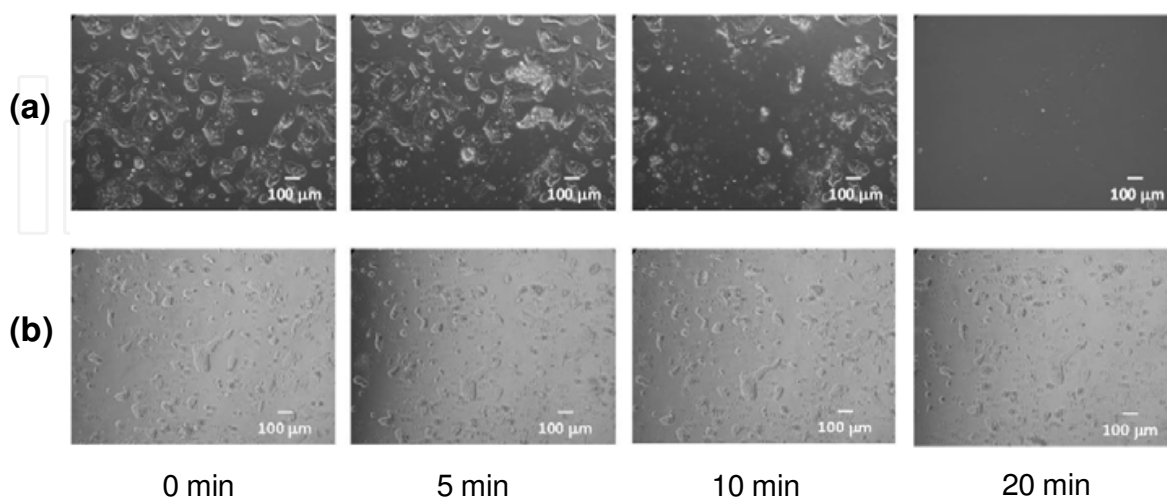


Fig. 13. Mouse ES cell detachment demonstrated on (a) a gelatin/PNIPAAm-PHB-PNIPAAm surface compared with (b) a gelatin-coated substrate. Reproduced with permission [Loh, 2009]

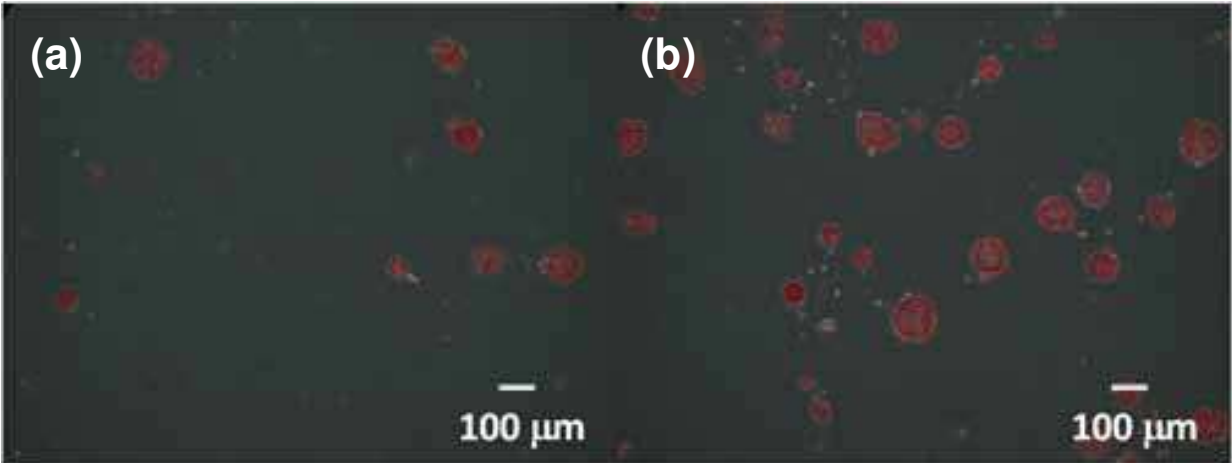


Fig. 14. Phase contrast microscope images mouse ES cells stained for alkaline phosphatase after being cultured for 3 days on the different surfaces: (a) Gelatin/PNIPAAm and (b) Gelatin/PNIPAAm-PHB-PNIPAAm. Reproduced with permission [Loh, 2009]

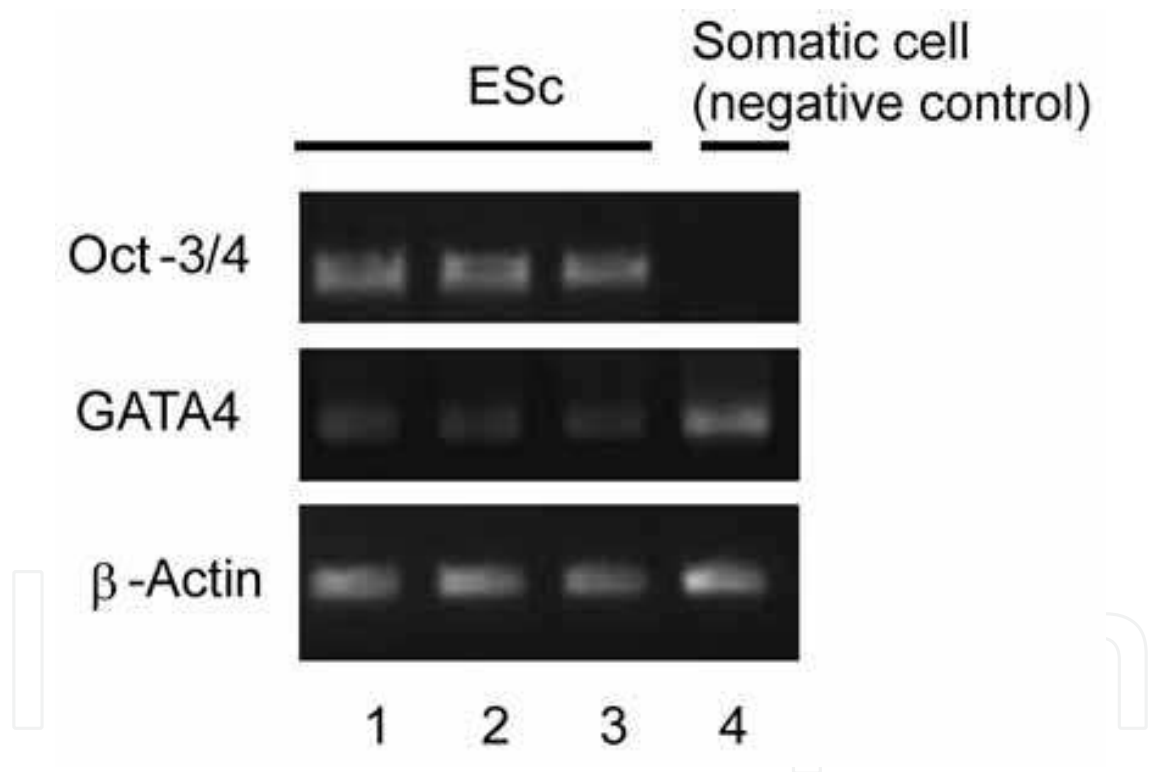


Fig. 15. Expression levels of  $\beta$ -actin, Oct3/4 and GATA4 in mouse ES cells cultured on the different substrates, measured by reverse transcription polymerase chain reaction. Lane 1, gelatin control; lane 2, gelatin/PNIPAAm-PHB-PNIPAAm; lane 3, gelatin/PNIPAAm and lane 4, STO mouse ES cells (negative control). Reproduced with permission [Loh, 2009]

In Figure 11b, we show a schematic diagram of the likely cell detachment process. The cells adhere on the substrate coated with copolymer micelles, which have a hydrophobic core and a collapsed PNIPAAm corona at 37 °C. When the substrate is cooled, the PNIPAAm segment relaxes and becomes more hydrophilic. This hydrophilic surface is not suitable for the attachment of the cells, and so they detach.

To confirm the undifferentiation state, first, the activity of alkaline phosphatase of mouse ES cells was measured by staining (Figure 14). Cells cultured on the different surfaces were tested for ALP activity after three days of culture. Staining for ALP was positive, indicating the undifferentiated state of the ES cells when cultured on the coated substrates.

Second, RT-PCR was performed to check the expression level of Oct3/4 and thus to confirm the undifferentiated state of the ES cells. The Oct3/4 bands of the cells cultured on the gelatin, gelatin/PNIPAAm-PHB-PNIPAAm and the gelatin/PNIPAAm surfaces were of almost equal intensity, as shown in Figure 15. The negative control used in this experiment was mRNA extracted from STO, mouse embryonic fibroblast cells. Next, we tested for the expression of GATA4. Cells express GATA4 during the early endodermal state or while differentiating to embryoid bodies [Kelly, 1993; Grepin, 1997]. We detected a weak band of GATA4 from the ES cells cultured on the gelatin, gelatin/PNIPAAm-PHB-PNIPAAm and gelatin/PNIPAAm surfaces. Overall, the ES cells showed a lower expression of GATA4 than the STO cells. Incorporation of the polymers as a surface coating reduced the intensity of the band compared with the pure gelatin surface. Taken in total, the ALP, western blotting and the RT-PCR results show that the mouse ES cells remained in an undifferentiated state after culture on these thermosensitive coatings.

## 5. Conclusion

We devised three kinds of biomaterials for *in vitro* expansion of ES cells. The first is chemically fixed feeder cells. The fixed feeder cells are easy for handling. With fixed feeder cells, ES cells could be cultured without contamination of other living cell, metabolism and alternation of feeder cells during the culture. The second is photoreactive polymers. By using the polymers Embryoid body like structure could be prepared. The third is thermoresponsive polymer. By using the polymer combined with gelatin, ES cell colonies could be detached from culture dish without deleterious enzymatic separation or separation into single cells or loss of extra cellular matirics. Each of the materials retained the ability to support cell proliferation and puluripotency based on molecular markers expressions.

There is no golden standard of feeder cells or coating material of culture dish yet, for regenerative medicine or stem cell research. Each of feeder cells or materials has advantages and disadvantages. Ideally a defined artificial substrate without any biologically derived components is desired for stem cell culture. Therefore, as a fundamental research, some different surfaces were prepared and biological interactions between ES cells were investigated. Not only biological properties but also physico-chemical properties significantly affected the behavior of stem cells. In future, by consideration on these results, new biomaterials should be designed and developed for stem cell culture.

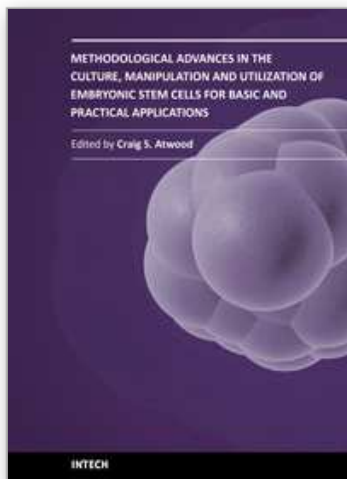
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