Simple autogeneic feeder cell preparation for pluripotent stem cells

Weizhen Li,1, Hiromi Yamashita,1, Fumiyuki Hattori,1, Hao Chen,a, Shugo Tohyama,a, Yusuke Satoh,a,c, Erika Sasaki,d, Shinsuke Yuasa,a, Shinji Makino,a, Motoaki Sano,a, Keiichi Fukuda,a,

⁎ Corresponding authors. F. Hattori is to be contacted at Division of Cardiology, Department of Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81 3 5363 3875. K. Fukuda, fax: +81 3 5363 3875.
E-mail addresses: hattori.fumiyuki.ef@asubio.co.jp (F. Hattori), kfukuda@sc.itc.keio.ac.jp (K. Fukuda).
1 Weizhen Li and Hiromi Yamashita equally contributed to this study.

1873-5061/S – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.scr.2010.09.003
rodents (Hearn, 2001; Nakatsuji and Suemori, 2002). Sasaki and co-workers recently established common marmoset ES (cmES) and iP cells, and green fluorescent protein (GFP)-transgenic marmosets (Sasaki et al., 2005; Sasaki et al., 2009; Tomioka et al., 2010), and described the efficient differentiation of neural cells from cmES cells (Sasaki et al., 2005). Chen et al. also reported successful differentiation of cardiomyocytes from cmES cells and described their characterization (Chen et al., 2008).

Recently, iP cells have been established in rodents (Takahashi and Yamanaka, 2006), nonhuman primates (Tomioka et al., 2010; Wu et al., 2010; Liu et al., 2008), and humans (Takahashi et al., 2007). Clinical applications for these cells are also eagerly awaited, since iP cells with a genetic background identical to that of the patient can be generated with less ethical concerns. Even though several improvements have been made to combat initial problems, clinical application of human iP (hiPS) cells is still controversial due to a number of safety concerns.

Common technical constraints for the therapeutic application of hES and hiPS cells also remain. One such limitation is avoiding the use of xenogeneic materials, since there is a risk of cross-transfer of potential pathogens and unexpected genes. To date, various xenogeneic factor-free culture methods have been developed to replace the MEFs used for culturing hES cells, such as immobilized MEFs (Choo et al., 2006), Matrigel (Xu et al., 2003; Lee et al., 2005) and immortalized feeder cells (Unger et al., 2009), suspension culture systems (Steiner et al., 2010; Singh et al., 2010; Olmer et al., 2010; Amit et al., 2010), and autogeneic feeder (AF) cells (Amit and Itskovitz-Eldor, 2006; Choo et al., 2008; Stojkovic et al., 2005; Wang et al., 2005), as well as several xenogeneic factor-free media (Akopian et al., 2010) which can be combined with xeno-free feeders and feeder-free methods. Nevertheless as shown in the report from the International Stem Cell Initiative, most xenogeneic factor-free culture systems based on feeder-free conditions are biased toward hES cell lines (Akopian et al., 2010), suggesting that the MEF feeder system remains the standard because it ensures stable and reliable maintenance for every pluripotent stem cell line. Also in our hands, the MEF feeder system is still the most reliable and general method for maintaining cmES and hES cells, and hiPS cells. Therefore, it is necessary to develop further options for alternative human feeder cells.

AF systems for hES cells have been reported by two groups; the first group derived AF cells via embryoid body (EB) formation (Stojkovic et al., 2005), while the second group generated a stable cell line from differentiated hES cells (Choo et al., 2008). In the present report, we describe a novel method for the preparation of AF cells derived from spontaneously differentiated cells for use in the routine maintenance of pluripotent stem cells. In addition, we report a common method for the preparation of AF cells for different nonhuman primate and human pluripotent stem cells.

Results

Under our routine experimental conditions, cmES, hES, and hiPS cells stably self-renew on MEFs. However a small fraction of each colony contains spontaneously differentiat-ed cells that have sprouted from the edges of the colonies. During routine passaging, we found that the weak trypsin and collagenase treatment detached preferentially the undifferentiated cells of the colonies, leaving the differentiated cells attached to the plate (Fig. 1a). Since the detached cells had features typical of fibroblasts, we expected that they could be used as AF cells. Previously, these cells would have been discarded, so we term our AF preparation the “cell recycling system.” The principle underlying this phenomenon is shown in Fig. 1b.

We cultured the residual cells for 2–4 weeks until they reached subconfluence. The cultivation period to the first passage varied depending on the initial concentration of differentiated cells. The period of time between the passages was approximately 3–5 days. Between the first and third passages, we estimated that the cells had a doubling time of about 20 h. To investigate the relationship between passage number and ability to maintain the undifferentiated state of the cmES cells, we seeded cmES cell clumps onto mitomycin C-treated cmAF cells of various passage numbers, and observed the morphology of the cells under the microscope (Fig. 1c).

We found that the cmAF cells that underwent up to three passages maintained the cmES cells without any obvious morphologic alteration of the cmES cells (Fig. 1c). In contrast, when we used cmAF cells after the fourth passage, we found a significant decrease in their maintenance capability. Therefore, we used cmAF cells at the third passage for routine culturing of cmES cells. cmAF cells that were freshly treated with mitomycin C could maintain cmES cells for 1 week; thereafter, they showed decreased viability and maintenance ability. To investigate whether residual MEFs were diluted during cmAF expansion, we performed immunohistochemical analysis for human nuclear antigen (Chen et al., 2008) on cultures of cmAF cells. We found no human nuclear antigen-negative cells (residual MEFs) by fluorescent microscopy (Fig. 1d), which meant that the MEFs had been eliminated during the three passages. The mitomycin C-treated and untreated cmAF cells were successfully stored in the long-term using slow-freezing methods. The viability of the recovered cmAF cells was typically 80 to 90%.

We prepared consecutive batches of cmAF cells from cmES cells that had been cultured on cmAF cells, and maintained the cmES cells in this system for more than 6 months. The cmES cells cultured on cmAF cells for 6 months expressed Oct-3/4, Nanog, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 2a). We also confirmed that the cmES cells possessed alkaline phosphatase activity, which is a typical feature of pluripotent stem cells (Fig. 2a). Cytogenetic analysis of the cmES cells that were long-term cultured on cmAF cells revealed that they retained the normal karyotype of 46XX (Fig. 2b).

To investigate the differentiation ability of the cmES cells cultured on cmAF cells, we induced cardiogenic and neurogenic differentiation. The cmES cells differentiated into cardiomyocytes via embryoid body formation (Fig. 3a). We partially dispersed the EBs and attached them to fibronectin-coated dishes. Immunohistochemical analysis revealed that the EBs expressed Nkx2.5 and sarcomeric α-actinin, indicating that they were cardiomyocytes (Fig. 3b, left panel). We induced neurogenic differentiation by serum withdrawal and retinoic acid stimulation. Thus, we observed protruding filamentous cells from the attached core of the EBs (Fig. 3b, right panel). We demonstrated immunofluorescence staining for βIII tubulin, 

W. Li et al.
which is a marker for mature neurons, and confirmed that some of the cells expressed βIII tubulin. Furthermore, to demonstrate the similarity of cmAF cells and MEFs, we analyzed the protein expression profiles of cmES cells cultured on cmAF cells and MEFs, taking advantage of antibody (protein) arrays that are applicable to a broad range of species from rodents to humans. We used the Panorama antibody microarray XP725 kit, which consists of 725 antibodies that have been validated by the manufacturer for studies of mouse and human samples. These antibodies represent families of proteins known to be involved in a variety of important biological pathways, including cell signaling, matrix processing, cell growth, and apoptosis. We analyzed Cy3 labeling of the total protein extracts from cmES cells cultured on cmAF cells or MEFs. Each Cy3-labeled protein was bound to an individual antibody-arrayed glass slide. The fluorescent signals were evaluated using a scanner (Fig. 3c, left). Few proteins had greater than twofold expression changes between cmES cells cultured on cmAF cells and MEFs, indicating that cmES cells cultured on cmAF cells and MEFs have similar protein expression profiles (Fig. 3c, right). We also compared cmES cells cultured on cmAF cells with purified common marmoset ES cell-derived cardiomyocytes as a control experiment. Several differences in protein expression were observed between the cmES cells and purified cardiomyocytes; these included proteins reported to be expressed in cardiomyocytes, such as histone deacetylase 2 (Lu and Yang, 2009), estrogen receptor, and in pluripotent stem cells, such as mitogen and stress activated kinase (Arthur and Cohen, 2000), C-src tyrosine kinase, and Coffilin (Fig. 3d).

Next, we applied our feeder preparation method to hES and hiPS cells (Fig. 4). The hAF and hiAF cells prepared from hES and hiPS cells were found to maintain hES and hiPS cells for more than 2 months, respectively (Fig. 4a). To investigate whether the MEFs were diluted during the expansion of hAF cells, we performed immunofluorescent staining for human nuclear antigens with analysis by FACS. Almost all the prepared hiAF cells were positive for human nuclear antigen (Fig. 4b). To further investigate whether the MEFs were diluted during the expansion of hAF cells, we used a stably GFP-expressing hES cell line for hAF cell preparation. We randomly observed five visual fields under the microscope. As a result, no GFP-negative cells (MEFs) were found in the prepared hAF cells (Supplementary Fig. 2). Taken together, these findings show that residual MEFs are eliminated in hiAF cells. Next, we investigated the pluripotency of hiPS cells maintained on hiAF cells by immunofluorescent stainings including microscopic observation (Supplementary Fig. 3) and FACS analysis of SSEA-4, TRA-1-81, Nanog, and Oct-3/4, and confirmed that almost all the hiPS cells expressed the four pluripotent markers (Fig. 4c). We also performed DNA expression array analysis of the hES and hiPS cells that were maintained long-term on hAF and hiAF cells, respectively. Equivalent mRNA expression levels of the pluripotency-related genes including oct-3/4, nanog, sox-2, lin28, and c-myc were observed. Global gene expression profiles were also quite similar in both cases (Fig. 4d, left). In contrast, two comparative expression profiles between hES and hiPS cells and their differentiating EBs indicated the marked existence of differentially expressed genes (Fig. 4d, right). In addition, a similar magnitude of difference to the results shown in the left panel of Fig. 4d was seen when comparing global gene expression profiles for hES and hiPS cells during different passage numbers (Supplementary Fig. 4). All these results show
that our hAF and hiAF cells can maintain hES cells in an undifferentiated state and in a condition quite similar to that achieved with MEFs.

Discussion

We have demonstrated the preparation of AF cells from three different cell sources using a common method. These AF cells succeeded in effectively maintaining their pluripotent stem cells.

In this study, we used antibody array analyses to characterize cmES cells. Quite similar protein expression profiles were observed between cmES cells cultured on cmAF cells and MEFs. However, in contrast, various differentially expressed proteins were observed in purified cmES cell-derived cardiomyocytes compared to cmES cells. These results validate the usefulness of this system, and indicate a similar efficacy of cmAF cells compared to MEFs for the maintenance of cmES cells. The mRNA expression profiles produced by global gene array analyses comparing hES cells cultured on hAF cells and MEFs and hiPS cells cultured on hiAF cells and MEFs revealed an overall high similarity in profiles; however, they were not perfectly identical. The differential gene expression profiles comparing different passage numbers of the same human pluripotent stem cells maintained with the same feeder cells showed a similar dispersion to those observed between human pluripotent stem cells maintained with MEF and AF cells. These results suggest that some allowable gene expression changes might spontaneously occur during long-term culture in pluripotent stem cells, although the genes related with pluripotency must be maintained.

Using our routine preparation of cmAF cells, approximately $1 \times 10^8$ cells can be obtained from a single 10-cm dish and three cell passages. This number of cmAF cells is sufficient to prepare 100 10-cm dishes for cmES cell culturing. In contrast, $1 \times 10^7$ MEFs are typically obtained from a single mouse embryo under our experimental conditions. Thus we believe that our AF system has a comparable cell yield to that of the MEF system.

As potential therapies using personalized iPSCs become possible, it may be reasonable to maintain an individual's hiPS cell line using their AF cells, because there would be no concerns of transfer of allogenic antigens or infectious viruses from the feeder cells. Even in the case of mass production of therapeutic cells from banked pluripotent stem cells, techniques have not yet been established for maintaining pluripotent stem cells under xenogeneic factor-free conditions at a reasonable cost.

Conclusions

The present study establishes an effective method for preparing AF cells which is applicable to cmES, hES, and hiPS cells. We believe that the results of the present study pave the way for the reliable and economic production of alternative feeder cells for pluripotent stem cells.

Materials and methods

Maintenance of undifferentiated cmES, hES cells, and hiPS cells

The cmES cells (cell line No. 20; Central Institute of Experimental Animals, Kawasaki, Japan), hES cells (khES-2; Institute for Integrated Cell-Material Sciences, Kyoto University), and hiPS cells (G4; Center for iPSC Cell Research and
Application, Institute for Integrated Cell-Material Sciences, Kyoto University) were maintained as described previously (Chen et al., 2008; Hattori et al., 2010). Briefly, cmES cells were maintained on feeder cells in cmES cell medium, which consisted of 80% Knockout Dulbecco’s modified Eagle’s medium (KO-DMEM; Invitrogen, Carlsbad, CA, USA), 20% Knockout Serum Replacement (KSR; Invitrogen), 0.1 mM nonessential amino acids (Sigma Chemical Company), 2 mM L-glutamine (Sigma, St. Louis, MO), 0.1 mM β-mercaptoethanol (Sigma), and 4 ng/mL basic fibroblast growth factor (Wako Pure Chemical Industries, Osaka, Japan). hES and hiPS cells were similarly maintained, except that Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 Ham’s (1/1 ratio, DMEM-F12; Sigma) was used instead of KO-DMEM. The cmES and hES cells were passaged every 5–7 days. Typically, we cultured pluripotent stem cells in 15-cm dishes (Becton–Dickinson, NJ, USA). Using this method, all reagent amounts were suitable for 15-cm dish cultures.

Passage of pluripotent stem cells

All cmES, hES, and hiPS cells were treated with 2 mL of 0.25% trypsin (Becton–Dickinson), 0.1% collagenase type 3 (Worthington Biochemical Corp., NJ, USA), 20% KSR, and 1 mM CaCl₂ in phosphate-buffered saline at 37 °C for 5–15 min, which resulted in disruption of the boundaries between the pluripotent stem cells and the feeder cells. Then, 5 mL of DMEM supplemented with 10% fetal bovine serum (FBS; Biowest, FL, USA) was added and the cells were gently pipetted several times, which detached all the pluripotent stem cell colonies and most of the feeder cells from the dish. The cells were separated into three fractions by size, <40 μm, between 40 and 100 μm, and >100 μm, using cell strainers with mesh pore diameters of 40 and 100 μm (Becton–Dickinson). These procedures are illustrated in Supplementary Fig. 1a. This process eliminated feeder cells (Supplementary Fig. 1b). The collected pluripotent stem cell colonies of the correct size (larger than 40 μm and smaller than 100 μm) were seeded onto a new plate with feeder cells.

Preparation of AF cells

A low number of differentiating cells remained on the culture dish after passage (Fig. 1a). These cells were the seeds of the AF cells, and they were propagated in DMEM (Wako) supplemented with 10% FBS. Typically, propagation to near confluence took 10–15 days for cmAF cells and 20–30 days for human AF (hAF) and iAF (hiAF) cells. For passaging, the cells were detached and dispersed by treatment with 0.25% trypsin-EDTA solution (TE; Invitrogen) at 37 °C for 10 min. A cell
strainer with mesh pore diameter of 40 μm (Becton-Dickinson) was used to eliminate the residual large cell clumps (diameter >40 μm). The purified AF cells were plated onto new 0.1% gelatin-coated dishes that contained DMEM supplemented with 10% FBS. For all the cmES, hES, and hiPS cells, confluence was achieved in 3–5 days, and these cells were subsequently passaged to the next generation using a 5-times dilution. The second or third generation of AF cells were treated with 10 μg/mL of mitomycin C at 37 °C for 3 h, and cryopreserved at -150 °C in Cellbanker solution (Mitsubishi Chemical, Tokyo, Japan). The cmAF, hAF, or hiAF cells recovered from the cryo-stocks were seeded onto 0.1% gelatin-coated dishes at a concentration of 2×10^6 cells per 15-cm dish.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2010.09.003.

Acknowledgments

This study was supported in part by research grants from the Special Coordination Funds for Promoting Science and Technology in the Japanese Ministry of Education, Culture, Sports, Science, and Technology and from the Japan-China Medical Association. This work was also in part supported by KAKENHI and New Energy and Industrial Technology Development Organization (NEDO).

References


