human cell lines. Such a database is indispensable with regard to the establishment of a set of global reference STR profiles for human cell lines and critical in support of the Standard.

Methodology. STR profiling was selected as the recommended authentication technology for inclusion in this Standard primarily because it is capable of resolving human cells to the individual level. In contrast, historically important authentication technologies such as karyotyping, isoenzyme analysis, immunotyping, and human leukocyte antigen typing do not have sufficient resolving (discriminating) power to enable unambiguous authentication of human cells to the individual level (see ATCC SDO Workgroup ASN-0002 2010 for a more detailed discussion of the relative discriminating power of these technologies). In addition, the STR profiling technology is commercially available in kit form and is rapid and economical. Masters et al. (2001) demonstrated that the technology can provide a universal reference standard for human cell lines. The STR profiling technology, as normally used, detects only human cells, and therefore additional methods may need to be used to detect contamination with non-human cells. There are a number of different commercial kits now available for STR profiling, and the users will be encouraged to follow the protocol specific to the kit being used and to refer to the Standard for additional methodological information. The most important aspects of the Standard will be the discussions on the numbers and types of loci to be evaluated, quality control of the data, interpretation of the results (matching criteria, loss of alleles, etc.), and implementation of a universal STR database.

Associated database. Associated with the issuance of the Standard will be the construction of a comprehensive and continuously updatable public database of STR profiles based on results subject to agreed-upon interpretation guidelines and quality control parameters. Comparison of STR profiles generated from individual cell stocks to such a database will help reduce the frequency of misidentification of human cells, enhance confidence in results, assure the user's ability to compare scientific results between laboratories, and verify that important data originated from intended samples. STR profiles submitted to the database may, at the request of the user, be verified by staff at the National Institutes of Standards and Technology (NIST). The user also will have the option of submitting STR profiles to the database without verification. The database will indicate which profiles have been verified by NIST. The STR database will be established and maintained under the auspices of the National Center for Biotechnology Information and NIST.

Timeline for completion of the Standard. The Standard, once drafted, will be submitted to the SDO Steering Committee for initial review (Fig. 1). After a nominal 14-d review period, the ASN-0002 workgroup will have a chance to respond to any comments provided by the Steering Committee. At this point, the Standard will be submitted for public review and comment. ANSI notifies the public via its weekly publication "ANSI Standards Action." Concurrent with this, the Standard will be sent to all ATCC SDO members for review and comment. At the end of the 45-d public review and comment period, the workgroup will review and respond to all negative comments, resolve differences, and notify unresolved objectors of their right to appeal, if necessary.

The final document will be submitted for review and approval as an American National Standard by the ANSI.

Once the consensus standard has been approved and published by ANSI, the workgroup will take appropriate actions to raise awareness throughout the biomedical community of the existence of the new standard.

Anticipated Flow and Impact

For newly developed human primary cell cultures and cell lines, including feeder layer-dependent human stem cells and tumor cells propagated through xenografting, an initial STR profile should be established on the earliest material, for example biopsy, frozen tissue, or formalin-fixed paraffin-embedded tissue. For feeder layer-dependent human stem cell preparations, DNA amplification and barcoding (e.g., Cooper et al. 2007), an isoenzyme analysis assay or an alternative species identification method may need to be performed to demonstrate that there are no cross-contaminating mouse feeder cells in the preparation. Additional testing may also be necessary in the case of tumor cell isolation from xenografts to demonstrate that there are no host cells remaining in the recovered tumor cell line.

For existing human cell lines, investigators will be encouraged to: (1) check the public database to see if the cell line is represented within the STR database; (2) perform an STR profile and compare the results to those within the STR database; and (3) ensure that the STR database indicates that this cell line is not misidentified. The Standard will provide the necessary matching criteria.

Continuous human cell lines which are manipulated within laboratories employing non-human cell lines may need to be monitored periodically for non-human cell cross-contamination using one of the cell species identification methods mentioned above (isoenzyme analysis or DNA amplification and barcoding).

To the degree to which the Standard is adopted and complied with, issuance of the Standard will have a significant beneficial impact on the quality and validity of research based upon the use of human cells.

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Check your cultures! A list of cross-contaminated or misidentified cell lines

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Mini Review

Continuous cell lines consist of cultured cells derived from a specific donor and tissue of origin that have acquired the ability to proliferate indefinitely. These cell lines are well-recognized models for the study of health and disease, particularly for cancer. However, there are cautions to be aware of when using continuous cell lines, including the possibility of contamination, in which a foreign cell line or microorganism is introduced without the handler's knowledge. Cross-contamination, in which the contaminant is another cell line, was first recognized in the 1950s but, disturbingly, remains a serious issue today. Many cell lines become cross-contaminated early, so that subsequent experimental work has been performed only on the contaminant, masquerading under a different name. What can be done in response—how can a researcher know if their own cell lines are cross-contaminated? Two practical responses are suggested here. First, it is important to check the literature, looking for previous work on cross-contamination. Some reports may be difficult to find and to make these more accessible, we have compiled a list of known cross-contaminated cell lines. The list currently contains 360 cell lines, drawn from 68 references. Most contaminants arise within the same species, with HeLa still the most frequently encountered (29%, 106/360) among human cell lines, but interspecies contaminants account for a small but substantial minority of cases (9%, 33/360). Second, even if there are no previous publications on cross-contamination for that cell line, it is essential to check the sample itself by performing authentication testing.

Key words: authentication, cell culture, cell lines, cross-contamination, DNA profiling, misidentification

Additional Supporting Information may be found in the online version of this article.

Novelty and Impact: This manuscript reviews the literature relating to cross-contamination of cell lines. Its novelty comes from the inclusion of a list of known cross-contaminated cell lines (over 300 lines named), allowing researchers to check their own cell lines with reference to the article. Recent developments in this field, including methods of authentication testing, are also discussed.

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Cell Lines as Model Systems

Continuous cell lines represent a readily accessible and easily studied resource for research into health and disease. These cell lines have acquired the ability to proliferate indefinitely if grown in the appropriate culture conditions; usually this is a rare event, since the majority of cells even in tumor tissue will cease proliferation after a limited number of cell divisions.[1] However, once established, a continuous cell line can be repeatedly passaged, reliably recovers from cryopreservation and retains many of the properties of its cell type or tissue of origin.[2,3] These advantages make continuous cell lines effective, and widely used, model systems for normal cellular processes and for a variety of disease states.

Cell lines are particularly attractive models for studying malignant disease. The genetic changes in tumor-derived cell lines closely resemble those of the tumors of origin.[4] Moreover, the genetic changes required to establish continuous cell lines from normal cells recapitulate many of the genetic changes occurring in cancer.[5,6] These genetic changes are required to overcome replicative senescence, in which normal cells continue to be metabolically active but are restricted from further division.[1] Cells able to overcome senescence continue

proliferating until their telomeres become so short that the chromosomes undergo fusion-breakage-bridge cycles and the ensuing genomic instability results in culture crisis. Occasionally (at a rate of ~1 in 10^7 cells), an immortalized cell will emerge from crisis and begin to divide again, yielding a continuous cell line.[1] The changes seen throughout this process have many parallels within cancer development, both for malignancy in general and when considering specific tumor types.[7,8]

Despite these advantages, numerous cautions have emerged from the literature regarding appropriate use of cell lines as model systems.[9,10] Even where cultures have been transformed through the introduction of specific genes, cell lines that have passed through replicative senescence and crisis are aneuploid, heteroploid and genotypically and phenotypically unstable, resulting in considerable heterogeneity within the culture.[10] This instability will cause changes in the characteristics of the cell line but a further consequence may result: alterations in a cell line can be accepted by the user as intrinsic to that culture when there is actually extrinsic contamination present.

Cell Line Cross-contamination and Misidentification

Cell lines become contaminated when a foreign cell line or microorganism is introduced without the handler's knowledge. Although we do not wish to minimize the problem of microbial contamination, we will focus on cell line cross-contamination in this article. Cross-contamination may arise due to several causes, including poor technique (spread *via* aerosols or accidental contact), use of unplugged pipets, sharing media and reagents among cell lines and use of mitotically inactivated feeder layers or conditioned medium, which may carry contaminating cells if not properly eliminated, for example, by freeze-thaw and filtration.[11] In addition, a cell line can be replaced by another as a result of misidentification by confusing cultures during handling, mislabeling or poor freezer inventory control. Simple errors during labeling of culture flasks, truncation of the cell line name or typographic errors in a published manuscript, can result in significant confusion for years after the event when another researcher attempts to use the same cell line for ongoing experimental work.[12]

Cross-contamination may occur "early," in which case the original cell line has probably never existed independently, or "late," where the tested sample has been overgrown but other stocks of the original may still exist.[13] Unfortunately, cell lines generally become cross-contaminated early, while still within the originating laboratory.[14] This is not surprising: cultures can remain in crisis for a prolonged period of time before emergence of an immortalized population and this is a time when a single cell, if introduced from a separate cell line, would rapidly take over the culture.

There are now a number of studies pointing out the severity of this problem and the need to take urgent action to minimize cross-contamination and its consequences.[9,15–17] Ten years ago, the German Collection of Microorganisms and Cell Cultures (DSMZ) published data from its identification testing of cancer cell lines submitted by various laboratories for deposit at the cell bank.[14] They found that 18% of 252 submitted cell lines were cross-contaminated with more than half of cases arising within only 6 laboratories. Subsequent work by the DSMZ, extending the number of cell lines tested (Fig. 1), shows that of 598 leukemia–lymphoma cell lines (the group provided with the most complete genetic data), 187 (31%) were contaminated with Mycoplasma and/or a second cell line with 38 (6%) of cell lines contaminated with both. These data suggest that poor practice within some laboratories results in contamination of multiple cell lines with multiple contaminants, which can then be disseminated more widely if these cultures are used by others.

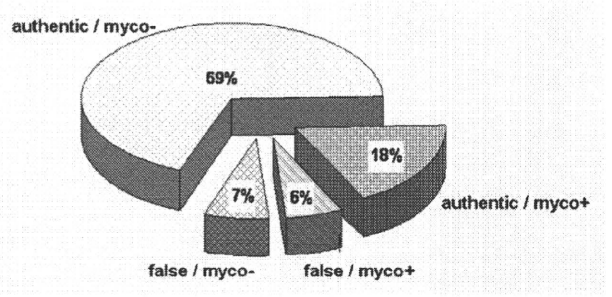


Figure 1. Rates of contamination for leukemia–lymphoma cell lines. Percentages of cross-contaminated and Mycoplasma-contaminated cell lines from a dataset of 598 leukemia and lymphoma cell lines analyzed by the German cell line bank DSMZ. "False/authentic" refers to the presence or absence of cross-contamination; "myco+/myco−" refers to the presence or absence of Mycoplasma contamination. Cell lines fall into the following categories: authentic/myco− (n = 411, 69%); authentic/myco+ (n = 108, 18%); false/myco− (n = 41, 7%) and false/myco+ (n = 38, 6%). (Courtesy of Hans Drexler, DSMZ.)

Other studies have pointed out that testing of cell lines is often infrequent, resulting in the failure to detect contaminated samples. John Ryan of Corning Life Sciences conducted surveys of seminar attendees in 1990, asking about Mycoplasma contamination; 50% were not currently performing testing and only 18% said they tested their cultures regularly. Almost 1 in 4 respondents (23%) had experienced Mycoplasma contamination, but with such a low level of testing, it is likely that the real figure was much higher.[18] Other data on cross-contamination were published in 2004 by researchers at the University of California, Berkeley, where Walter Nelson-Rees worked on this problem in the 1970s, focusing on the HeLa cell line.[19] Of 483 respondents to a questionnaire on cell line usage, 35% were using cell lines obtained from another laboratory rather than a cell line repository, but almost half of all respondents performed no testing for cross-contamination.[20]

A practical example of the consequences of cell line contamination can be found in a recent study published by Berglind *et al.*[21] The authors analyzed data within the UMD_p53 (2007) database, which includes information on the p53 status of 1,211 cell lines. Discrepancies were found in p53 status for 23% (88/384) of cell lines where data have been published by 2

independent laboratories. It is likely that many of these discrepancies arose due to work with cross-contaminated samples; the authors noted that many groups rely on previously published reports of a cell line's p53 status,[21] resulting in further confusion when interpreting results from these cell lines.

Cell banks have the expertise to detect such cross-contamination, and have been proactive in publishing reports of cross-contaminated cell lines,[22,23] in publishing test results online[24] and in developing new detection methods.[25–27] Unfortunately, however, cell banks have also reported reluctance from many researchers to deposit cell lines for distribution.[28] Such repositories specialize in the detection of cross-contamination and it is unlikely that most laboratories have comparable resources in this regard. In addition, many researchers obtain cell lines from one another, rather than approaching the originator or purchasing the cell line from a cell bank performing quality control testing. This may be faster or cheaper than obtaining cultures from a reputable source but the practice makes contamination more prevalent and harder to detect.

Practical Responses

Having defined the problems, it is time to focus on what can be done. Several cancer-related journals, including the International Journal of Cancer, have recently responded to these issues by changing their policies to require evidence of authentication with all submitted manuscripts using continuous cell lines.[29,30] Their response underscores the need for laboratories to come to grips with cell line cross-contamination and misidentification. Every researcher involved in cell culture will have cell lines currently in culture, stored in liquid nitrogen or may be commencing work on a new cell line. Put practically, how can you know if your cell lines are cross-contaminated?

There are 2 important answers to this question:

1. Check the literature, for example, by searching the PubMed database using the cell line name and "cross-contamination."
2. Check your cultured cells. Unless a cell line has come directly from a repository or other laboratory performing identification testing, it should be tested on arrival, and all cultures should be periodically tested while in use, before cryopreservation and when thawed from liquid nitrogen.[31] A variety of methods are available for authentication; for human cell lines, short tandem repeat (STR) profiling is the current international reference standard and is recommended as an easy and economical way to confirm cell line identity by comparison to donor tissue or to other samples of the cell line held by laboratories worldwide.[26]

Checking the Literature: A List of Cross-Contaminated Cell Lines

A 2004 survey of abstracts within the PubMed database would suggest that inappropriate usage of cross-contaminated cell lines is increasing,[20] despite many years of publication on this issue. It is possible that many researchers simply cannot find existing references to cross-contamination so, to make this already published work more accessible, we have surveyed the literature and other online resources for references to cell line contamination. The resulting list of cross-contaminated cell lines is included as Electronic Supporting Information.

To generate this list, the authors examined the PubMed database, references within other articles relating to this topic and the websites of 5 cell banks: the American Type Culture Collection (ATCC), DSMZ, European Collection of Cell Cultures (ECACC), Japanese Collection of Research Bioresources and the RIKEN Bioresource Center Cell Bank. A Wikipedia list of contaminated cell lines was also accessed (http://en.wikipedia.org/wiki/List_of_contaminated_cell_lines). Cross-contaminated cell lines are listed by name along with their species and cell type (both claimed and actual), the name of the contaminating cell line where identified, the reference in which this was reported and the PubMed ID number where available. Notes are also included for some cell lines. The list is made available in Excel spreadsheet or PDF format for easy accessibility.

The cell lines listed within this database are divided into 2 tables. Supporting Information Table 1 contains those cell lines where cross-contamination occurred as an early event, and thus where there is no original material remaining. Supporting Information Table 2 contains those cell lines where it is thought cross-contamination occurred as a late event and where original stocks may still exist. A full list of references is also given.

The current list of cross-contaminated cell lines (version 6.4) contains 360 cell lines, 346 in Supporting Information Table 1 and 14 in Supporting Information Table 2, drawn from 68 references. Cell lines affected are primarily human, although cultures from at least 8 other species are included, and come from a wide spectrum of tissue types. The cell or tumor type is given within the list where known; extensive work has been done by some cell banks and laboratories in this area to characterize the actual cell type or tumor type.[22,32] In some cases, this work has shown that a cell line carries the correct name but its cell or tumor type has been incorrectly identified, for example, the cell line RPMI-6666 was initially thought to have come from Hodgkin lymphoma but is now known to be an EBV-positive B-lymphoblastoid cell line.[22]

Common features for cross-contaminating cell lines within the current list are summarized in Table 1. It can be seen that most cross-contamination events have arisen from within the same species but a substantial minority (9%, 33/360) involved cross-contamination from a second species. For the intraspecies contaminants, all of those detected were human but it is likely that this relates to the difficulty of detecting intraspecies contaminants for nonhuman species. The commonest contaminant remains the HeLa cell line

Table 1. Cross-contaminating cell lines

Type of contaminant	Number of cell lines affected
Intraspecies	
Human	324
Nonhuman	0
Interspecies	33
Correct name—incorrect cell type (misidentified)[1]	3
Total	360
Contaminating cell line—12 most frequent	**Number of cell lines affected**
HeLa (human cervical adenocarcinoma)	106
T-24 (human bladder carcinoma)	18
HT-29 (human colon carcinoma)	12
CCRF-CEM (human acute lymphoblastic leukemia)	9
K-562 (human chronic myeloid leukemia)	9
U-937 (human lymphoma)	8
OCI/AML2 (human acute myeloid leukemia)	8
Hcu-10 (human esophageal carcinoma)[2]	7
M14 (human melanoma)	7
HL-60 (human acute myeloid leukemia)	6
PC3 (human prostate carcinoma)	6
SW-480, SW620 (human colon carcinoma)[3]	6

[1]For additional misidentified cell lines see Drexler *et al.*[22] [2]Hcu-10 carries the same genetic identity as Hcu-18, Hcu-22, Hcu-27, Hcu-33, Hcu-37 and Hcu-39; it is unclear which is the correct identity (see Electronic Supporting Information for reference). [3]SW480 and SW620 come from the same donor and therefore carry the same genetic identity (see Electronic Supporting Information for reference).

(29%, 106/360), followed by T-24 (5%, 18/360) and HT-29 (3%, 12/360).

It is important for such a list to be continually updated and feedback is welcome for this purpose. An earlier version of the database was released online by ECACC[31]; 6 cell banks have now agreed to make the database available online and to update this information where necessary. Current website addresses for access to the list of cross-contaminated cell lines are given in Table 2. In future, it is envisaged that the current list of misidentified cell lines will be included in a new initiative improving access to authentication data. The Standard Development Organization at the ATCC is in the process of producing an international standard for human cell line identification based on STR profiling (ATCC SDO Workgroup ASN-0002, manuscript submitted). Strict criteria for STR profiles derived from cancer cell lines are being developed. One consequence of this initiative is that funding is being sought for a quality controlled and curated cell line database with free access into which the database described here will be incorporated.

Table 2. Websites for ongoing access to the list of cross-contaminated cell lines

Cell bank	Website address
ATCC	http://www.atcc.org/
CellBank Australia	http://www.cellbankaustralia.com/
DSMZ	http://www.dsmz.de/
ECACC	http://www.hpacultures.org.uk/collections/ecacc.jsp
JCRB	http://cellbank.nibio.go.jp/
RIKEN Bioresource Center Cell Bank	http://www.brc.riken.go.jp/lab/cell/english/guide.shtml

Checking Your Cultures: Authentication of Cell Lines

Even if a search of the literature shows no indication that a cell line is contaminated, it is still essential to test the sample that you are working with. Authentication testing should be considered in a positive light, as an essential part of good cell culture practice[33] and as an assurance for researchers, funding bodies and journals that the cell line used is a valid experimental model.[17]

There are a number of methods for testing cell line identity. When the issue of cross-contamination was first identified, HeLa contaminants were detected through a combination of isoenzyme and chromosomal analysis.[19,34] Both techniques continue to be used but there are also many newer molecular approaches. Commonly used authentication methods are summarized in Table 3; what factors should be considered when choosing between these methods?

The expertise of the laboratory holding the cell line is an important factor. For example, laboratories with experience in cytogenetics would have the skills to identify species through karyotype analysis and cell lines through the presence or absence of appropriate markers.[35] Although this is an older approach, it still allows clear identification of cell lines, and many cell banks have published karyotypic information on their cell lines to allow comparison to well-characterized stocks. It should be noted that tumor-derived cell lines can be surprisingly difficult to harvest for cytogenetic analysis[35] and are typically heteroploid making interpretation difficult: the experience of the operator is important for success.

The species of cell lines held within the laboratory is also important. Although some authentication methods can be used on more than 1 species, molecular methods such as STR profiling are only successful for a single species; other species will simply fail to amplify.[26] This may not be an issue for laboratories working only with human samples but clearly is a significant factor for groups working with rodent cell lines. In this regard, multilocus DNA fingerprint analysis has a clear advantage, since probes are able to hybridize to a wide variety of species.[25] Unfortunately, although successful within a single laboratory, it can be challenging to compare DNA fingerprints across several experimental runs, and it is difficult to exchange data among laboratories or for cell

Table 3. Commonly used methods for authenticating cell lines

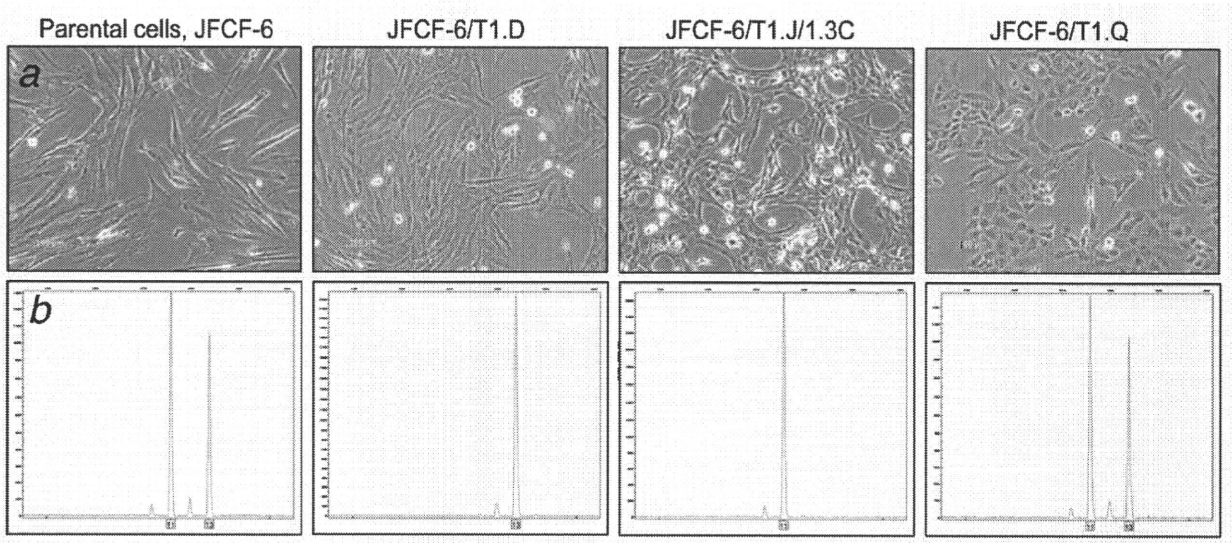
Name	Description	Purpose	References
Chromosomal analysis/karyotyping	Involves preparation of a metaphase spread with chromosome banding and painting to identify chromosome number and markers	Separates species, plus individual cell lines if detailed analysis performed	Ref. 35
Isoenzyme analysis	Biochemical method separating isoenzymes by electrophoresis; isoenzyme mobility may vary within or across species. Kits available include the Authentikit gel electrophoresis system	Separates species, sometimes individuals	Refs. 36,37
Multilocus DNA fingerprint analysis	Molecular method detecting variation in length within minisatellite DNA containing variable numbers of tandem repeat sequences. Analysis is by Southern blot hybridization using probes 33.6 and 33.15, M13 phage DNA, or oligonucleotide sequence	Separates individual cell lines across multiple species	Refs. 25,38
Short tandem repeat (STR) profiling	Molecular method detecting variation in length within microsatellite DNA containing variable numbers of short tandem repeat sequences. Analysis is by PCR with comparison to set size standards; usually available in a kit format allowing amplification of up to 16 loci	Separates individual cell lines within a single species	Refs. 26,39
Polymerase chain reaction (PCR) fragment analysis	Molecular method involving amplification of specific genes or gene families, aiming to detect variations in exon/intron sequence, transcript splicing, or the presence of pseudogenes. Genes examined include the aldolase gene family and the beta-globin gene	Separates species only	Refs. 40,41
Sequencing of "DNA barcode" regions	Involves sequencing of a DNA fragment from the mitochondrial gene cytochrome *c* oxidase subunit I, with comparison to sequence obtained from online databases. This "DNA barcode" has been shown in practice to distinguish a broad range of animal species	Separates species only	Refs. 27,42

banks to publish such fingerprints online. It is advisable to always compare the test sample to a known sample within the same experiment, ideally using DNA from the blood or tissue of the original donor.

The obvious advantage of STR profiling lies in the use of control samples to generate a numerical code for each sample, which precisely identifies that cell line and which can be readily shared and published online. It is primarily for this reason that STR profiling is recommended as an international reference standard for human cell lines[26] and accepted within the legal system for human identity testing.[39] STR profiling is based on the presence of STRs within the human genome that exist at variable lengths throughout the population. Each of the repeat regions to be analyzed (usually tetra or pentanucleotide repeats in noncoding sequence) is amplified by PCR using primers carrying fluorescent tags and electrophoresed in a sequencing gel; the precise length of each allele is determined and compared with size standards and controls. This allows identification software to assign a number to each allele at that locus (see, *e.g.*, Fig. 2). The combination of multiple loci—classically 13, as used in the FBI Laboratory's Combined DNA Index System (CODIS)—gives sufficient data to uniquely identify that individual.

STR profiles for individual cell lines and panels have now been reported by many laboratories (*e.g.*, Ref. 44) and are published online by several cell banks. However, there are some cautions to be aware of when using this approach. It is accepted within the forensic field that tumor samples are not as genetically stable as other tissue sources for STR profiling, because of loss of heterozygosity and microsatellite instability.[45,46] This is even more evident in tumor-derived cell lines, where evolution or genetic drift continues to occur with passage.[47] When searching an online database of STR profiles from cell lines, the user needs to look for close matches and not just identical matches; most studies would agree that 80% similarity is an appropriate threshold for declaring a match when comparing cell line profiles.[26,44] There may also be a significant start-up cost if testing in-house; in addition to an STR kit, access to methods for DNA extraction, precise quantitation, fragment analysis and software for STR profile identification is required.

The fact that STR profiling is only suitable for distinguishing cell lines of a single species has led to the need to re-examine authentication of nonhuman cell lines. Laboratory rodent samples will always be difficult to identify precisely due to inbreeding; laboratories working with rat or mouse cultures may wish to examine strain identity rather than authentication of individual cell lines, particularly if they have expertise in single nucleotide polymorphism (SNP) or single sequence length polymorphism (SSLP) analysis,



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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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Database of misidentified cell lines

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Dear Sir,

Much of current cancer and cell biology research depends on the use of cell lines cultured from normal and malignant tissue. However, ever since the time when continuous cell lines were first established, there has been a problem of the more vigorous lines contaminating and overgrowing more slowly growing cultures. This has been compounded by confusion of one cell line with another by mislabeling in routine culture or during and after cryopreservation. The result is that some 15–20% of cell lines in current use may not be what they are claimed to be. This has prompted a number of recent reports in the literature[1–7] and discussions at scientific meetings. One of the main conclusions is that there needs to be a way to alert scientists using established and frequently propagated cell lines that there is a significant risk that they may be using cell lines which are not what they need them to be. This issue of International Journal of Cancer will address this problem and wants to increase the awareness of authors submitting their work for publication and of reviewers considering the merit of the work. Restrictions and conditions will be imposed regarding proof of authentication of cell lines used and advice given on how to authenticate cell lines (see editorial and letter by W. Dirks). My purpose in this letter is to notify the scientific community of the existence and free availability of a list of cell lines which are known or suspected to be falsely identified or cross contaminated. This will allow scientists embarking on a project or reviewers considering the work for publication, to have access to a data source which will advise them on the respective cell line's authenticity. This list is available for download from: http://www.hpacultures.org.uk/services/celllineidentityverification/misidentifiedcelllines.jsp by following the link after my and Amanda Capes-Davis's names. It has been compiled from quality assurance carried out by a number of cell banks (ATCC, CellBank Australia, sDSMZ, ECACC, JCRB, and RIKEN) and published on their websites, from an entry in Wikipedia, and from reports in the scientific literature. It must be emphasized that while many of the cell lines listed are clearly and incontrovertibly not what they are supposed to be, original and authentic stocks of other lines may yet exist. Where this is believed to be the case the line is included in the second table. This list will be published (Capes-Davis *et al.*, ms in preparation).

I would request that anyone who uses this list and finds that some misidentified cell lines have been omitted or that some cell lines reported as misidentified do have authentic stocks available should contact me (i.freshney@ntlworld.com), and I will arrange to have the database updated.

The recommended procedure for anyone contemplating the use of cell lines is as follows:

- Check that the cell line that you intend to use is not listed in the above database.
- Ensure that the cell line is obtained from a properly authenticated source (and that may not be the originator), preferably from one of the recognized cell banks.
- Authenticate cell lines received from a nonauthenticated source on receipt (see letter of W. Dirks, this issue, and instruction for authors of IJC).
- Repeat authentication at intervals of 3–6 months for cell lines used for an extended study, before cryopreservation, and after thawing for further use.

It may not be possible to eliminate misidentification entirely, as new examples will continue to appear, but following these precautions should reduce the frequency and minimize the spread of the problem.

Yours sincerely,
R. Ian Freshney

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Cell line cross-contamination initiative: an interactive reference database of STR profiles covering common cancer cell lines

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[2] RIKEN—Cell Engineering Division, Tsukuba, Japan
[3] JCRB—Japanese Collection of Research Bioresources, Osaka, Japan
[4] ATCC—American Type Culture Collections, Manassas, VA

Dear Sir,

Recent reports[1–4] demonstrate the growing perception in the scientific community that cross contamination (CC) of mammalian cell lines represents a major risk for generating false scientific data. The level to which research has been compromised by the use of contaminated or misidentified cell lines has become a major concern for scientists, granting agencies, and, increasingly, scientific journals. In 2007, a group of cell biologists led by Roland M. Nardone petitioned the United States Secretary of Health and Human Services to develop an active program for cell line authentication.[5] They stressed that research and teaching tools in diverse fields of science and industry would be unimaginable without cell cultures. Despite the key importance of cell cultures, only little consensus exists regarding the technical means by which cell line identity can be controlled and how to follow through the results of any such testing.

The key problems of CC are known and chronic in nature: neglecting guidelines for quality control and disregarding adequate cell culture techniques are the main reasons why cell lines have been misidentified or cross contaminated. The incidence of CC in directly and indirectly provenanced cell lines alike[1,3] implies that the majority of false cell lines are perpetrated in originators' own laboratories, presumably by failures during the establishment of new cell lines. A plethora of reports unmasking bogus cancer cell lines, including members of the NCI-60 panel used to generate reference baseline transcriptional drug responses has triggered calls for remedial action.[5,6] Nevertheless, standard authentication procedures for testing cell line identity have yet to be defined.

Short tandem repeat (STR) microsatellite sequences are highly polymorphic in human populations, and their stability throughout the lifespan of individuals renders STR profiling (typing) ideal for forensic use. STR typing has served as a reference technique for identity control of human cell lines at Biological Resource Centers (BRCs) since the turn of the millennium.[7] Ideally, authentication involves coincident STR typing of paired donor and derived cell line samples. However, this ideal is met by a few recently established cell lines only. Most widely used cell lines are decades old and their identification is largely retrospective and multidisciplinary, combining diverse criteria such as uniqueness and the congruence of STR profiles across independent samples with the consistency of observed karyotypes with those reported by the originators.

The DSMZ as well as the ATCC, JCRB, and RIKEN repositories have generated large databases of STR cell line profiles. By using the same microsatellite loci at these BRCs, individual databases could be merged, thereby facilitating interactive searches. This work was piloted at the DSMZ to generate an international reference STR profile database for human cell lines. To render it user friendly, a simple search engine for interrogating STR cell line profiles has now been made available on the homepages of JCRB and DSMZ (http://cellbank.nibio.go.jp/cellbank_e.html, http://www.dsmz.de/STRanalysis). Registered users simply login at the search-site on the DSMZ homepage and will be guided. Aided by simple prompts, users can input their own cell line STR data to retrieve best matches with authenticated cell lines listed on the database.

Once the problem of false negatives due to discrepant representation of single STR alleles, *e.g.*, by losses of heterozygosity and bottlenecking selection—has been tackled and unambiguous search results are produced, human cell lines will need to be consistent with consensus STR reference data sets. STR profiles of all human cell lines distributed by DSMZ, JCRB, and RIKEN and one-third of the cell lines distributed by ATCC are now publicly accessible on interactive databases where match criteria have been arbitrarily set to 95%. Inevitably, reference profiles remain subject to revision until all commonly held cell lines have been STR typed across participating repositories. At present, about 2,342 such cell lines have been STR typed and are represented as reference sets on the database.

The authors of this article are currently participating in an international workgroup organized by the ATCC Standards Development Organization, (ATCC SDO) to develop a standardized methodology (protocols and procedures for STR analysis) for authenticating human cell lines. An additional

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goal of the workgroup is to establish a global database for STR profiles of human cell lines. The development of the consensus standard offers a new tool to the cell biology community that will foster reproducibility and comparability of cell lines used in different laboratories. Armed with these tools, online verification of cell line identity should prove a vital weapon to combat the havoc of cell line cross contamination which has dogged cancer research since inception.

Yours sincerely,
Wilhelm G. Dirks
Roderick A. F. MacLeod
Yukio Nakamura
Arihiro Kohara
Yvonne Reid
Herbert Milch
Hans G. Drexler
Hiroshi Mizusawa

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Growth factor-defined culture medium for human mesenchymal stem cells

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ABSTRACT Human bone marrow-derived mesenchymal stem cells (hMSCs) are potential cellular sources of therapeutic stem cells as they have the ability to proliferate and differentiate into a wide array of mesenchymal cell types such as osteoblasts, chondroblasts and adipocytes. hMSCs have been used clinically to treat patients with graft *vs.* host disease, osteogenesis imperfect, or alveolar cleft, suggesting that transplantation of hMSCs is comparatively safe as a stem cell-based therapy. However, conventional culture medium for hMSCs contains fetal bovine serum (FBS). In the present study, we developed a growth factor-defined, serum-free medium for culturing hMSCs. Under these conditions, TGF-β1 promoted proliferation of hMSCs. The expanded hMSC population expressed the human pluripotency markers SSEA-3, -4, *NANOG*, *OCT3/4* and *SOX2*. Furthermore, double positive cells for SSEA-3 and a mesenchymal cell marker, CD105, were detected in the population. The potential to differentiate into osteoblasts and adipocytes was confirmed. This work provides a useful tool to understand the basic biological properties of hMSCs in culture.

KEY WORDS: *mesenchymal stem cell, serum-free culture, TGF-β1*

Introduction

Bone marrow-derived cells can differentiate into osteoblasts *in vitro* and *in vivo* (Friedenstein *et al.*, 1966) and thus are considered a useful source of stem cells for bone regeneration. Recently, many studies have reported that human bone marrow contains a distinct cell fraction referred to as multipotent mesenchymal stem cells (hMSCs) which can give rise to a wide array of mesenchymal cell types, including bone, fat, and cartilage"(Pittenger *et al.*, 1999). However, hMSCs can differentiate along some ectodermal and endodermal cell lineages such as neuronal cells and liver cells (Pittenger *et al.*, 1999; Dezawa *et al.*, 2004; Dezawa *et al.*, 2005). Further, a recent study reported that hMSCs have the ability to generate the multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). It has been estimated that hMSCs comprise about 0.001 to 0.01% of total bone marrow mononuclear cells (Pittenger *et al.*, 1999). For use in cell-based therapies, hMSC populations require extensive *in vitro* expansion to obtain sufficient numbers. The conventional culture medium for hMSCs is composed of a basal nutrient medium supplemented with fetal bovine serum (FBS) (Haynesworth *et al.*, 1992; Lennon DP, 1996). Although these traditional culture conditions provide robust undifferentiated hMSC expansion, the ill-defined components of FBS is undesirable for clinical applications and also hampers analysis of the cell biological mechanisms that control cell behavior.

Abbreviations used in this paper: hES cells, human embryonic stem cells; hMSCs, human mesenchymal stem cells.

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We and others previously described serum-free media consisting of minimum essential components suitable to propagate and accurately analyze the characteristics of differentiated cells (Hayashi and Sato, 1976; Furue and Saito, 1998; Sato *et al.*, 2002; Furue *et al.*, 2005; Furue *et al.*, 2008; Hayashi *et al.*, 2010). One of these media, hESF9, supports the serial cultivation of undifferentiated human embryonic stem (hES) cells in the absence of feeder cells and thus provides an experimental system for elucidating cellular responses to specific environmental stimuli (Furue *et al.*, 2008; Na *et al.*, 2010). For example, either FGF-2 or heparin promotes proliferation of hES cells in a concentration-dependent manner although these effects were not detected under conventional culture conditions. Thus, a defined serum-free medium consisting of minimum essential components should be useful in elucidating hES/iPS cell responses to specific cues that control self-renewal, differentiation, and lineage selection (Furue *et al.*, 2010).

Because hMSCs have multipotent properties similar to hES cells, we speculated that hMSCs should be able to grow in similar culture conditions as hES cells. In the present study, we demonstrated that addition of TGF-β1 to the defined serum-free medium for hES cells supports the robust proliferation of hMSCs. The hMSC population expanded in the absence of serum expressed the mesenchymal cell markers CD44, CD73, CD90, and CD105. Further, they expressed human pluripotency surface markers, SSEA-3, -4, TRA-2-54, and also the transcription factors of *NANOG*, *OCT3/4*, and *SOX2*. We show that the serum-free expanded hMSCs can differentiate into osteoblasts and adipocytes. This work sets the stage for serum-free hMSC cell culture and thereby provides a useful tool to understand the basic biological characteristics of hMSCs.

Results

In this study we used a human bone marrow-derived hMSC line designated UE7T-13 (JCRB 1154). The life span of these cells was prolonged by infecting them with a retrovirus containing human papillomavirus E7 and telomerase reverse transcriptase (hTERT) cDNAs (Mori *et al.*, 2005; Shimomura *et al.*, 2007; Ishii *et al.*, 2008; Takeuchi *et al.*, 2007). We first tested the ability of hESF9 medium, which we had developed for use with hES cells, to support the growth of UE7T-13 cells. The cells were harvested using trypsin/EDTA, from cultures in conventional medium containing 10% FBS (POWERDBY10) and transferred to 0.1% gelatin-coated dishes in hESF9 medium. However, UE7T-13 cell growth was quite slow. We then investigated the effects of various growth factors on proliferation of the cells. UE7T-13 cells were seeded on 0.1% gelatin in hESF9 in the absence of FGF-2 and heparin (hESF9(-/-)), containing increasing concentrations of FGF-1, FGF-2, TGF-β1, activin A, or leukemia inhibitory factor (LIF) (Fig. 1). Both FGF-1 and FGF-2 promoted UE7T-13 proliferation in a dose-dependent manner, and the greatest effect was seen at 10 ng/ml FGF-2. Neither LIF nor activin A affected on UE7T-13 cell proliferation, but TGF-β1 slightly stimulated UE7T-13 proliferation. Next all five factors (FGF-1, FGF-2, TGF-β1, activin A, and LIF) or four factors with increasing concentrations of heparin were added to UE7T-13 cultures (Fig. 2). When either FGF-2 or TGF-β1 was withdrawn from the cultures, the cell numbers decreased significantly. Heparin promoted cell proliferation in a dose-dependent manner. This result suggested that addition of FGF-2 and TGF-β1 to hESF9(-/-) medium, is critical for UE7T-13 proliferation, and heparin also enhanced cell growth. hESF9 medium supplemented with TGF-β1 was designated hESF10.

L-ascorbic acid-2-phosphate (Asc 2-P) in hESF9 medium supported hES cells. However, it is known to promote hMSC cell differentiation into osteoblasts. Therefore, we examined whether the presence of Asc 2-P in hESF10 medium promoted osteoblastic differentiation of UE7T-13 cells. We analyzed the expression of *bone sialoprotein* (*IBSP*), *osteocalcin* (*BGP*), *osteonectin* (*SPOCK2*), and *osteopontin* (*SPP1*) in UE7T-13 cell cultured in hESF10 with or without Asc 2-P and in conventional medium (Fig. 2). These osteoblast genes were expressed at significantly lower levels in cells cultured in the serum-free media than in those cultured in the conventional medium. These results suggest that the serum-free medium is suitable for hMSC maintenance. *IBSP* gene expression was higher in the cells cultured in the

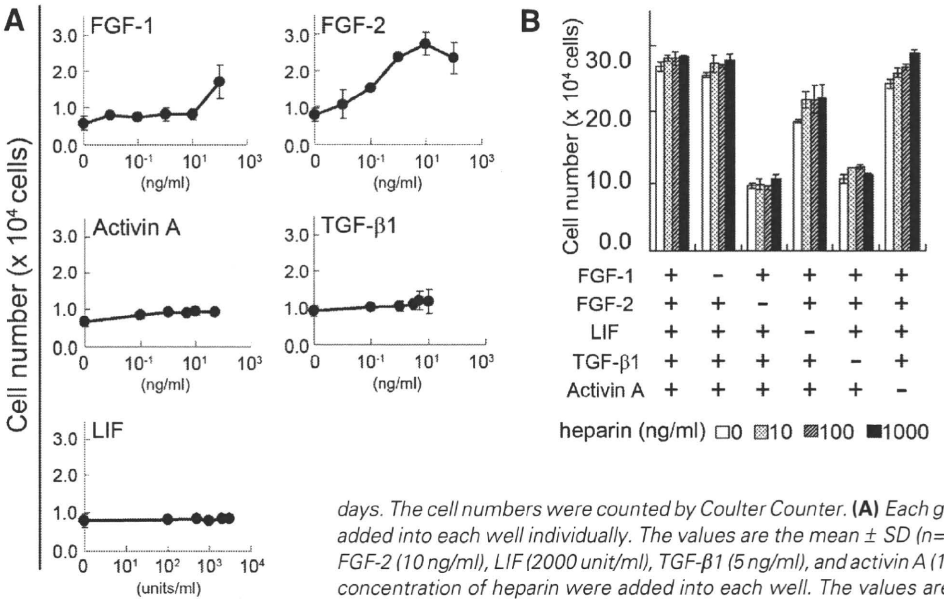

Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. *After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-) at 1 x10⁴ cells per well and cultured for 6 days. The cell numbers were counted by Coulter Counter.* **(A)** *Each growth factor at indicated concentration was added into each well individually. The values are the mean ± SD (n=3).* **(B)** *All five factors of FGF-1 (100 ng/ml), FGF-2 (10 ng/ml), LIF (2000 unit/ml), TGF-β1 (5 ng/ml), and activin A (10 ng/ml) or four factors of them with varying concentration of heparin were added into each well. The values are the mean ± SD (n=3).*

presence of Asc 2-P. These results suggested that Asc 2-P promoted differentiation of UE7T-13 cells into osteoblasts. We removed Asc 2-P from hESF10 medium for hMSCs, and designated the new formulation D-hESF10.

To confirm the characteristics of UE7T-13 cells expanded in the absence of serum, we performed flow cytometry with antibodies to markers for hMSCs and pluripotent cells (Fig. 3A). Cells grown in D-hESF10 medium were positive for CD44, CD73, CD90, CD105, and TRA-2-54 (tissue non-specific alkaline phosphatase antibody), but negative for CD45 (a marker of all hematopoietic cells) and CD56 (a neural cell adhesion molecule). We further stained the cells with antibodies to CD105 and SSEA-3 (Fig. 3B). The immunocytochemical analysis showed that SSEA-3$^+$/CD105$^+$ double positive cells were present in the UE7T-13 population grown in D-hESF10 although cells positive for either CD105 or SSEA-3 were also detected in the population. The cell growth rate in D-hESF10 was comparable to that in conventional culture conditions (Fig. 4).

We subsequently examined the properties of UE7T-13 cells serially passaged in D-hESF10 medium. The morphology of serum-free expanded UE7T-13 cell populations was comparably small, spindle-shaped cells compared with that in conventional medium (Fig. 5A). The expression of hMSC and hES cell pluripotency markers were determined by real-time PCR analysis (Fig. 5B) in UE7T-13 cells cultured for 4 passages in D-hESF10 medium. The expression of hMSC markers, *CD105*, *THY1*, and *integrinβ1* (*ITGB1*), and the hES cell pluripotency markers, *OCT3/4* (*POU5F1*) and *NANOG* were similar in the cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. *SOX2* expression was significantly higher in cells cultured in D-hESF10 compared with cells cultured in conventional culture conditions. On the other hand, the expression levels of *IBSP*, *BGP*, *SPOCK2*, and *SPP1* were significantly lower in cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. These results suggest that serum-free expanded UE7T-13 cells retain an undifferentiated phenotype.

We determined the differentiation capacity of the serum-free expanded UE7T-13 cells. After the UE7T-13 cells were cultured in D-hESF10 for 7 passages, the cells were cultured in medium designed to induce differentiation into osteoblasts or adipocytes (Fig. 6). Culturing in osteoblastic differentiation medium induced the formation of nodules that stained positive with Alizarin red, suggesting that the cells had the potential to differentiate into osteoblasts. When the cells were cultured in

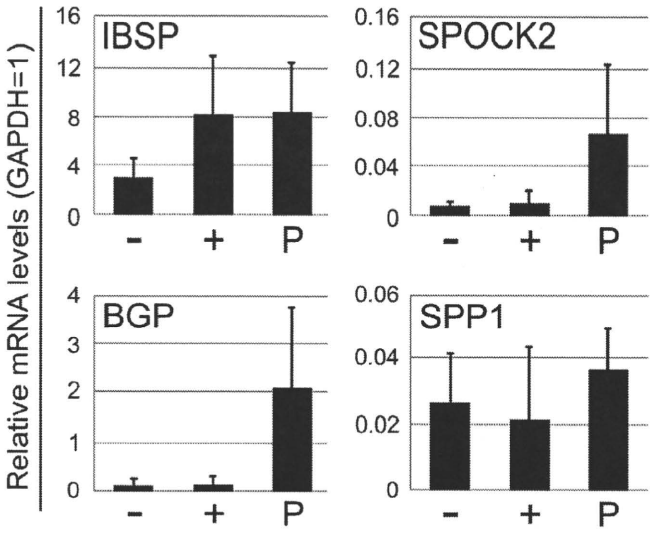

Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. *The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression was normalized by the amount of* GAPDH. *The values are the mean ± SD (n=3).*

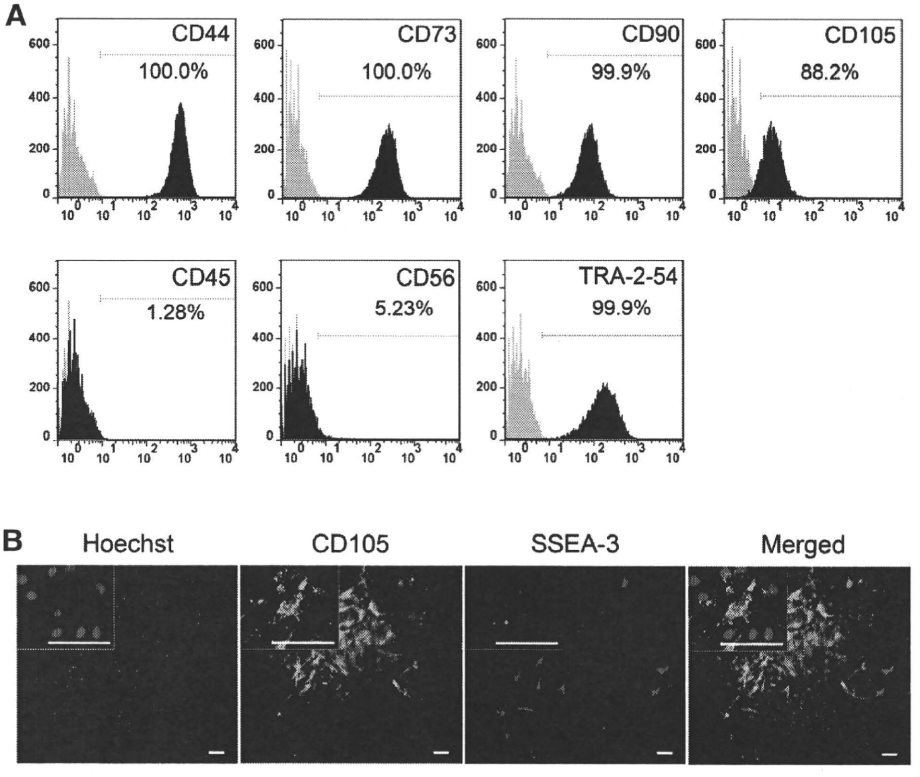

Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) *Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells.* **(B)** *Immunocytochemical analysis of SSEA-3 and CD105 expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days. Scale bars, 100 μm.*

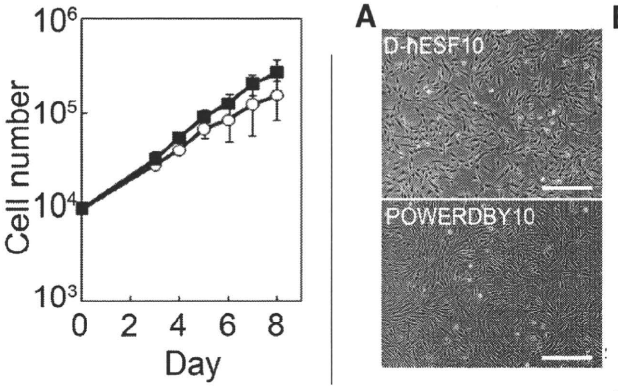

Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. *The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density of 1x10^4 cells per well. Cell numbers were counted every day. The values are the mean ± SD (n=3).*

adipocytic differentiation medium, Oil red O-positive cells appeared. Taken together these results suggest that the serum-free expanded UE7T-13 cells have maintained the capacity to differentiate into osteoblasts or adipocytes.

Discussion

Developing clinical serum-free media for maintaining and expanding human stem cells is a major research topic in regenerative medicine. Our current results indicate that it is possible to culture hMSCs on gelatin in a defined medium, designated D-hESF10, in which human recombinant insulin, human transferrin, a low concentration of fatty acid-free bovine albumin conjugated with oleic acid, FGF-2, and TGF-β1 are the protein components. The basal medium ESF was developed for mouse ES cells (Furue *et al.*, 2005). For hES cell culturing, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) was removed from ESF but Asc 2-P was added (Furue *et al.*, 2008). For propagating hMSCs, Asc 2-P was removed from the hES cell culture medium because we found that Asc 2-P increased osteoblastic marker expression in hMSCs. These findings indicated that signaling by Asc 2-P in hMSCs is different from that in hES cells.

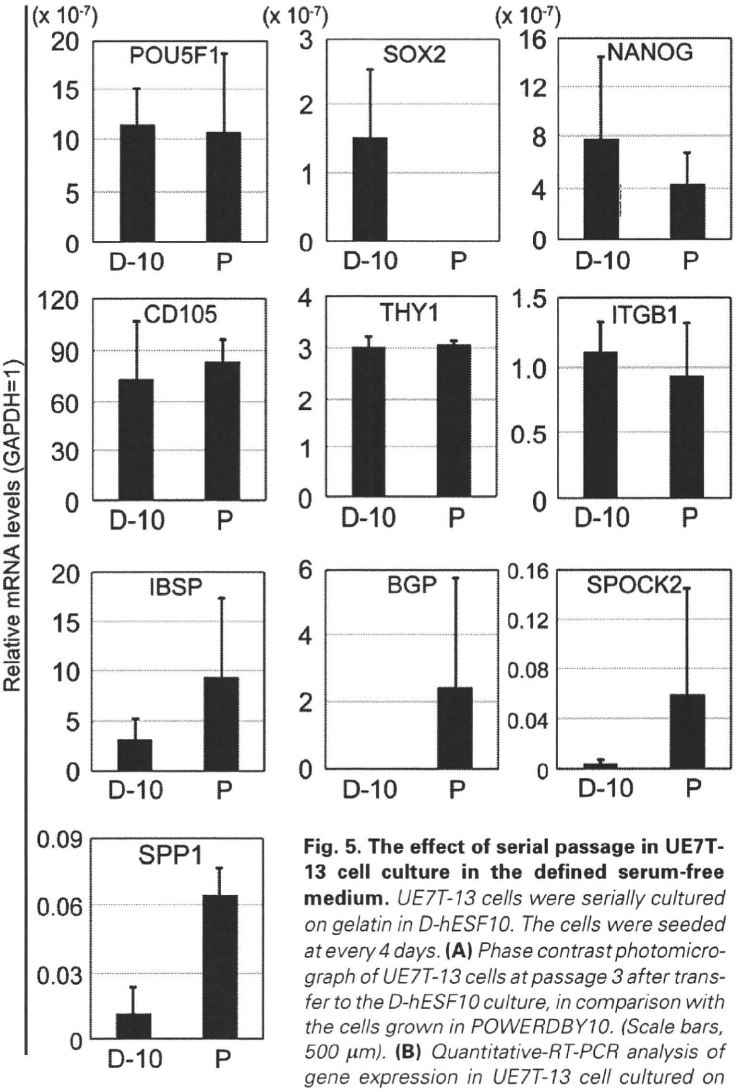

Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. *UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days.* **(A)** *Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture, in comparison with the cells grown in POWERDBY10. (Scale bars, 500 μm).* **(B)** *Quantitative-RT-PCR analysis of gene expression in UE7T-13 cell cultured on gelatin in D-hESF10 at passage 4 (D-10), in comparison with the cells grown in POWERDBY10 (P). The name of each gene is noted in each bar graph. Gene expression was normalized with respect to* GAPDH. *The values are the mean ± SD (n=3).*

FGF-2 is a heparin-binding growth factor which stimulates the proliferation of a wide variety of cells. The biological activity of FGF-2 is efficient in the concentration range of 0.1 to 10.0 ng/ml. Addition of FGF-2 has been shown to increase the growth rate and life span of hMSCs from different species (Tsutsumi *et al.*, 2001; Benavente *et al.*, 2003), suggesting that FGF-2 play an important role in self-renewal of hMSCs. In hES cells, FGF-2 is a crucial to maintain the undifferentiated state (Amit *et al.*, 2004; Hoffman and Carpenter, 2005). We previously reported that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hES cells in serum-free without feeders (Furue *et al.*, 2008). In this study, we found that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hMSCs in a serum-free medium. These findings suggest that they share the same signal pathway to support self-renewal. Heparin at 1 mg/ml promoted hMSC cell proliferation, and we previously reported that heparin at 1 mg/ml inhibited hES cell proliferation. Thus the sensitivity to heparin is different between hMSCs and hES cells.

The TGF-β1 pathway has been reported to be important in hMSC differentiation into the osteogenic and chondrogenic lineages (Li and Xu, 2005; Kulterer *et al.*, 2007). While we have shown that TGF-β1 alone did not promote cell proliferation of hMSCs, the combination with FGF-2 and heparin enhanced cell proliferation of hMSCs. Chase *et al.* reported the combination of TGF-β1, FGF-2, and PDGF-BB in a commercial serum-free medium for the expansion of hMSCs although the optimal concentrations of these factors were not disclosed. The cell growth rate in D-hESF10 medium was similar with that in the conven-

tional culture conditions suggesting that addition of TGF-β1 and FGF-2 is sufficient to replace serum in supporting hMSC cell growth. A culture medium consisting of the minimum components necessary to support survival and proliferation would be beneficial to understand the characteristics of naïve hMSCs. Therefore, we think that addition of PDGF-BB is not crucial for an hMSC culture medium.

Several studies reported that two distinct cell morphologies are seen in early-passage hMSC cultures: small, spindle-shaped cells that are rapidly self-renewing and large, flat cells that replicate slowly and appear more mature (Mets and Verdonk, 1981; Colter *et al.*, 2001; Sekiya *et al.*, 2002). The morphology of serum-free expanded UE7T-13 cell population contained comparably small, spindle-shaped cells. However, specific undifferentiated markers of hMSCs have not been identified yet (Pochampally *et al.*, 2004). Further, although the cells are cloned, cells within an individual colony are heterogeneous in morphology, growth rates, and efficiency with which they differentiate (Mets and Verdonk, 1981; Bruder *et al.*, 1997; Colter *et al.*, 2001). The International Society for Cellular Therapy (ISCT) has proposed three criteria to define hMSCs (Dominici *et al.*, 2006). hMSC population must be positive at least for several antigens such as CD105, CD73, and CD90, and negative for CD45. CD105 is usually used to identify an hMSC population. Many studies reported that hMSCs also expressed hES cell pluripotency markers, SSEA-3, -4, NANOG, OCT3/4, and alkaline phosphatase (Pochampally *et al.*, 2004; Roubelakis *et al.*, 2007; Battula *et al.*, 2008; Conrad *et al.*, 2008; Pang *et al.*, 2010). We also detected the expression of NANOG, OCT3/4, and SOX2. These findings suggested that hES cell pluripotency markers may be universal stem cell markers in humans. Dezawa's group recently reported that double positive CD105 and SSEA-3 cells have the ability to generate multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). We also confirmed the existence of CD105 and SSEA-3 double positive cells in the hMSC population expanded in D-hESF10. In this study, we confirmed the differentiation potential of hMSCs to generate osteoblasts or adipocytes, but in the future we will examine the ability of hMSCs to generate cells from all three germ layers.

To facilitate the transition of human stem cell biology from basic research to clinical application all the components of maintenance and differentiation media should be publicly disclosed so they can be evaluated by many researchers. A commercial xeno-free serum-free medium for hMSCs was reported recently (Chase *et al.*, 2010). However, the non-disclosure of components is problematic as the medium formulation cannot be usefully modified or improved. Because all the components of D-hESF10 medium are disclosed here, the medium can be modified to study signaling pathways involved in maintaining multipotency and to develop differentiation protocols.

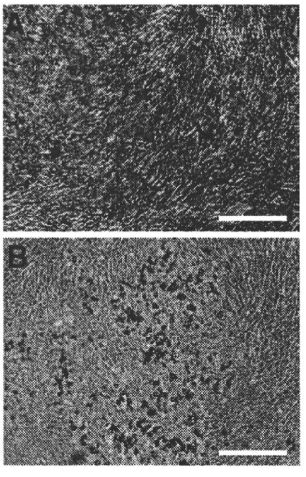

Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. *The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium.* **(A)** *Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were stained with Alizarin Red S (red).* **(B)** *Adipocytic differentiation was induced in adipocytic medium for 24 days. The cells were stained by Oil red O staining (red). Scale bars: 500 μm.*

Materials and Methods

Cell Cultures

An immortalized hMSC line UE7T-13 (Mori *et al.*, 2005) (JCRB 1154, JCRB Cell Bank, Osaka, Japan) was used in this study. Cells were maintained on 100 mm dish (BD Falcon, Oxnard, CA) in POWERDBY10 (MED-SHIROTORI, Tokyo, Japan) that was also used in the experiments as a control medium. The cells were harvested with 0.25% trypsin in 1 mM EDTA-4Na.

Serum-free Cell Culture Media

hESF9 comprises ESF basal medium (Furue *et al.*, 2005) without HEPES supplemented with nine defined factors: Asc 2-P, 6-factors (human recombinant insulin, human transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA)), bovine heparan sulfate sodium salt, and human recombinant FGF-2 (Sigma, St. Louis, MO), as described previously (Furue *et al.*, 2008) (Supplementary Table 1). ESF basal medium without HEPES supplemented with Asc 2-P (hESF-GRO), and ESF basal medium without HEPES and Asc 2-P (hESF-DIF) were purchased by the Cell Science & Technology Institute (CSTI, Sendai, Japan). All other reagents were from Invitrogen (Carlsbad, CA) and Sigma. D-hESF10 medium consists of hESF-DIF medium supplemented with 6-factors, FGF-2, heparin, and TGF-β1 (R&D Systems, Minneapolis, MN). To harvest cells, 0.25% trypsin in 1 mM EDTA-4Na was used and the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). For differentiation into osteoblasts or adipocytes, the cells were cultured according to the instruction by the suppliers (Lonza, Basel, Switzerland). The differentiated cells were stained by Alizarin Red S (Wako Pure Chemical Industries, Osaka, Japan) or Oil Red O (Wako).

Cell proliferation

Before the serum-free experiments, cells grown in POWERDBY10 were incubated by in hESF9 medium without heparin and FGF-2 (hESF9(-/-)) overnight to starve the effect of serum. Cells were replaced at the cell density of $1x10^4$ cells /well on 24-well plate (BD Falcon) coated with 0.1% porcine gelatin solution (Millipore, Billerica, MA) and cultured in hESF9(-/-) medium in the presence of varying growth factors. The cell numbers were counted by Coulter Counter (Beckman Coulter, Hialeah, FL).

Gene expression

A detailed reverse transcription-polymerase chain reaction (RT-PCR) protocol was described previously (Furue, *et al.*, 2005). Total RNA was extracted from hMSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the provider's instructions. Q-RT-PCR was carried out using the TaqMan gene expression Master Mix on in ABI PRISM 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the supplier's instructions (ABI). Specific primers-probe set were listed in Supplementary Table 2. Expression levels were all normalized by the expression level of *GAPDH*. The relative level of each gene in cDNA of undifferentiated hES cells was defined as "1." The KhES-3 cell line was used as a control; the cells were obtained from the Institute for Frontier Medical Science, Kyoto University, and the Review Board of the National Institute of Biomedical Innovation approved this research.

Antigen expression

For *in situ* immunocytochemisty, the cells were immunostained with antibodies, as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, fluorescence images were acquired using by IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, England). Flow cytometry was performed with BD FACS Canto flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, the labeled primary antibodies were used, but the biding of anti-SSEA-3, anti-CD56, and Tra-2-54 antibodies was visualized with RPE-conjugated goat anti-mouse Ig (Dako, Carpinteria, CA) or Alexa Fluor 647 goat anti-rat IgM (Invitrogen). The primary antibodies used are listed in Supplementary Table 3.

Acknowledgements

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