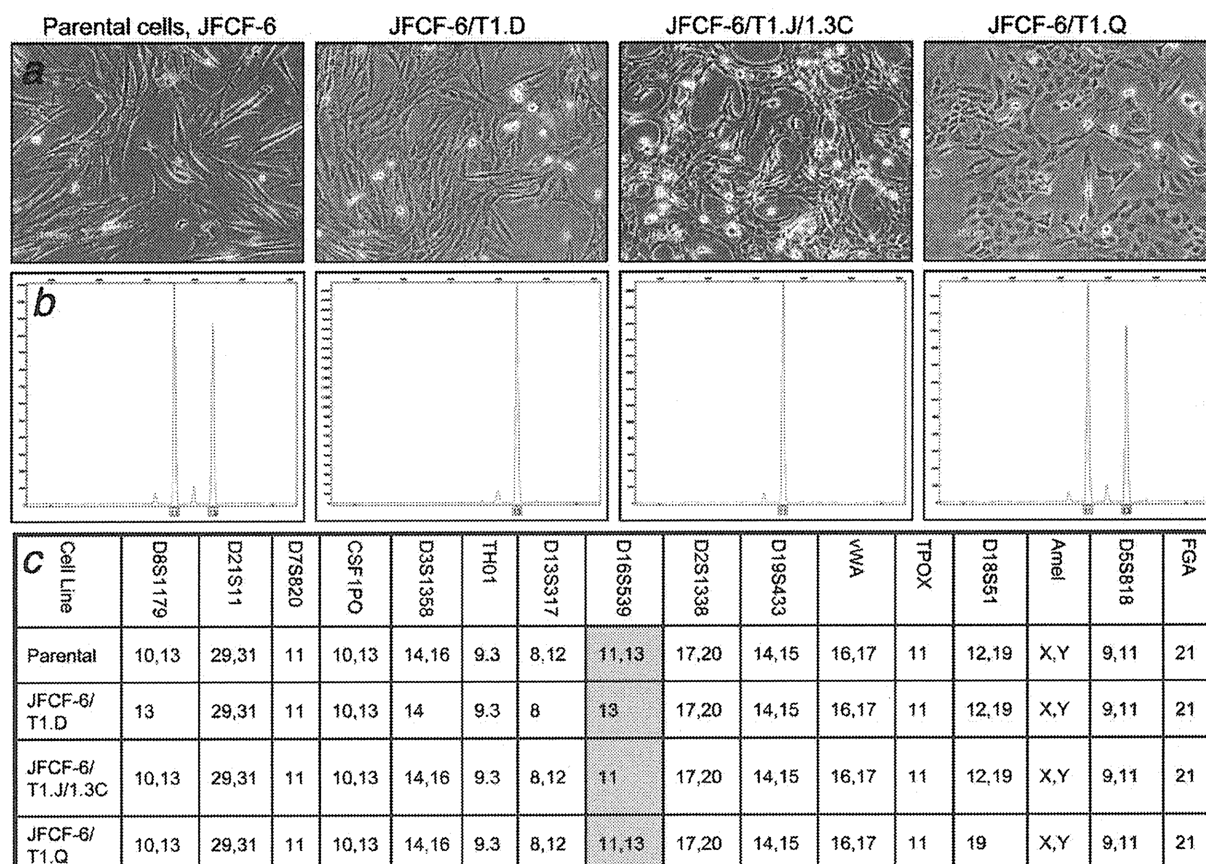

Cell Line	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	Amel	D5S818	FGA
Parental	10,13	29,31	11	10,13	14,16	9.3	8,12	11,13	17,20	14,15	16,17	11	12,19	X,Y	9,11	21
JFCF-6/ T1.D	13	29,31	11	10,13	14	9.3	8	13	17,20	14,15	16,17	11	12,19	X,Y	9,11	21
JFCF-6/ T1.J/1.3C	10,13	29,31	11	10,13	14,16	9.3	8,12	11	17,20	14,15	16,17	11	12,19	X,Y	9,11	21
JFCF-6/ T1.Q	10,13	29,31	11	10,13	14,16	9.3	8,12	11,13	17,20	14,15	16,17	11	19	X,Y	9,11	21

Figure 2. Example of STR profile generation and interpretation. An example of STR profiling is given for the JFCF-6 cell fibroblast strain and 3 of its immortalized derivatives, JFCF-6/T1.D, JFCF-6/T1.J/1.3C and JFCF-6/T1.Q.[43] Derivatives were established after transfection with SV40 early region DNA and were handled by CellBank Australia through its Culture and Return service. DNA from each culture was amplified using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems, Mulgrave, Australia), which includes primers for 16 STR loci. Amplified sequence was analyzed using an ABI PRISM 3100 Genetic Analyzer and data files were assessed using GeneMapper ID software (Applied Biosystems). (*a*) Photographs taken of each culture, comparing parental cells to the morphology of each derived cell line (scale bar = 100 μm). Each derivative has a markedly different morphology, showing the need for authentication testing to confirm that derivatives correspond to the parental strain. (*b*) Examples of STR peak amplification for the D16S539 locus of each culture. Amplification varies at this locus due to genetic drift during establishment of the 3 JFCF-6–derived cell lines. The peaks shown correspond to specific allele sizes known to exist at this locus and confirmed using size standards and controls supplied with the kit (data not shown). (*c*). STR profiles for JFCF-6 and derived cell lines; the locus shown in B, D16S539, is highlighted in grey. Despite the differences seen due to genetic drift, the profiles for derived lines closely match the parental cell strain and all of these cultures are correctly identified.

which can be used for strain identification.[48,49] SNP analysis can also be used to identify individual samples[50] and has been used for cell line authentication,[51] making it a method of great promise for application to human and nonhuman samples alike. Laboratories working on specific cell types may be able to use expressed markers for identification, as 1 laboratory has done recently, publishing a technique for identification of hybridomas based on sequencing of light-chain variable regions.[52]

A simple method has recently emerged to help detect interspecies contamination. The term DNA barcoding here refers to amplifying a specific 648 bp fragment of the mitochondrial gene, cytochrome C oxidase subunit I (COI), using primers developed by Folmer *et al.*[53] Sequence divergences within this fragment allow species discrimination across almost all animal phyla.[42] Although debate is ongoing as to whether DNA barcoding is sufficient for assignment of species in taxonomic terms,[54] it is clear that the technique can readily identify the species of an unknown specimen if compared with previously sequenced reference material in online databases.[55] DNA barcoding has been tested for species identification of cell lines[27] and its use would reduce the incidence of interspecies cell line

contamination, found here to cause almost 1 in 10 of all published cross-contamination events.

Whatever the authentication method used, it should be clearly recorded within the researcher's experimental notes, and the result should be linked if possible to the laboratory's liquid nitrogen records, so that quality control for frozen vials is clearly evident. When publishing experimental work, the Material and Methods section should include the correct and full name of the cell line used, its origin (with appropriate references), the source of the cultures used and details of authentication testing.

Conclusions

Cell line contamination is a serious issue that detracts from the use of cell lines as model systems to help us understand a broad range of diseases, including cancer. Responding practically by checking each cell line before it is used, searching for previous references and authenticating the sample itself is worthwhile and will reduce the risk and subsequent consequences of contamination long-term.

Acknowledgements

The authors gratefully acknowledge the work of many cell banks and laboratories working in this area, and those responsible for compiling the list in Wikipedia, and regret that there is insufficient space to include all references here. A complete list of publications on cross-contamination can be found in the Electronic Supporting Information. Elsa Moy is thanked for her work in handling the cell lines shown in Figure 2. CellBank Australia was established by a joint venture of the Children's Medical Research Institute, Cure Cancer Australia Foundation and National Breast Cancer Foundation, and by an Enabling Grant of the National Health and Medical Research Council of Australia.

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Human Cell 2010; **23**: 113–118 doi: 10.1111/j.1749-0774.2010.00091.x

CELL LINE

Establishment of induced pluripotent stem cells from human neonatal tissues

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Abstract

Following the success in establishing human induced pluripotent stem (iPS) cells, research into various applications of the cells derived from human iPS cells has begun in earnest. The use of iPS cell-derived cells in clinical therapies is one of the most exciting of the possible applications. However, the risk of tumorigenicity is the biggest potential obstacle to use iPS cell derivatives in the clinic. It should be noted that the human cells used to generate iPS cell lines may have acquired genetic mutations and these might influence the tumorigenicity of the cells. In particular, the cells of older people have a higher risk of genetic mutations than those of younger people. Here, we show that iPS cells could be derived from short-term cultures of neonatal tissues. The established human iPS cells expressed various markers of undifferentiated cells and formed teratoma in immunodeficient mice. The human iPS cells derived from neonatal tissues may represent a clinical material possessing less tumorigenicity.

Key words: clinical application, neonatal tissue, induced pluripotent stem cells.

INTRODUCTION

The development of a method to generate human induced pluripotent stem (iPS) cell lines[1–4] has stimulated a considerable number of studies into the potential applications of these cell lines. The use of iPS cell-derived cells in clinical therapies is one of the most exciting of the possible applications. However, the risk of tumorigenicity is the biggest potential obstacle to use iPS cell derivatives in the clinic. Initially, the method for producing iPS cell lines involved the integration of exogenous genes into the host genome. Recent modifications to the methodology have obviated the need for retention of exogenous genes to generate iPS cell lines.[5–7] These methodological changes may reduce the problem of potential tumorigenicity.

However, even when iPS cells are established with methods that avoid the integration of exogenous genes, the risk of tumorigenicity of iPS cell derivatives remains similar to that of embryonic stem (ES) cell derivatives. One potentially problematic aspect of iPS cells compared with ES cells is that they are established from somatic cells that may have already acquired genetic mutations. In addition, the cells of older people have a higher risk of genetic mutations than those from younger people. This potential problem has to be taken into account prior to the use of iPS cell derivatives in the clinic.

Although neonatal tissues such as umbilical cord, fetal membrane, and placenta are readily available,[8] they are usually discarded if they are not required for immediate use. Provided the mother of a neonate agrees to allow the neonatal tissues to be used in basic research

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Received 22 July 2010; accepted 21 August 2010

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and/or clinical applications, these tissues could providea useful resource without the complicating factor of critical ethical concerns. Here, we show that iPS cells can be derived from short-term cultures of neonatal tissues.

MATERIALS AND METHODS

Cell sources of iPS cells

Fibroblast-like cells derived from human umbilical cord (HUC-F2, HUC-Fm and HUC-5 derived from three different neonates) and from human fetal membrane (HFM-1 derived from a neonate) were obtained from the Cell Engineering Division of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and were cultured in minimum essential medium-α (MEM-α; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Sigma, St Louis, MO, USA).

iPS cell generation

pMXs retroviral vectors were obtained from addgene (Cambridge, MA, USA) and used to express Oct3/4, Sox2, Klf4, and c-Myc. To produce the recombinant pseudo-type retrovirus, plasmid DNA was transfected into 293T cells along with the *gag-pol* expression plasmid (pCAGGS *gag-pol*) and the vesicular stomatitis virus G glycoprotein (VSV-G) *env* expression plasmid (pMD/G VSV-G) by FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA), and supernatant from the transfected cells was collected to infect the cells from neonatal tissues.

To produce the recombinant pseudo-type lentivirus, human Oct3/4 and c-Myc cDNAs were obtained as Human Fetus Marathon-Ready cDNA and Human Bone Marrow Marathon-Ready cDNA, respectively, from Clontech (Mountain View, CA, USA) and human Sox2 and Klf4 cDNAs were obtained from German Science Center for Genomic Research. The cDNAs were inserted into the pENTR/D-TOPO entry vector plasmid (Invitrogen, Carlsbad, CA, USA) and verified by DNA sequencing. The cDNAs in pENTR/D-TOPO were then transferred to the pCSII-EF-MCS-IRES2-Venus lentiviral vector plasmid using the Gateway LR clonase (Invitrogen). The VSV-G-pseudotyped lentiviral vectors were produced by transient transfection of three plasmids, the packaging plasmid (pCAG-HIVgp), the VSV-G- and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev), and the lentiviral vector plasmid into 293T cells. Human neonatal tissue cells were transduced with lentiviral vectors and nearly 100% of transduction efficiency was confirmed by fluorescence microscopy for Venus expression. Six to ten days after transduction, the cells were harvested by trypsinization and 5–10 × 10^4 cells were replated on mouse embryonic fibroblast (MEF) feeder cells in a 100 mm dish.

Viral infection and iPS cell generation were performed essentially as has been described previously.[1] Generated iPS cells were maintained essentially as has been described previously.[9]

Feeder cells

The iPS cells were established and maintained on MEF feeder cells. MEFs were obtained from 14 day embryos of ICR mice as previously described.[10] Pregnant ICR mice were obtained from Charles River Japan (Tsukuba, Ibaraki, Japan). MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 10% FBS (Sigma) and were treated with Mitomycin C (10 μg/mL) for 1.5–2.0 h prior to their use as feeder cells.

Alkaline phosphatase staining and immunocytochemistry

Alkaline phosphatase staining was performed using an alkaline phosphatase substrate kit 4 (Vector Laboratories, Burlingame, CA, USA). Immunocytochemistry was performed essentially as described previously.[9] Primary antibodies used were SSEA-4 (Millipore, Billerica, MA, USA), Tra-1-60 (Millipore), Tra-1-81 (Millipore), Oct3/4 (Santacruz, Santa Cruz, CA, USA), and Nanog (Reprocell, Yokohama, Kanagawa, Japan). Secondary antibodies used were Alexa Fluor 546 anti-mouse IgG (Molecular Probes, Eugene, OR, USA) to detect SSEA-4, TRA-1-60, TRA-1-81 and Oct3/4, and Alexa Fluor® 546 anti-rabbit IgG (Molecular Probes) to detect Nanog.

Karyotype analysis

Karyotype analysis was performed essentially as described previously.[9]

Teratoma formation assay

The teratoma formation assay was performed as described previously,[1] except that the cells were transplanted into the sub-capsular space of the testis.

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Short tandem repeat polymorphism analysis

Short tandem repeat (STR) polymorphism analysis was carried out on genomic DNA using a PowerPlex1.2 kit (Promega, Madison, WI, USA), which is polymerase chain reaction (PCR) based.[11]

RESULTS

Generation of iPS cells from human neonatal tissue cells

Approximately 2 weeks after initiating the generation of iPS cells, some granulated colonies appeared that were dissimilar to ES cells in morphology. We observed distinct types of colonies that were flat and resembled ES cell colonies 3–5 weeks after initiation of culture. Eventually, all of the human cell types tested provided ES-like colonies in their cultures. We picked some representative colonies from each culture and placed these in fresh cultures. All of the colonies continued to proliferate when grown on MEFs in primate ES cell medium containing basic fibroblast growth factor (FGF). Of note, iPS cells established with lentiviral vector containing Venus did not express Venus at all, i.e. the expression of exogenous genes was silenced in the established iPS cells.[1]

The human iPS cell lines generated in this study are described in Table 1. All of the cell lines were morphologically similar to human ES cells, with the exception of

Table 1 List of information regarding established human iPS cell lines

Cell name	Source cells	Introduced genes	Vector origin	Origin	Karyotype	Teratoma
HiPS-RIKEN-1A	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XX	Observed
HiPS-RIKEN-1B	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XX	ND
HiPS-RIKEN-1C	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-1D	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-1E	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-1F	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2A	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-2B	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2C	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2D	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2E	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2F	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-12A	HUC-5	OcT3/4, Sox2, Klf4	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-12B	HUC-5	OcT3/4, Sox2, Klf4	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-13A	HUC-5	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-13B	HUC-5	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-11A	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-11B	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-3A	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	46, XY	Observed
HiPS-RIKEN-3C	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	ND	ND
HiPS-RIKEN-3D	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	ND	ND
HiPS-RIKEN-3E	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	ND	ND
HiPS-RIKEN-4A	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	46, XY, t(6;9)(p22;q32)	Observed
HiPS-RIKEN-4B	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4C	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4D	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4E	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4F	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4G	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4H	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND

HUC-F2, HUC-Fm, and HUC-5 are fibroblast-like cells derived from human umbilical cord. HFM-1 are fibroblast-like cells derived from human amniotic membrane. The cellular origins of human induced pluripotent stem (iPS) cells were confirmed using short tandem repeat polymorphism analysis (see text and Materials and methods). Transplants of iPS cells into immunodeficient mice that generated teratomas with all three types of germ layer are indicated by "observed". ND, not done.

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HiPS-RIKEN-3C (data not shown). Although HiPS-RIKEN-3C continued to proliferate for more than one month, this line did not appear to be comprised of ES-like cells (data not shown). The nature of these apparently non-ES-like cells in HiPS-RIKEN-3C remains to be determined.

Expression of gene markers of the undifferentiated state

Alkaline phosphatase activity and the expression of stage specific embryonic antigen-4 (SSEA-4), tumor-related antigen-1–60 (TRA-1-60), TRA-1-81, Oct3/4, and Nanog were detected in all of the iPS cell lines (Fig. 1), with the exception of HiPS-RIKEN-3C.

Teratoma formation

To evaluate the *in vivo* pluripotency of these new iPS cells, we transplanted the cells into the sub-capsular space of the testis of immunodeficient mice. Tumor formation was screened at about 8 weeks after transplantation. Histological examination of the tumors showed the presence of ectoderm-, mesoderm-, and endoderm-derived tissues (Fig. 2).

Authentication of the origin of the new iPS cell lines

To confirm that the newly generated iPS cell lines were derived from neonatal tissue cells, we compared the results of an STR polymorphism analysis of the original neonatal tissue cells and the new iPS cell lines (data not shown). All of the iPS cell lines were confirmed to be derived from the source cells (Table 1). Interestingly, we also found that the amniotic membrane cells, HFM-1, were a mixture of cells from two individuals. Since we also possessed umbilical cord cells from the neonate, we were able to confirm that the neonate provided some of the cells. It is highly likely that the other contributor was

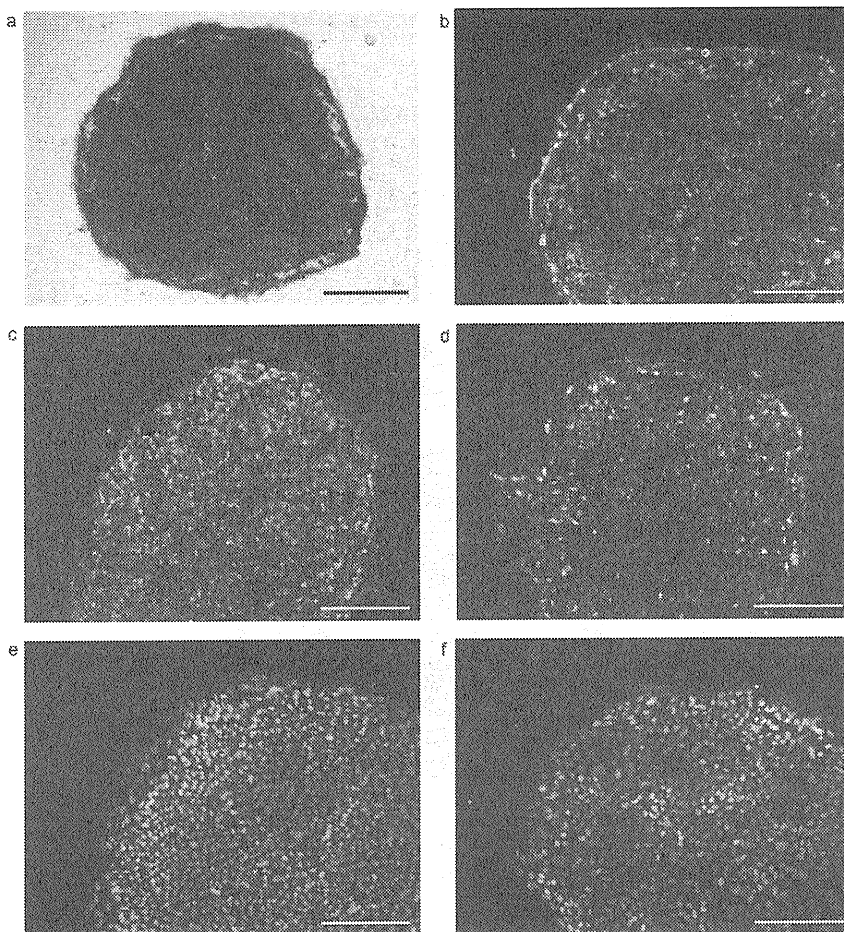

Figure 1 Expression of marker genes for the undifferentiated embryonic stem (ES) cell-like state in cells of the clone HiPS-RIKEN-1A. (a) alkaline phosphatase. (b) SSEA-4. (c) Tra-1-60. (d) Tra-1-81. (e) Oct3/4. (f) Nanog.

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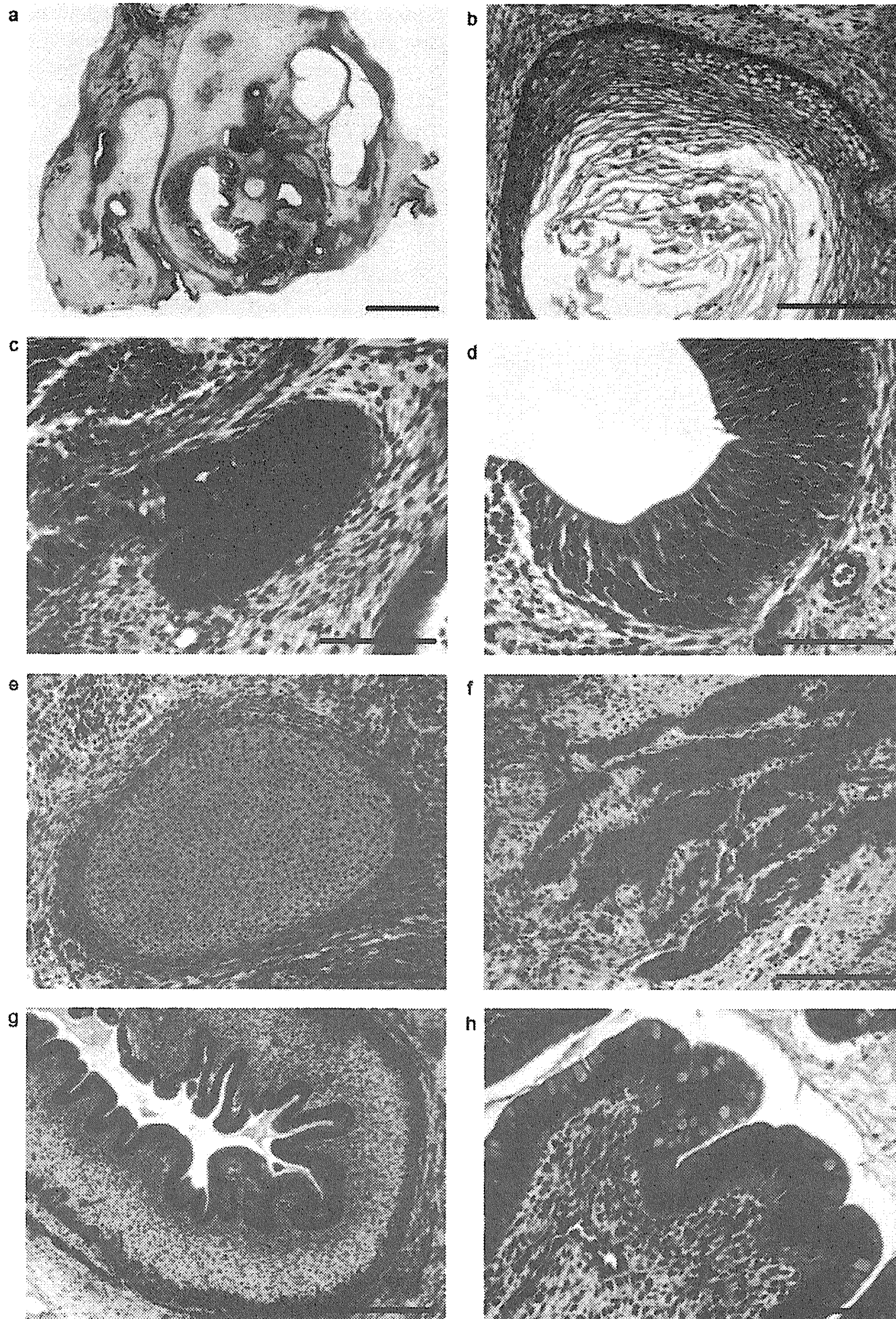

Figure 2 Teratomas derived from human induced pluripotent stem (iPS) cells. Cells from clone HiPS-RIKEN-1A were transplanted into the sub-capsular space of the testis of immunodeficient mice. A hematoxylin and eosin stained section from a resulting tumor is shown. (a) Whole teratoma. (b) Epithelial tissue. (c) Pigmented cells. (d) Neural epithelium. (e) Cartilage. (f) Muscle. (g) Intestinal tract. (h) Intestinal epithelium.

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the mother. Thus, the iPS cell lines produced from HFM-1 were derived from both neonatal and maternal cells (Table 1).

DISCUSSION

In contrast to iPS cell lines produced using inbred mouse strains, human iPS cell lines are derived from individuals who possess different genetic backgrounds. This factor needs to be taken into account when considering differences in characteristics among human iPS cell lines. Additionally, it should be noted that the human cells used to generate iPS cell lines may have acquired genetic mutations and these might also influence the characteristics of the cell lines.

Acquired mutations are of interest, for example in an oncology laboratory, when iPS cell lines are derived from a patient's cells to analyze aspects of their pathology. By contrast, acquired genetic mutations are an obstacle to the clinical use of iPS cell lines for regenerative medicine. It will therefore be necessary to establish validated "clinical grade iPS cell lines", possibly using cells with a low risk of carrying acquired genetic mutations, for example, neonatal tissue cells.

However, the use of neonatal tissue cells is not free of potential problems. Thus, for example, we cannot exclude the possibility of late-onset diseases that may occur in the neonate's future. The development of the technology for whole genome sequencing may resolve this issue by enabling the prior estimation of the risk of disease.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

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Development of a Simple Method to Determine the Mouse Strain from which Cultured Cell Lines Originated

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Subject areas: Resources

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Reviewer: Atsushi Yoshiki, RIKEN BioResource Center, Japan; Arihiro Kohara, National Institute of Biomedical Innovation, Japan

Editor: Yeonhee Lee, Seoul Women's University, Republic of Korea

Received December 14, 2010;
Accepted December 17, 2010;
Published December 29, 2010

Citation: Nakamura, Y., et al. Development of a Simple Method to Determine the Mouse Strain from which Cultured Cell Lines Originated. IBC 2010, 2:14, 1-6.
doi: 10.4051/ibc.2010.2.4.0014

Funding: This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Competing interest: All authors declare no financial or personal conflict that could inappropriately bias their experiments or writing.

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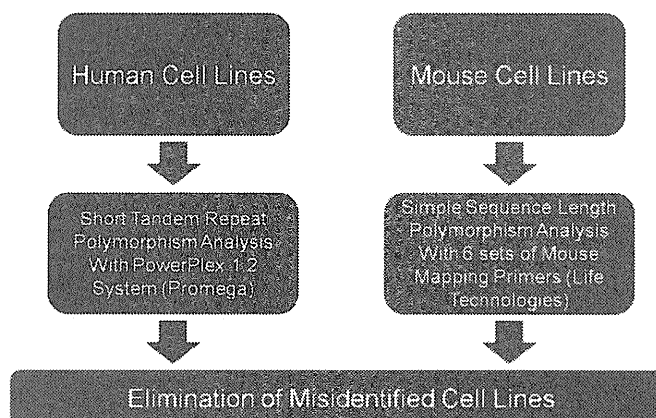
SYNOPSIS

Misidentification of cultured cell lines results in the generation of erroneous scientific data. Hence, it is very important to identify and eliminate cell lines with a different origin from that being claimed. Various methods, such as karyotyping and isozyme analysis, can be used to detect inter-species misidentification. However, these methods have proved of little value for identifying intra-species misidentification, and it will only be through the development and application of molecular biological approaches that this will become practical. Recently, the profiling of microsatellite variants has been validated as a means of detecting gene polymorphisms and has proved to be a simple and reliable method for identifying individual cell lines. Currently, the human cell lines provided by cell banks around the world are routinely authenticated by microsatellite polymorphism profiling. Unfortunately, this practice has not been widely adopted for mouse cells lines. Here we show that the profiling of microsatellite variants can be also applied to distinguish the commonly used mouse inbred strains and to determine the strain of origin of cultured cell lines. We found that approximately 4.2% of mouse cell lines have been misidentified; this is a similar rate of misidentification as detected in human cell lines. Although this approach cannot detect intra-strain misidentification, the profiling of microsatellite variants should be routinely carried out for all mouse cell lines to eliminate inter-strain misidentification.



An SSLP analysis using the 6 MIT markers described in this study was sufficient to distinguish the common and popular inbred mouse strains such as C57BL/6, BALB/c, C3H/He, 129/Sv and DBA/2. Although this approach cannot detect intra-strain misidentification, it should be routinely carried out for all mouse cell lines to eliminate inter-strain misidentification.

Keywords: cell bank, cross-contamination, microsatellite polymorphism, misidentification, quality control, short tandem repeat polymorphism, simple sequence length polymorphism

Introduction

Cultured cell lines have proved a valuable resource in all fields of the life sciences and have been utilized in many types of biological study. Currently, however, stringent analyses to check the identity of a cell culture are not always included as part of the culture protocol routine. This has led to misidentification or cross-contamination of cell lines going undetected. As a result, the published literature contains a number of reports that are based on wrongly identified cell lines[1]. Despite numerous publications warning of inter- and intra-species misidentification of cell lines[2-6], the problem of misidentification continues to occur at an extremely high rate[7-10]. Therefore, articles pointing out misidentification of cell lines continue to be published[11-14].

Inter-species contamination can be detected by various methods, such as karyotyping and isozyme analysis. However, it was not possible to detect intra-species misidentification prior to the development of molecular biology techniques that make use of the genetic differences between cell lines to facilitate their identification. One such method makes use of microsatellite polymorphisms to develop diagnostic profiles for cell lines[15]. Microsatellite polymorphisms result from differences in the numbers of a repeating unit of 1-7 base pairs; these variants are also called short tandem repeat (STR) polymorphisms or simple sequence length polymorphisms (SSLPs). These polymorphisms have been extensively used in forensic science. Gene profiling using STR polymorphisms (STR profiling) has been shown to be an efficient and reliable means for identifying individual human cell lines[16,17] and is now performed routinely in the major cell banks around the world. What about mouse cell lines; do they also suffer from problems of misidentification? This question prompted us to establish a method to authenticate the identities of mouse cell lines.

Results and Discussion

The mouse genome possesses a huge number of microsatellite polymorphisms, similarly to the genomes of humans and other mammalian species. The so-called MIT markers developed at the Massachusetts Institute of Technology (MIT) Whitehead Institute[18] in the mouse have been extensively developed and utilized in various fields of research. Information on microsatellite polymorphisms, including data on MIT marker sizes in 47 mouse strains, is publicly available on the Center for Inherited Disease Research Web site (http://www.cidr.jhmi.edu/mouse/mouse_resources.html). It is possible to amplify a number of polymorphic microsatellite loci using commercially available sets of primers. The PCR products are analyzed simultaneously with size standards using automated fluorescent detection techniques. The result is a simple numerical code that corresponds to the lengths of the PCR products amplified at each locus, and is accurate to less than one base pair.

Our aim was to establish a simple method that can be used for routine analysis in our cell bank work. Thus, we sought to establish a method using the smallest practical number of polymorphic loci. First, we screened more than 500 microsatellite primers covering the autosomes and the X chromosome and selected the 24 MIT markers that exhibited the most distinct differences between inbred mouse strains (Figure 1, Table 1), mostly larger than 10 bp on electrophoretic patterns[19]. We then performed an SSLP analysis of 40 mouse strains using the 24 selected MIT markers (Table 1) and concluded that 6 MIT markers would be sufficient to distinguish the common and popular inbred strains such as C57BL/6, BALB/c, C3H/He, 129/Sv and DBA/2 (Table 2).

An SSLP analysis, using the 6 MIT markers described above, has now been adopted by the Cell Engineering Division of the RIKEN BioResource Center (RIKEN Cell Bank) to exclude misidentification among the cultured mouse cell lines that we currently provide. However, this analysis can detect inter-strain but not intra-strain misidentification.

The screening of the RIKEN Cell Bank indicated that 97.7% (334 lines out of 342 lines) of the mouse cell lines were derived from

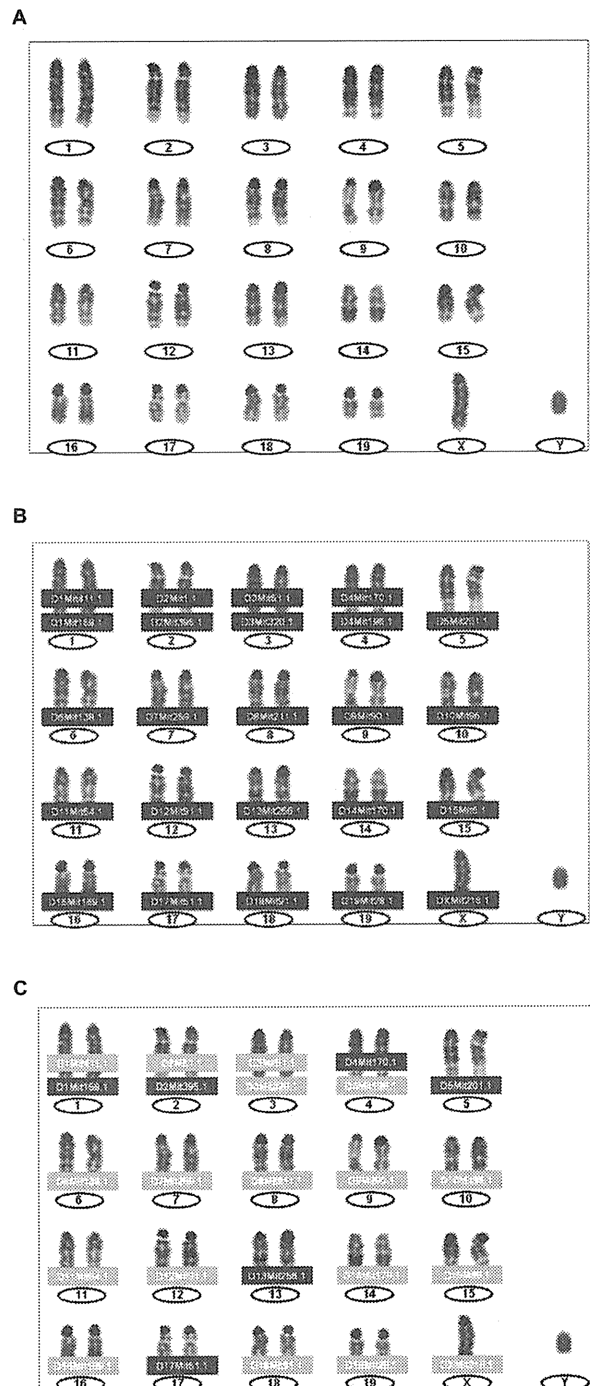


Figure 1. Distribution of the analyzed simple sequence length polymorphisms (SSLPs) loci in the mouse genome. (A) Karyotype of the mouse, 40XY. (B) The chromosomal distributions of the screened 24 loci are indicated. (C) The 6 loci selected as being sufficient for identification of common inbred mouse strains are indicated by the red background.

Table 1. Results of the SSLP analyses of 40 inbred mouse strains using the 24 loci indicated in Figure 1B

Strain Name	origin	Mit159_1	Mit411_1	Mit1_1	Mit395_1	Mit51_1	Mit320_1	Mit170_1	Mit196_1	Mit201_1	Mit138_1	Mit259_1	Mit211_1	Mit90_1	Mit95_1	Mit54_1	Mit91_1	Mit256_1	Mit170_1	Mit6_1	Mit169_1	Mit51_1	Mit91_1	Mit28_1	Mit216_1
C57BL/6JMs	Ms	203	104	217	130	240	110	226	104	98	192	241	150	110	201	148	153	109	203	134	234	157	132	153	126
C57BL/6J	JAX	203	104	217	129	240	110	226	104	98	192	241	150	110	201	148	158	109	203	134	234	157	132	153	126
C57BL/6J Jcl	Jcl	203	104	217	129	240	110	226	104	98	192	241	150	110	201	148	159	109	203	134	234	157	132	153	126
C57BL/6N Jcl	Jcl	203	104	213	129	240	110	226	104	98	192	241	150	106	201	148	153	109	203	134	234	157	132	153	126
C57BL/6N Crj	Crj	203	104	213	129	240	110	226	104	98	192	241	150	101	201	148	153	109	203	134	234	157	132	153	126
C57BL/10SnJ	Ms	203	104	213	130	240	110	226	104	98	196	241	160	106	201	148	153	109	203	134	234	157	132	155	126
C57L/J	Ms	142	97	213	130	240	110	226	104	98	192	217	150	106	181	148	158	109	188	134	234	163	130	157	126
C58/J	Ms	142	104	213	123	240	110	226	104	98	211	217	160	106	181	148	142	109	186	134	234	153	122	157	126
CBA/J	Ms	185	97	213	123	240	101	236	104	92	211	246	162	98	181	119	151	76	203	138	238	140	132	162	126
DBA/1J	Ms	142	97	213	135	258	101	236	104	92	207	248	160	103	181	144	159	76	202	138	234	153	132	162	126
DBA/2J	JAX	142	97	213	135	258	105	242	104	92	207	248	160	126	181	144	153	76	197	138	234	155	132	162	126
BALB/cJ	JAX	142	97	213	135	254	103	242	116	94	211	225	162	106	181	147	151	88	206	134	234	155	130	162	132
BALB/cANMs	Ms	142	97	213	135	254	103	242	116	94	211	225	164	106	181	147	151	88	204	134	234	155	130	162	132
AKR/J	Ms	203	97	213	161	258	103	236	116	104	211	225	166	106	193	119	153	76	206	134	234	140	132	162	126
C3H/HeJ	JAX	185	104	213	124	240	103	236	116	92	215	246	160	106	181	146	151	78	206	127	232	140	130	162	126
C3H/HeNCrj	Crj	185	104	213	124	240	103	236	116	92	211	246	160	106	181	144	151	78	206	127	232	140	130	162	126
A/J	JAX	185	104	213	135	240	103	226	116	106	211	246	164	106	181	119	151	88	202	136	234	153	144	153	132
A/WySnJ	Ms	185	104	213	135	240	103	226	116	106	211	246	164	106	181	119	151	88	202	136	234	153	132	153	132
129/SvJ	Ms	191	104	215	157	246	103	226	116	94	211	239	162	106	181	147	142	76	202	108	234	169	132	153	132
SM/J	Ms	193	104	209	135	254	103	236	104	98	211	217	160	106	181	147	151	76	206	136	234	157	132	157	126
FVB/N	Jcl	191	104	224	161	254	108	234	104	102	211	244	160	112	181	148	150	88	209	134	250	142	124	162	128
SJL/J	JAX	203	104	219	161	254	108	242	104	106	211	248	160	108	181	147	149	88	202	134	234	153	130	153	126
SJL/JMs	Ms	203	104	219	161	254	108	242	104	106	211	246	160	108	181	147	149	88	202	134	234	153	130	153	126
SWR/J	Ms	203	97	213	161	258	103	232	104	104	215	244	162	106	181	148	150	92	203	131	262	142	130	162	126
CE/J	Ms	185	97	215	161	258	103	245	116	106	215	248	164	98	181	151	145	76	197	131	232	140	132	162	132
GRS/A	AichiCC	203	86	217	123	258	103	242	116	104	211	246	160	106	181	148	151	76	203	131	232	167	130	162	128
[illegible]	Nga	191	97	213	161	254	108	242	116	104	203	249	160	97	181	148	151	88	209	136	240	153	126	153	132
[illegible]	Nga	191	97	213	135	254	103	242	116	92	207	225	160	106	181	147	151	108	203	131	240	157	130	153	132
[illegible]	Stm	185	97	213	123	258	103	232	104	98	207	244	160	106	181	147	151	108	203	131	232	153	124	153	132
MF1	Ms	142	97	226	129	326	99	226	104	92	344	217	160	128	201	119	114	100	220	108	278	170	130	155	135
CAST/Ei	Ms	168	111	229	138	232	91	247	106	102	236	227	182	107	187	135	114	90	174	108	311	165	142	160	136
[illegible]	Ms	144	97	230	140	288	105	228	106	108	249	219	156	112	201	123	114	92	204	108	286	172	227	176	118
[illegible]	Ms	166	97	228	100	238	91	249	104	98	221	258	191	121	185	173	114	86	174	117	311	220	147	160	139
[illegible]	Ms	142	97	228	142		87	226	110	108	284	217	175	124	201	121	114	98	204	108	236	149	120	172	133
[illegible]	Ms	176	106	224	142	230	91	226	106	100	280	217	141	84	197	139	114	90	197	106	266	151	138	172	121
[illegible]	Ms	142	97	222	129	313	89	226	104	96	332	217	168	124	204	121	114	92	218	108	278	170	120	155	140
M. spretus	RPCI	176	97	179	122	252	132	279	91	114	247	221	160	88	134	157	118	90	201	110	205	134	128	178	134
SPRET/Ei	JAX	180	97	190	124		106.5	269	91	114	266	213	185	95		151	116	90	205	110	204	134	115	170	139
PWK	RPCI	142	111	217	142	281	91	242	106	102	280	217	164	88	199	138	114	100	203	106	274	164	132	185	123
[illegible]	Ms	139	97	196	114		89	253	89	120	322	229	152	117		150	116	67	99	126		156	114	164	92

In each column (at each polymorphic locus), fragments of similar length are indicated by the same color.

Table 2. SSLP analysis of 5 common and popular mouse strains using the 6 selected loci indicated in Figure 1C

Sample	D1 Mit159.1	D2 Mit395.1	D4 Mit170.1	D5 Mit201.1	D13 Mit256.1	D17 Mit51.1
C57BL/6	203.2	129.7	226.0	98.9	100.3	157.3
BALB/c	141.8	135.5	242.5	94.9	88.3	155.1
C3H/He	185.1	123.8	236.4	92.5	78.4	140.1
129/Sv	191.5	157.3	226.1	94.8	76.6	163.7
DBA/2	141.8	135.3	242.6	92.6	76.5	155.2

the common inbred strains (e.g., Table 3); the remaining 2.3% appeared to be derived from non-inbred mice. Cell lines derived from an F1 hybrid of two different strains show different alleles at each locus that correspond with those of their parental strains (Table 4). With regard to the cell line UV.CC3-11.1 (RCB2074), which was established from an F1 hybrid from the cross BALB/c x C3H/He, only the BALB/c allele at the D5Mit201.1 locus could be detected (Table 4). The C3H/He allele has been replaced by the BALB/c allele during culture, i.e., so-called loss of heterozygosity (LOH) appears to have occurred.

For some cell lines of uncertain provenance, we were able to identify the originating strains by SSLP analysis. For example, we found that LLC[20], PU5-18[21], and MBT-2[22] were derived from the C57BL/6, BALB/c, and C3H/He strains, respectively (Table 5). Interestingly, the LLC cell line had a deletion mutation at the locus D13Mit256.1 and only this mutated allele was detectable. Presumably, LOH had occurred subsequent to the deletion mutation.

Approximately 4.2% (14 lines out of 334 lines) of the mouse cell lines derived from common inbred strains were misidentified (Table 6), i.e., the strains were different from those claimed by the depositors of the cell lines. This rate of misidentification is similar to that reported for human cell lines[17]. As an example of misidentification, the cell line TSt-4 was registered as being C57BL/6-derived; however, SSLP analysis indicated that the cell line was derived from the BALB/c strain (Table 7). LOH following a deletion mutation was detected at the D17Mit51.1 locus of the TSt-4 cell line. Similarly, LOH following a deletion mutation was also detected in the MC3T3-E1[23] cell line at locus D1Mit159.2 (Table 8). In general, it is inevitable that aberrations such as point mutations, deletion mutations and LOHs accumulate in cell lines following long term culture. Thus, the optimum strategy is to culture the cell for as short period as possible not only in the cell bank but also for ordinary laboratory work.

Table 3. SSLP analysis of three C57BL/6-derived cell lines, B6mt-2, MEDEP-BRC5, and UV.B6-4.1 and three BALB/c-derived cell lines, RAW264, J774.1, and UV.BAL-7.1

Sample	D1 Mit159.1	D2 Mit395.1	D4 Mit170.1	D5 Mit201.1	D13 Mit256.1	D17 Mit51.1
C57BL/6	203.2	129.7	226.0	98.9	100.3	157.3
B6mt-2	203.2	129.8	226.0	98.7	100.3	157.0
MEDEP-BRC5	203.2	130.0	226.0	98.9	100.6	157.0
UV.B6-4.1	203.6	130.1	226.0	98.9	100.8	157.4
BALB/c	141.8	135.5	242.5	94.9	88.3	155.1
RAW264	141.8	135.3	242.5	94.6	88.3	155.0
J774.1	142.2	135.7	242.6	94.4	88.5	154.8
UV.BAL-7.1	141.8	135.3	242.4	94.6	88.2	155.1

The analyses authenticated the origins of the six cell lines.

Table 4. SSLP analysis of a mouse cell line derived from a (BALB/c x C3H/He) F_1 Mouse

Sample	D1 Mit159.1	D2 Mit395.1	D4 Mit170.1	D5 Mit201.1	D13 Mit256.1	D17 Mit51.1
BALB/c	141.8	135.5	242.5	94.9	88.3	155.1
C3H/He	185.1	123.8	236.4	92.5	78.4	140.1
UV.CC3-11.1	142.0 185.2	135.5 123.9	242.6 236.4	94.8	88.4 78.5	155.2 140.2

At locus D5Mit201.1 only the allele corresponding to that of BALB/c was detected.

Table 5. SSLP analysis of the LLC, PU5-18, and MBT-2 mouse cell line

Sample	D1 Mit159.1	D2 Mit395.1	D4 Mit170.1	D5 Mit201.1	D13 Mit256.1	D17 Mit51.1
C57BL/6	203.2	129.7	226.0	98.9	100.3	157.3
LLC	203.4	129.7	226.1	98.4	98.5	157.0
BALB/c	141.8	135.5	242.5	94.9	88.3	155.1
PU5-18	141.5	135.0	242.5	94.2	87.6	155.0
C3H/He	185.1	123.8	236.4	92.5	78.4	140.1
MBT-2	184.7	123.2	236.3	92.2	77.8	139.8

The mouse strains from which the cell lines originated were not registered by the depositors. The SSLP analysis indicated that they were derived from C57BL/6, BALB/c, and C3H/He, respectively.

Table 6. List of cell lines that the strains were different from those claimed by the depositors of the cell lines

RCB No.	Cell Name	Registered Strain	Result	Comment
RCB0792	T88-M	DBA/2J	C3H/He	Providing
RCB1144	DA-3	BALB/c	DBA/2	Providing
RCB2116	TSt-4	C57BL/6	BALB/c	Providing
RCB2117	TSt-4/G	C57BL/6	BALB/c	Providing
RCB2118	TSt-4/G-DLL1	C57BL/6	BALB/c	Providing
RCB2119	TSt-4/N	C57BL/6	BALB/c	Providing
RCB2120	TSt-4/N-DLL1	C57BL/6	BALB/c	Providing
RCB2633	MM46 CEA-2	C3H/He	Unknow	Providing
RCB2634	MM46-APR-MUC1 cl.1	C3H	Swiss	Providing
RCB2195	FVB-2	Swiss FVB	129/Sv	Stopped provision
RCB2196	ICRmt-1	ICR	Unknow	Stopped provision
RCB2617	MM46	C3H	Swiss	Stopped provision
RCB2632	BALB/3T3aP R-MUC1 clone 16	BALB/c	Swiss	Stopped provision
RCB2647	BALB/3T3	BALB/c	Swiss	Stopped provision

Table 7. Misidentification of a cell line identified using SSLP analysis

Sample	D1 Mit159.1	D2 Mit395.1	D4 Mit170.1	D5 Mit201.1	D13 Mit256.1	D17 Mit51.1
C57BL/6	203.2	129.7	226.0	98.9	100.3	157.3
TSt-4	142.0	135.5	242.7	94.5	88.4	152.3
BALB/c	141.8	135.5	242.5	94.9	88.3	155.1

The mouse cell line TSt-4 was registered as a C57BL/6-derived cell line, but our SSLP analysis showed it was derived from the BALB/c strain.

Table 8. SSLP analysis of the mouse cell line MC3T3-E1

Sample	D1 Mit159.1	D2 Mit395.1	D4 Mit170.1	D5 Mit201.1	D13 Mit256.1	D17 Mit51.1
C57BL/6	203.2	129.7	226.0	98.9	100.3	157.3
MC3T3-E1	199.4	139.6	255.9	98.4	100.4	157.0

Loss of heterozygosity following a deletion mutation appears to have occurred at locus D1Mit59.1.

The C57BL/6N and C57BL/6J substrains were shown to have 11 single nucleotide polymorphisms (SNPs) in a study using mouse MD Linkage Panel 1449 SNPs (Illumina)[24]. It is therefore possible to distinguish cell lines derived from the C57BL/6N and C57BL/6J substrains using these 11 SNP.

The relatively high rate (4.2%) of misidentification of cell lines derived from common inbred mouse strains strongly suggests that intra-strain misidentification is also likely to have occurred. For example, although a cell line may be registered as being derived from colon cancer cells, an error may have occurred and the cell line was actually derived from another cancer (a similar phenomenon also applies to human cell lines). It is impossible to identify the originating tissue by microsatellite polymorphism analysis. In this context, a profiling analysis based on gene expression using many cell lines may be useful for authenticating the originating tissues of cultured cell lines.

Conclusion and Prospects

We have established a simple and reliable method to identify the common inbred mouse strains from which cultured mouse cell lines are derived. With respect to intra-strain misidentification, such as errors regarding the originating tissue type, it will be necessary to develop other analytic techniques, for example, gene expression profiling analysis. Other types of OMICS analysis, such as whole genome sequencing, will also be useful for authentication of cell lines. In this context, bioinformatics will become increasingly important for the quality control of cultured cell lines.

Materials and Methods

Mouse cell lines

All mouse cell lines that the Cell Engineering Division of the RIKEN BioResource Center (http://www.brc.riken.jp/lab/cell/english/) has collected, 342 cell lines in total, were subjected to SSLP analysis. We selected the latest preserved stock cells and those that were preserved immediately after deposition (token stock cells) for this analysis.

DNA preparation

DNA was prepared from approximately 2×10^6 cells using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

SSLP analysis

Multiplex PCR reactions for the SSLP analysis were carried out using the following fluorescent dye-linked primers (Mouse Mapping Primers, Life Technologies, Carlsbad, CA, USA): D1Mit159.1 (VIC) on chromosome 1, D2Mit395.1 (6-FAM) on chromosome 2, D4Mit170.1 (6-FAM) on chromosome 4, D5Mit201.1 (VIC) on chromosome 5, D13Mit256.1 (NED) on chromosome 13, and D17Mit51.1 (NED) on chromosome 17. VIC, 6-FAM, and NED are green, blue, and yellow fluorescent dyes, respectively.

PCR was performed with 2.4 ℓ of genomic DNA (25 ng/ ℓ) and 1.25 units AmpliTaq Gold (Applied Biosystems, Foster, CA, USA) in a 15 μl reaction volume using the GeneAmp PCR system 9700 (Applied Biosystems). Samples were amplified under the following conditions: an initial incubation at 95°C for 12 min was followed by 10 cycles of 94°C for 20 sec, 55°C for 20 sec, 72°C for 30 sec, and 20 cycles of 89°C for 20 sec, 55°C for 20 sec, 72°C for 30 sec, and finally incubation at 72°C for 10 min.

Labeled products were detected by electrophoretic size fractionation on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). A size control PCR was performed and the products were subjected to electrophoretic size fractionation as an internal control with each analysis. The end result for each cell line was an electropherogram with each allele represented as one or two peaks. As expected, one peak was detected at each locus in cell lines derived from inbred mouse strains. Samples that failed to give measurable peaks at all loci were reanalyzed using a different concentration of DNA or using newly prepared DNA.

Acknowledgements

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Int J Hematol (2011) 93:5–9
DOI 10.1007/s12185-010-0742-2

Red blood cell production from immortalized progenitor cell line

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Received: 21 November 2010 / Revised: 24 November 2010 / Accepted: 30 November 2010 / Published online: 25 December 2010
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Abstract The supply of transfusable red blood cells (RBCs) is not sufficient in many countries. If immortalized erythroid progenitor cell lines able to produce transfusable RBCs in vitro were established, they would be valuable resources. However, such cell lines have not been established. We have developed a robust method to establish immortalized erythroid progenitor cell lines following the induction of hematopoietic differentiation of mouse embryonic stem (ES) cells and have established many immortalized erythroid progenitor cell lines so far. Although their precise characteristics varied among cell lines, each of these lines could differentiate in vitro into more mature erythroid cells, including enucleated RBCs. Following transplantation of these erythroid cells into mice suffering from acute anemia, the cells proliferated transiently, subsequently differentiated into functional RBCs, and significantly ameliorated the acute anemia. Considering the number of human ES cell lines that have been established so far and the number of induced pluripotent stem cell lines that will be established in future, the intensive testing of a number of these lines for establishing immortalized erythroid progenitor cell lines may allow the establishment of such cell lines similar to the mouse erythroid progenitor cell lines.

Keywords Erythrocyte · Erythropoiesis · Red blood cell · Transfusion therapy

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1 Introduction

Organ and cell transplantation therapy is now a standard therapy. However, the supply of organs or cells is not necessarily sufficient all over the world. Hence, production of artificial organs and in vitro production of transplantable cells have been studied in earnest. At the moment, transfusion therapies involving red blood cells (RBCs), platelets, and neutrophils depend on the donation of these cells from healthy volunteers. Unpredictable adverse results derived from the current transfusion therapy system such as contamination of hazardous viruses and transfusion-related acute lung injury (TRALI) may be eliminated by the development of new technologies in the future. On the other hand, lack of supply of transfusable materials by the current system will become a severe problem in advanced nations including Japan, since in those countries the ratios of aged generations who have more opportunities to require transfusion therapies are increasing while the ratios of younger generations who can donate transfusable materials are decreasing. In this situation, research and development to produce transfusable blood materials is very important and should be carried out more earnestly. In particular, since RBC transfusion is now routine and indispensable for many clinical purposes, in vitro production of transfusable RBC is an urgent theme.

2 RBC production from hematopoietic stem cells

The hematopoietic stem cells present in bone marrow and umbilical cord blood are promising materials for in vitro production of RBCs and this has stimulated interest in the development of in vitro procedures for the generation of functional RBCs from these tissues [1–3]. Umbilical cord

blood cells are of particular interest as they are readily available but are usually discarded. Provided the mother of a neonate consents to use of the umbilical cord blood, this material can provide a useful resource without any further complicating critical or ethical concerns.

It was reported that human erythroid cells (nucleated cells) produced on a large scale ex vivo could differentiate in vivo into enucleated RBCs [1]. This study demonstrated that erythroid progenitor cells produced in vitro from hematopoïetic stem and progenitor cells could have a clinical application as an alternative method for transfusing terminally differentiated RBCs. More recently, the same group described an ex vivo methodology for producing fully mature human RBCs from hematopoietic stem cells [2]. The enucleated RBCs produced by this approach are potentially even more valuable as they should be functional immediately after transfusion without requiring the long latency period for enucleation normally necessary for erythroid cells.

The mechanism of erythroblast enucleation, a critical step in RBC production, has not yet been fully elucidated [4, 5]. The role of interactions between erythroblasts and other cells, such as macrophages, in this process is a controversial topic [6–10]. Macrophages in retinoblastoma gene (Rb)-deficient embryos are unable to physically interact with erythroblasts and RBC production is impaired in these embryos [9]. In addition, in vitro production of enucleated RBCs from immature hematopoietic stem/progenitor cells proceeds efficiently in the presence [2] but not in the absence [1] of feeder cells.

Of note, however, enucleation can apparently be initiated in vitro in erythroblasts that have been induced to differentiate in vivo to a developmental stage that is competent for nuclear self-extrusion [10, 11]. Moreover, we have developed a method to produce enucleated RBCs efficiently in vitro without use of feeder cells [3]. The culture system has allowed erythroid cells to differentiate to a developmental stage competent for nuclear self-extrusion [3]. Taken together, although it has generally been thought that efficient enucleation of erythroblasts is largely dependent on signals mediated by cells in their local environment [6–9], the interaction of erythroblasts with other cells is not necessary for efficient erythroblast enucleation [3]. Signals mediated by humoral factors appear to be sufficient for the efficient autonomous completion of erythroblast enucleation [12].

Since culture without the use of feeder cells is technically easier and less expensive, the method we established [3] has the potential to be a cost-effective means of producing transfusable RBCs on a large scale from immature hematopoietic stem/progenitor cells. Currently, however, cost factors mean that it is not yet realistic to produce RBCs on a large scale, approximately 200 ml or more, using our in vitro culture system. In particular, patents on the growth factors used in the culture system are a major obstacle, because these growth factors are very expensive, at least at the moment. After the relevant patents expire, our in vitro culture system will become a more realistic scenario.

3 RBC production from embryonic stem (ES) cells

ES cells possess the potential to produce various differentiated cells able to function in vivo and thus represent another promising resource for RBC production. Furthermore, since ES cell lines are immortalized, they can be used repeatedly and have potential to produce abundant differentiated cells in the quantities required for clinical use. However, it will be important to carry out routing screening of the ES cell lines for de novo chromosomal aberrations and/or genetic mutations that may arise in vitro, before these long-term cell cultures are applied in the clinic. Unsurprisingly, there is now a widespread and enthusiastic debate on standardization of the characteristics of ES cells for regenerative medicine protocols that exploit these cell lines. In my opinion, since chromosomal aberrations and genetic mutations are inevitable in long-term cell cultures, only ES cell lines that have been cultured for a limited period, e.g., less than 30 passages, should be selected for clinical use.

Hematopoietic cells, including those in the erythroid lineage, have been generated from mouse ES cells [13–16], non-human primate ES cells [17–19], and human ES cells [20–25]. We have also established a long-term in vitro method for culturing hematopoietic cells derived from ES cells of the non-human primate, the common marmoset [26]. Recently, abundant production of enucleated RBCs from human ES cells was reported [27].

Taken together, we can now produce mature RBCs by in vitro culture of ES cells or the hematopoietic stem/progenitor cells present in umbilical cord blood. In practice, however, the efficiency of RBC generation varies with the quality of the ES cell line or the umbilical cord blood sample. Since ES cell lines can be utilized repeatedly, derivation of RBCs from ES cells appears to be more practical. However, even with optimal experimental procedures and the most appropriate ES cell line, the generation of abundant RBCs directly from primate ES cells is a costly and time-consuming process [26, 27]. If human erythroid progenitor cell lines can be established that have efficient production of mature RBCs, they would provide a much more useful resource than ES cell lines.

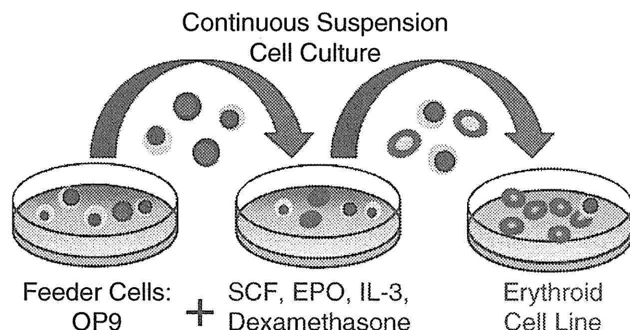

Fig. 1 Culture protocol to establish immortalized erythroid progenitor cell lines. Hematopoietic cells derived from ES or iPS cells are cultured on feeder cells, OP9, in the presence of stem cell factor (SCF), erythropoietin (EPO), interleukin-3 (IL-3) and dexamethasone

4 Establishment of mouse RBC progenitor cell lines able to produce transfusable RBCs

Several mouse and human erythroid cell lines have been established. However, to the best of our knowledge, there is no cell line that can efficiently differentiate into enucleated RBCs. It is generally difficult to establish hematopoietic cell lines from adult hematopoietic stem and progenitor cells as both are sensitive to DNA damage and are unable to maintain the lengths of telomere repeats on serial passage [28]. In contrast, ES cells are relatively resistant to DNA damage and maintain telomere lengths on serial passage [28]. Therefore, these characteristics of ES cells may be advantageous for the establishment of cell lines since differentiated cells derived from ES cells may retain them.

Recently, we developed a robust method to obtain differentiated cell lines following the induction of hematopoietic differentiation of mouse ES cells (Fig. 1), and established five independent hematopoietic cell lines using this method [29]. Three of these lines exhibited characteristics of erythroid cells, and they were designated mouse ES cell-derived erythroid progenitor (MEDEP) cell lines. Although their precise characteristics varied, each of the MEDEP lines could differentiate in vitro into more mature erythroid cells, including enucleated RBCs (Fig. 2). Following transplantation into mice suffering from acute anemia, MEDEP cells proliferated transiently and subsequently differentiated into functional RBCs. Treated mice showed a significant amelioration of acute anemia. In addition, MEDEP cells did not form tumors following transplantation into mice. This report was the first to demonstrate the feasibility of establishing erythroid cell lines able to produce mature RBCs [29]. At present, the mechanism underlying the establishment of differentiated cell lines from ES cells has not been elucidated. Nevertheless, our data clearly indicate that useful erythroid cell lines can be reproducibly obtained from mouse ES cells.

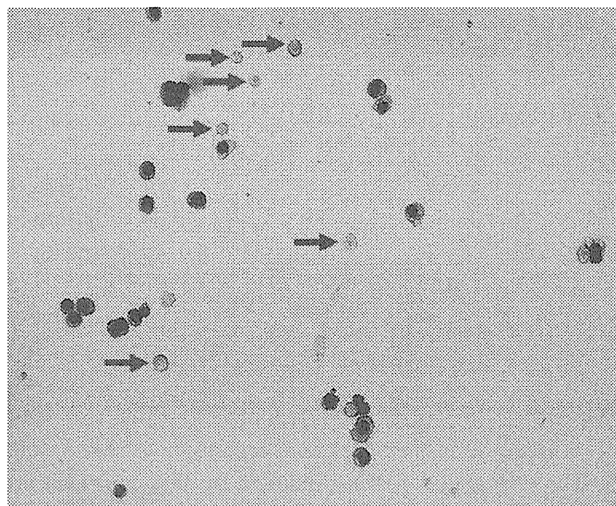

Fig. 2 Red blood cells (RBCs) produced from immortalized mouse progenitor cell line. *Arrows* indicate enucleated RBCs

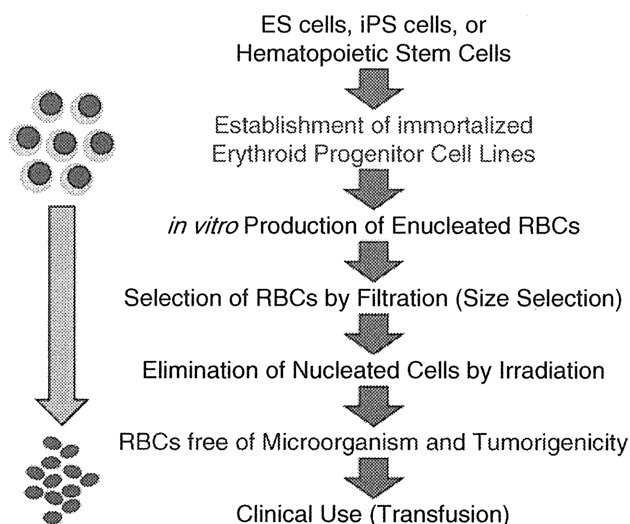

Fig. 3 Strategy to utilize red blood cells (RBCs) produced in vitro from immortalized erythroid progenitor cell lines in the clinic

5 Establishment of RBC progenitor cell lines from human ES cells or human induced pluripotent stem (iPS) cells

The reproducible establishment of MEDEP cell lines described above strongly suggests that similar erythroid cell lines could also be established from human ES cells. We, therefore, sought to establish human erythroid progenitor cell lines. The methods used to induce hematopoietic cells from ES cells and to culture the induced hematopoietic cells are similar to those established for MEDEP cell lines, with the exception that the corresponding human factors were applied and IL-3 was not used at all. Exclusion of IL-3 was based on our finding that

the compound was not necessary for establishment of MEDEP cell lines [29].

Initially, we used three human ES cell lines, KhES-1, KhES-2 and KhES-3, that had been established in Japan. Although we were able to induce hematopoietic cells from all three lines, the efficiency of production of hematopoietic cells was extremely low compared to the cases of mouse ES cells. As a result, we have yet been successful in establishing immortalized cell lines from the three human ES cell lines.

During the course of the experiments using these human ES cell lines, a breakthrough discovery in the field of regenerative medicine was reported, namely, the establishment of human iPS cells [30] following that of mouse iPS cells [31]. This discovery prompted us to establish human iPS cells, since the characteristics of pluripotent stem cells, such as ES cells, differ among cell lines. In other words, we speculated that we could obtain iPS cell lines that could have the ability to differentiate into hematopoietic cells efficiently. We were able to establish a number of human iPS cell lines using fibroblast-like cells derived from neonatal tissues [32]. Fortunately, we were able to induce abundant numbers of hematopoietic cells from some of these iPS cell lines and also to establish immortalized hematopoietic cell lines from the induced hematopoietic cells (data not shown). Currently, we are investigating the characteristics of these immortalized hematopoietic cell lines. Some seem to be erythroid cell lines.

6 Clinical application of human RBC progenitor cell lines

We reported that MEDEP cells did not exhibit tumorigenicity in vivo [29]. Nevertheless, the tumorigenic potential of any human erythroid cell line will need to be thoroughly analyzed prior to clinical use [33, 34]. In general, immortalized cell lines are not necessarily homogenous in karyotype, even after cloning. The emergence of cells possessing abnormal karyotypes is often observed following continuous culture of immortalized cell lines. Indeed, although the vast majority of the MEDEP cells in each cell line could differentiate into mature erythroid cells and transplantation of these cells significantly ameliorated anemia, the MEDEP lines included many cells possessing abnormal karyotypes [29]. Hence, it may be advisable to engineer the cells in such a way that they are eliminated if a malignant phenotype arises for any reason [35].

Alternatively, the use of terminally differentiated cells that no longer have the capability of proliferating should allow clinical applications of ES cell derivatives without the associated risk of tumorigenicity. Thus, e.g., RBCs lack nuclei following terminal differentiation, and are highly unlikely to exhibit tumorigenicity in vivo. As such, even if the original ES/iPS cells or the immortalized erythroid progenitor cell lines derived from them possessed abnormal karyotypes and/or genetic mutations, they might nonetheless be useful for clinical applications, provided that they can produce enucleated RBCs. Since enucleated RBCs are much smaller than normal nucleated cells, enucleated RBCs produced in vitro could be selected by size prior to use in the clinic so as to exclude nucleated cells, e.g., by filtration. In addition, X-ray irradiation might be useful for eradicating any contaminating nucleated cells without affecting the enucleated RBCs (Fig. 3).

Another potential obstacle to the clinical use of ES cell derivatives is that of immunogenicity [36, 37]. Transplanted MEDEP cells could not ameliorate acute anemia in mouse strains other than those from which each individual cell line was derived or in immunodeficient mice [29], suggesting immunological rejection in heterologous strains. Hence, the direct clinical application of immortalized erythroid cell lines will require use of many cell lines that express different major histocompatibility (MHC) antigens. However, in vitro-generated mature RBCs need to be compatible with ABO and RhD antigens alone (8 types in total). Moreover, RBCs lacking all of A, B and RhD antigens can be transfused into the vast majority of patients around the world. Hence, the establishment of a human erythroid cell line lacking the genes to produce A, B and RhD antigens would be a very useful resource for clinical application.

7 Conclusion

We propose that by utilizing ES cells or iPS cells it will be possible to establish human erythroid progenitor cell lines able to produce enucleated RBCs. RBCs produced by in vitro culture of such erythroid cell lines could be applied in the clinic following size selection and elimination of nucleated cells by irradiation (Fig. 3).

Acknowledgments This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

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