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PERSPECTIVES

MODELS OF CANCER SERIES — SCIENCE AND SOCIETY

Cell line misidentification: the beginning of the end

American Type Culture Collection Standards Development Organization Workgroup ASN-0002

Abstract | Cell lines are used extensively in research and drug development as models of normal and cancer tissues. However, a substantial proportion of cell lines is mislabelled or replaced by cells derived from a different individual, tissue or species. The scientific community has failed to tackle this problem and consequently thousands of misleading and potentially erroneous papers have been published using cell lines that are incorrectly identified. Recent efforts to develop a standard for the authentication of human cell lines using short tandem repeat profiling is an important step to eradicate this problem.

Cell lines are used extensively in biomedical research as *in vitro* models. The validity of the data obtained often depends on the identity of the cell line, particularly when it is being used as a surrogate for the tissue of origin. Surprisingly, the frequency of cell line misidentification is high, and consequently the ascribed origin of a cell line is often incorrect. This problem has been known for over 50 years and has been described as the most compelling quality-control issue confronting the scientific community[1]. Based on analyses of cell lines submitted to international cell banks, the incidence of misidentification in 1977 was 16%[2] and in 1999 was 18%[3]. Until recently, the authenticity of cell lines used in biomedical research has received little attention. This Science and Society article has been written by the members of the American Type Culture Collection (ATCC) Standards Development Organization (SDO) Workgroup ASN-0002 (BOX 1), a working group currently developing a standard for human cell line authentication. The ATCC SDO was formed in 2007 to develop best practices (standards) for use in the life sciences and to promote their use globally, using a consensus-driven process that balances the viewpoints of industry, government, regulatory agencies and academia. We expect that the draft standard (BOX 2) will be available for public review and comment in 2010 and subsequently the final draft will be approved by the American National Standards Institute (ANSI).

Here we describe the causes and scientific effects of cell line misidentification, its history and the efforts taken to solve the problem. The various methods currently available for authenticating cell lines are discussed and a recommendation is made for the use of short tandem repeat (STR) profiling for authenticating human cell lines. Perhaps of the greatest importance, a universal database of human cell line STR profiles is under construction.

Discovery of cell line misidentification

Misidentification of human and animal cell cultures is a long-standing problem, and awareness of this problem dates back to the 1950s (TIMELINE). Karyotyping and immunological approaches were first used for cell line authentication[4–6]. Extensive species misidentification was reported, leading to the establishment of a bank of authenticated cell lines at the ATCC in 1962.

Misidentification within species could not be detected in 1962, but in 1966 Stanley Gartler (FIG. 1a) introduced the concept of biochemical polymorphisms to distinguish human cell lines on the basis of their isozyme expression. At the Second Decennial Review Conference on Cell, Tissue and Organ Culture in 1966, Gartler reported that 18 human cell lines supposedly of independent origins were all HeLa cells[7], the first human cancer cell line to be established in culture[8]. The examples included cells claimed to be derived from normal intestinal epithelium (Int-407), normal amnion (WISH), normal liver (Chang liver), laryngeal cancer (Hep-2) and oral cancer (KB). The HeLa cell line was derived from a glandular cervical cancer in a female patient named Henrietta Lacks and, because of its celebrated status, was distributed internationally and passed from laboratory to laboratory. Then, as today, many scientists were oblivious to the possibility of cross-contamination. HeLa cells are particularly robust and fast-growing and consequently can rapidly overgrow other cells.

Denial and complacency

There was resistance and some hostility to Gartler's findings — even among scientists "who should have known better", according to Gartler — but one scientist, Walter Nelson-Rees (FIG. 1b), took particular note of Gartler's talk. Nelson-Rees ran a cell bank at Berkeley under contract for the National Cancer Institute. With his colleagues he developed karyotyping methods for authenticating cell lines and in a series of papers he showed there was extensive cross-contamination among the supposedly unique cultures sent to the bank (for example, see REF. 9). Nelson-Rees's work showed widespread cross-contamination by HeLa cells and for some years all cell lines were under suspicion of being HeLa cells until proven otherwise. He developed methods for cell identification and raised awareness of the problem in the scientific literature and through correspondence with individual scientists affected by the problem. Nelson-Rees's last contribution to the subject was published in 2009, soon after his death[10].

When Nelson-Rees first published his findings, some scientists ignored or denied the evidence and continued to publish papers containing false information[11]. As a consequence, Nelson-Rees felt that he had no option but to highlight the papers (and consequently the individuals) using cross-contaminated cell lines. At that time (and possibly today), Nelson-Rees's behaviour was

regarded as unscientific and he was attacked by many colleagues. He was branded a self-appointed vigilante and his contract terminated by the National Institutes of Health (NIH) in 1981. After this, cell line misidentification went largely unchecked and the problem escalated. For the next 10–20 years, cell banks distributed many cell lines under their false names.

Estimating how much misleading and erroneous research is attributable to cross-contamination or misidentification of cell lines has been difficult. The use of misidentified cultures increased about 10-fold in the PubMed database (see Further information for a link) between 1969 and 2004, and the papers that used cultured cells increased only 2–2.5-fold during the same time period[12,13]. By 2004, HeLa was just the tip of the iceberg, and many other cell lines masqueraded under various guises in laboratories worldwide.

A survey that profiled active cell culture workers found that of 483 respondents, 32% used HeLa cells, 9% unwittingly were using HeLa contaminants, only 33% of the investigators tested their cell lines for authenticity and 35% obtained their cell lines from other laboratories rather than from a major repository[12].

Although complacency and, in some cases, denial have been the primary responses to cell line misidentification over the past five decades, a few individuals have devoted a great deal of personal effort into remediation of the problem. Among the largely independent efforts were letters to editors from concerned individuals requesting that readers be alerted about the problem, and that authors be required to provide evidence that the cell lines used in their studies were neither cross-contaminated nor misidentified. These efforts were largely ignored in the period after Nelson-Rees's contract was terminated, despite the development of DNA-fingerprinting techniques, which brought new and more reproducible methods that once again revealed the extent of cell line misidentification in the early 1990s[14].

Roland Nardone (FIG. 1c) started the second crusade in 2004. He gained the support of Joseph B. Perrone, who was then Vice President for Standards at ATCC and provided ideas and the matching outrage needed to fuel the crusade. Together with other concerned scientists, Nardone developed a comprehensive and coordinated initiative that simultaneously sought to raise awareness of the nature and magnitude of the problem and canvassed the involvement of individuals and organizations concerned or affected by the problem[1,15]. Such organizations included the NIH, the Howard Hughes

Box 1 | **Authors and members of workgroup ASN-0002**

- Christine Alston-Roberts, Standards Specialist, ATCC, 10801 University Boulevard, Manassas, VA 20110, USA
- Rita Barallon, Ph.D., Service Business Manager, Life and Food Sciences Life Sciences, LGC, Queens Road, Teddington, Middlesex, TW11 0LY, UK
- Steven R. Bauer*, Ph.D., FDA/Center for Biologics Evaluation and Research, Chief, Cell and Tissue Therapy Branch, Division of Cellular and Gene Therapies, Office of Cellular, Tissue and Gene Therapies, NIH Building 29B 2NN10 HFM-740, 8800 Rockville Pike, Bethesda, MD 20892, USA
- John Butler, Ph.D., Biochemical Science Division (831), Advanced Chemical Science Laboratory (227), Room B226, NIST, 100 Bureau Drive, Stop 8312, Gaithersburg, MD 20899-8312, USA
- Amanda Capes-Davis, Ph.D., CellBank Australia, Children's Medical Research Institute, Westmead, New South Wales, Australia
- Wilhelm G. Dirks, Ph.D., Molecular Biology, DSMZ — German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7b, 38124 Braunschweig, Germany
- Eugene Elmore, Ph.D., Project Scientist, Department of Radiation Oncology, University of California, Medical Sciences I, B146D, Irvine, CA 92697, USA
- Manohar Furtado, Ph.D., Vice President, R & D, Applied Markets Division, Applied Biosystems, 850 Lincoln Centre Drive, MS404-1, Foster City, CA 94404, USA
- Liz Kerrigan, Director, Standards and Certification, ATCC, 10801 University Boulevard, Manassas, VA 20110, USA
- Margaret C. Kline, Research Biologist, Biochemical Science Division (831), Advanced Chemical Science Laboratory (227), Room B226, National Institutes of Standards and Technology,100 Bureau Drive, Stop 8312, Gaithersburg, MD 20899-8312, USA
- Arihiro Kohara, Ph.D., Scientist, National Institute of Biomedical Innovation, Department Biomedical Services, Laboratory of Cell Cultures, 7-6-8 Saito-Asagi, Ibaraki, Osaka, Japan 567-0085
- Georgyi V. Los, M.D., Ph.D., Honorary Fellow, Neuroscience Training Program, University of Wisconsin-Madison, 1300 University Avenue, Madison, WI 53706, USA
- Roderick A.F. MacLeod, Ph.D., Cytogenetics Laboratory, DSMZ — German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7b, 38124 Braunschweig, Germany
- John R. W. Masters, Ph.D., FCRPath, Professor of Experimental Pathology, University College London, 67 Riding House Street, London, W1W 7EJ, UK
- Mark Nardone, Director, Bio-Trac Program, The Foundation for the Advanced Education in the Sciences at the National Institutes of Health, Bethesda, MD 20892, USA
- Roland M. Nardone, Ph.D., Professor Emeritus, Catholic University of America, Cell and Molecular Biology, 620 Michigan Avenue NE, Washington, DC 20064, USA
- Raymond W. Nims, Ph.D., Consultant, RMC Pharmaceutical Solutions Inc., 2150 Miller Drive, Suite A, Longmont, CO 80501, USA
- Paul J. Price, Ph.D., CSO, Research and Development, Room B-33, D-Finitive Cell Technology, 1023 Wappoo Rd, Charleston, SC 29407, USA
- Yvonne A. Reid, Ph.D., Collection Scientist, Cell Biology Collection, ATCC, 10801 University Boulevard, Manassas, VA 20110, USA
- Jaiprakash Shewale, Ph.D., Director, Biology, Applied Markets/Genetic Systems, Life Technologies, 850 Lincoln Centre Drive, Foster City, CA 94404, USA
- Anton F. Steuer, Ph.D., Principal Scientist, BioReliance, 14920 Brochart Road, Rockville, MD 20850, USA
- Douglas R. Storts, Ph.D., Head of Research, Nucleic Acid Technologies, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA
- Gregory Sykes, Biologist, ATCC, 10801 University Blvd., Manassas, VA 20110, USA
- Zenobia Taraporewala*, Ph.D., FDA/Center for Biologics Evaluation and Research, Reviewer, Division of Cellular and Gene Therapies, Office of Cellular, Tissue, and Gene Therapies, 1401 Rockville Pike, Room 200N, Rockville, MD 20892, USA
- Jim Thomson, Innovation and Support Team, LGC, Queens Rd, Teddington, TW11 0LY, UK

*S. R. B. and Z. T. did not contribute as authors to this Perspective.

Medical Institute, heads of funding organizations and their attorney generals, leaders of professional societies and editors of science journals.

Copies of a white paper, 'Eradication of cross-contaminated cell lines: a call for action' (subsequently published by Nardone in 2007 (REF. 15)) were distributed to thousands of scientists. The white paper presented what seemed to be a straightforward solution: funding agencies would require cell line authentication as a condition for the receipt of funds and journals would have a similar requirement for manuscripts submitted for publication. This approach was met initially with indifference. Nevertheless, over a period of 4 years, several substantial milestones were reached[1]. An open letter[16] to Michael O. Leavitt, Secretary of Health and Human Resources, led the NIH to re-examine the situation. On November 28 2007, the NIH published an addition to its guidelines for research in the form of a notice regarding authentication of cultured cell lines calling for diligence and more careful peer review[17].

Two factors have driven this progress. One is heightened awareness. The other is the outrage of scientists angered by the failure of funding agencies and journals to address the problem and allowing it to fester and amplify for 50 years. Many scientists now accept the need for a standardized method of human cell line authentication to satisfy the new requirements. ASN-0002 will be the first step towards a universally adopted standard.

Box 2 | **ATCC SDO standards development process**

- American Type Culture Collection (ATCC) Standard Development Office (SDO) Consensus Standards Partnership (CSP) members recommend a new standard.
- Recommendation forwarded to ATCC SDO steering committee for review and vote.
- Project Initiation Notification System (PINS) published in *American National Standards Institute (ANSI) Standards Action* for 30-day public comment period, concurrent with CSP (ATCC SDO members) review.
- Recommendation for workgroup chair(s) sent to ATCC SDO steering committee for vote.
- Workgroup established; (ASN-0002), which includes stakeholders from academia, industry and government, and proceeds to draft the standard (see BOX 1 for members of the workgroup).
- ASN-0002 workgroup forwards draft standard to steering committee for internal review. Workgroup edits draft standard and forwards to ANSI and CSP (ATCC SDO membership) for concurrent 45-day public review.
- ASN-0002 workgroup responds to all comments and resolves any differences. If there are no substantial changes to the standard, the standard is submitted to the ANSI board of standards review for final action and publication as an ANSI-approved standard.

Examples and impact

Cross-contamination and misidentification have a long history with many examples, but it is difficult to judge which have been the most substantial and costly.

The classic case already described is contamination by HeLa cells, of which there are several examples (see REFS 7,9 for example). It is astonishing that many of these cell lines have continued to be used under their false descriptions in respected journals for over 40 years after they were first shown to be HeLa cells (BOX 3).

T24 is another fast-growing cell line that has contaminated many supposedly distinct bladder cancer cell lines (BOX 3). ECV304 was originally claimed to be a spontaneously transformed human normal endothelial cell line, but later shown to be T24 bladder cancer cells[18]. Surprisingly, the demonstration that ECV304 cells are not endothelial cells had little effect on its use as a model for endothelial cells in publications (FIG. 2).

The putative human prostate cancer cell lines TSU-Pr1 and JCA-1 are also derived from T24 bladder cancer cells[19]. These findings were published in *Cancer Research*, but that did not prevent TSU-Pr1 cells being used as a prostate cancer cell model in a later paper in *Cancer Research* (BOX 3).

DNA-fingerprinting analysis revealed that the NCI/ADR-RES cell line was actually an ovarian tumour cell line, OVCAR-8, rather than a breast cancer cell line. Around 300 papers have been published using the incorrect identification of the NCI/ADR-RES cell line[20]. NCI/ADR-RES is included in the NCI60 panel of cell lines, which has been subject to STR profiling (discussed below)[21].

A paper describing misidentification of oesophageal cell lines stated "Experimental results based on these contaminated cell lines have led to ongoing clinical trials recruiting EAC [oesophageal adenocarcinoma] patients, to more than 100 scientific publications, and to at least three National Institutes of Health cancer research grants and 11 US patents"(REF. 22).

The consequences of widespread misidentification and cross-contamination of cell lines are immeasurable. In addition to the waste of millions of dollars of public money, time and intellectual resources, there is the loss of confidence in published work, and the integrity of science suffers.

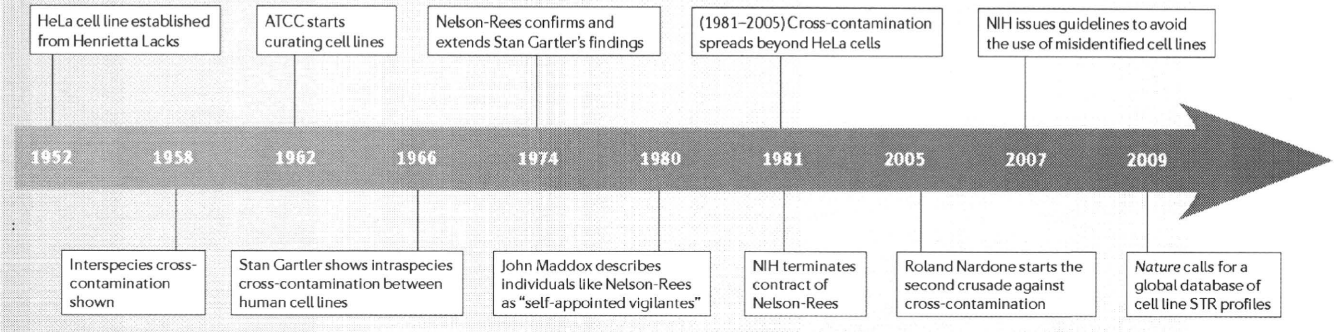
Over 50 years of suppression. Why?

Three constituencies share responsibility for cell line misidentification — individual scientists, scientific journals and funding agencies. For most of the past 50 years it is only individual scientists who have addressed the issue. Nevertheless, it is hard to escape the conclusion that many scientists have knowingly used misidentified cell lines in publications (for example, the evidence in FIG. 2). Furthermore, authors are often reluctant to publish corrections to the literature based on cell line misidentification.

John Maddox, the editor of *Nature* in 1980, wrote an editorial about a high-profile case of cross-contamination entitled 'Responsibility for trust in research' (REF. 23). With an almost complete lack of insight into the problem he suggested that "there is no reason to suppose that the few cases [of cross-contamination] that have come to light are in any sense the tip of the iceberg". In the same editorial, scientists like Nelson-Rees were vilified, as the article made the point that it would be tragic if these civilized habits (that is, truth in research) "were to be corrupted by the activities of self-appointed vigilantes". The history of cell line cross-contamination indicates that truth and trust are not as universal among the scientific community as many scientists wish to believe.

The responses of editors of scientific journals to the problem continue to be illuminating. There have been hundreds of papers in scientific journals describing examples of misidentification and, until recently, no remedial action has been taken to eradicate the problem by journals or funding agencies. The editor of an influential tissue culture journal was asked to consider introducing authentication as a requirement for publication and replied that it would be financial suicide. Editors of other journals also refused to consider such quality-control measures on the basis that introducing such a hurdle to publication would substantially reduce the number of authors willing to submit manuscripts to their journal.

Timeline | **Key milestones in the effort to address cell line misidentification**



ATCC, American Type Culture Collection; NIH, National Institutes of Health; STR, short tandem repeat

Over the past 2 years attitudes have begun to change, with journals, such as *In Vitro Cellular and Developmental Biology*, *International Journal of Cancer*, *Cell Biochemistry and Biophysics* and the American Association for Cancer Research (AACR) journals, demanding that all cell lines are authenticated before publication. *Nature* has indicated that first the funding organizations have to demand authentication and provide the necessary funds. Once they do, *Nature* will require cell line identification prior to publication[24]. In the meantime, the funding organizations continue to ignore the problem.

The constituency with the most power to maintain standards in science is the funding agencies. Surprisingly, these have been resistant to addressing or even acknowledging the problem of cell line misidentification. For example, the NIH advisory note issued in 2007 ignores the fact that individual scientists and reviewers have failed to overcome this problem. As an editorial in *Nature* pointed out, the advisory note merely enforces the status quo[24].

Attempts to address the problem by individual scientists have met with unhelpful responses from funding bodies, which have tended to deny or belittle the problem. A recent public statement by a senior scientist from Cancer Research UK made light of cell line misidentification, saying that "this issue raises its head every few years". Funding bodies seem to be threatened by the issue and are resistant to engaging with scientists who try to address the problem and often attempt to disparage and discredit those who try to find a solution.

Any of the major funding organizations that support biomedical research in the United States or United Kingdom could have eradicated cell line misidentification during the past 10 years for less than the cost of the average project grant by funding the measures outlined in this Science and Society article. Yet, these funding agencies have repeatedly ignored and in some cases suppressed debate, and continued to provide grants for research using false cell lines. There could be wider implications concerning the role of funding agencies in the control of scientific misrepresentation and fraud.

Zero tolerance of cell line misidentification is needed from both journals and funding agencies. There are signs that Nardone's crusade is gaining influence and the standard for human cell line identification will be tangible evidence of Nardone's legacy.

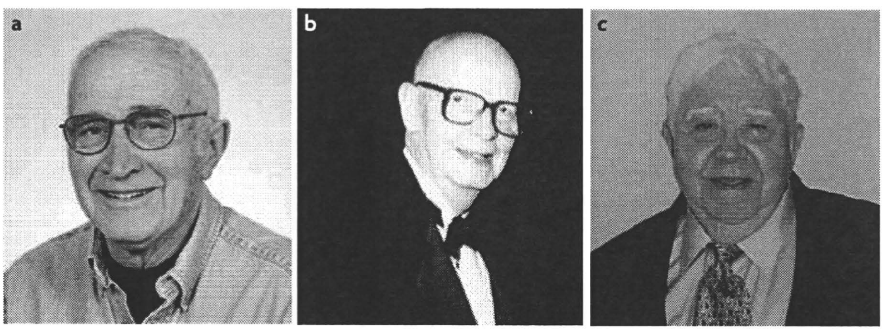


Figure 1 | **Pioneers of awareness of cell line misidentification. a** | Stanley Gartler **b** | Walter Nelson-Rees **c** | Roland Nardone

Causes of cell line misidentification

Most cell lines are established in academic environments in which tissue culture is often regarded as a technique requiring little skill and essential facilities, such as flow cabinets and incubators, are used without restriction. In these circumstances, it is not surprising that attempts to establish new cell lines often lead to cross-contamination. Among 550 leukaemia and lymphoma cell lines submitted to the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; German Collection of Microorganisms and Cell Cultures; please see Further information for a link) cell bank, 59/395 (15%) submitted by originators and 23/155 (15%) submitted by secondary sources were false[25]. Presumably most of the cell lines submitted by the secondary sources had also been cross-contaminated or misidentified by the originators.

There are many causes of cell culture misidentification and every laboratory is at risk. Perhaps the most straightforward cause is mislabelling of a cell culture vessel during routine manipulation. Factors contributing to this error include operator workload, lack of attention, or distractions during manipulation of cell lines.

Cross-contamination of a culture and subsequent overgrowth by the contaminating cell type is another frequent cause of cell line misidentification. The chances of this occurring are increased by the use of shared reagents, repeated use of the same pipette during re-feeding operations and manipulation of multiple cultures at the same time without adequate isolation of one cell type from another. When cross-contamination happens, one cell type may

rapidly outgrow the other, leading to a pure culture of the contaminating cells in four or five passages[26].

Intentional co-cultivation during propagation of human stem or primary cells using a feeder layer derived from another species (such as mouse 3T3 cells) can result in cross-contamination and overgrowth of the human cell line. Normally, feeder cells are rendered incapable of proliferating, but if the growth arrest procedure is inadequate, the feeder cells can proliferate and displace the human cells. Somatic cell hybridization is unusual but can occur, as found in the human mantle cell lymphoma line NCEB-1, which carries seven mouse chromosomes[27].

Xenografting can also lead to cell line cross-contamination and misidentification[28]. Recovered cell lines from xenografts can be replaced by cells derived from the host animal.

In general, cross-contamination results in the complete and rapid displacement of the less fit cell type. Two cell lines cannot co-exist in the same culture environment for extended periods unless there is a symbiotic relationship, which as far as we know has never been reported. Consequently, cell mixtures are discovered rarely. The only known situation in which a cell population contains a stable mixture of genomes over many passages is following somatic cell hybridization.

Simple, cheap quality-control measures can prevent or at least minimize the consequences of misidentification. Misidentification is rife because of a combination of lack of awareness and the failure to include quality-control measures. The extensive quality-control measures demanded of the biopharmaceutical industry and mandated in the applicable regulatory documents are believed to have contributed to the relatively low frequency of cell line misidentification reported in this industry[29].

Box 3 | **Examples of the use of cell lines under false descriptions**

The examples discussed below were picked at random from PubMed searches. The impact of the false descriptions ranges from minor to invalidation of the conclusions. The individual authors have been failed by peer review. The papers indicate that the editors and some of the reviewers of these journals (and by inference most scientific journals) are unaware of the extent of cell line misidentification, and indicate a general lack of awareness throughout the scientific community.

HeLa cervical cancer cells

- Int-407 (described as "non-transformed intestinal epithelial cells") in *Br. J. Cancer* **101**, 1596 (2009), *EMBO J.* **22**, 5003 (2003) and *J. Biol. Chem.* **280**, 13538 (2005)
- WISH (described as "non-transformed amniotic epithelial cells") in *Mol. Pharmacol.* **69**, 796 (2006), *Endocrinology* **147**, 2490 (2006) and *J. Biol. Chem.* **278**, 31731 (2003)
- Chang liver (described as "normal liver cells") in *Oncogene* **28**, 3526 (2009), *Proteomics* **14**, 2885 (2008) and *J. Biol. Chem.* **279**, 28106 (2000)
- HEp-2 (described as "laryngeal cancer") in *Investig. New Drugs* **26**, 111–118 (2008), *Carcinogenesis* **29**, 1519 (2008) and *J. Biol. Chem.* **283**, 36272 (2008)
- KB (described as "oral cancer") in *Biochem. Pharmacol.* **73**,1901–1909 (2007), *Clin. Cancer Res.* **14**, 8161(2008) and *J. Biol. Chem.* **280**, 23829 (2005)
- HeLa, Int-407 and HEp-2 cells were used as three distinct cell lines in the same study in *Cancer Res.* **69**, 632 (2009)

The scientists that use these cell lines sometimes use them under their false descriptions in many publications. For example, one group has used Int-407 as a model of normal intestinal cells since 1988 and during the past 10 years has published in the *Biochemical Journal* (2 papers), *Biochemical Society Transactions*, *British Journal of Cancer*, *Cancer Research* (2 papers), *Carcinogenesis*, *Experimental Cell Research* (3 papers), *Gastroenterology* (2 papers), *Journal of Biological Chemistry* (3 papers), *Journal of Cell Physiology*, *Journal of Cell Science* (3 papers), *Oncogene*, *PLoS One* and several other journals.

T24 bladder cancer cells

In 1999, ECV304 cells (originally described as spontaneously immortalized normal endothelial cells) were shown to be T24 cells[18].

Yet, many papers continue to describe ECV304 cells as endothelial, for example *Nature Immunol.* **6**, 497 (2005) and *Nature Biotechnol.* **25**, 921 (2007). Some studies use ECV304 cells in endothelial research without claiming that they are endothelial cells, but not stating that they are T24 bladder cancer cells, such as *Proc. Natl Acad. Sci. USA* **106**, 6849 (2009). Some studies have used T24 and one or more of its cross-contaminants as distinct bladder cancer cell lines, for example *J. Urol.* **181**, 1372–1380 (2009). Some studies describe ECV304 as bladder cancer cells, but fail to state that they are T24 cells, such as *J. Biol. Chem.* **285**, 555–564 (2010).

In *Cancer Research* in 2001, it was shown that TSU-Pr1 are T24 bladder cancer cells (*Cancer Res.* **61**, 6340–6344 (2001)). In the same journal, less than 3 years later, TSU-Pr1 cells were used as a prostate cancer model (*Cancer Res.* **64**, 1058–1066 (2004)). TSU-Pr1 continue to be used in some studies as a model for prostate cancer, such as *Endocrinology* **147**, 530–542 (2006) and *Cancer Cell* **5**, 67 (2004).

Detection of cross-contamination

Many methods have been used to detect cross-contamination, including isoenzyme analysis, karyotyping, human leukocyte antigen (HLA)-typing, immunotyping and DNA fingerprinting. These methods can authenticate a cell line, but with differing levels of ambiguity and powers of discrimination (Supplementary information S1 (table)). However, the data produced by these methods are not sufficiently reproducible between laboratories to allow any of them to be used for a standardized reference database.

Many laboratories have adopted STR profiling to identify human cell lines. STR profiling is the method used by forensic analysts and depends on the simultaneous amplification of multiple stretches of polymorphic DNA in a single tube. STR loci consist of repetitive DNA sequences that have varying numbers of repeats. Each STR locus can be amplified and the amplified products labelled with fluorophores of different colours, making the products easy to distinguish by size and colour (FIG. 3). STR analysis is rapid, inexpensive, amenable to automation and generates reproducible data in a format suitable for a standard reference database. For the quick, unambiguous authentication of cell lines, STR analysis has the greatest value.

STR profiling — potential and limitations

DNA repeat sequences of 3–5 bases have been used routinely for paternity testing, forensic casework, and the identification of victims of mass disaster for more than two decades[30–33]. Consequently, STR profiling was applied to cell line identification[34–36]. There are several advantages to using STRs for the authentication of human cell lines (Supplementary information S2 (box)).

Cancer cell lines contain many genetic alterations, and therefore the criteria used to compare them using STR profiling must be different to those used for normal tissue (Supplementary information S3 (box)). Cancer cells often show loss of heterozygosity (that is, loss of an allele, which cannot be distinguished easily from homozygosity) and can contain multiple copies of alleles owing to DNA duplication. Similarly, during culture, cancer cell lines can lose or more rarely gain a copy of an allele (for examples, see REF. 34). Consequently, sub-lines of the same cell line may not have identical STR profiles.

Comparing identical alleles, a threshold of 75% identity has distinguished all known cross-contaminated cell lines in published datasets, and no two cell lines thought to be derived from different individuals showed more than 50% identity[21,34]. Consequently, there is a comfortable cushion of 25% between cell lines that are unique and those that show evidence of cross-contamination. Any cell line found with an identity level between 50 and 75% should be regarded with suspicion.

Major issues in the interpretation of genotypes from human cell lines include heterozygote peak height imbalance (that is the peak height or area of one allele is much larger than the peak height or area of the second allele), multiple alleles at a locus, and allele dropout (no amplification product of the expected size). Cancer cell lines are aneuploid and consequently STR profiles typically show heterozygous peak height imbalances and/or multiple alleles at one or more loci.

The cost of genotyping is a major concern, but trivial in relation to the cost of the work being done with the cell line. The cost of STR profiling includes DNA extraction, polymerase chain reaction (PCR) amplification of STR loci, separation of amplified products by capillary electrophoresis and data analysis. Increasing the number of STR loci, for example, from 6 to 15 would achieve a much higher power of discrimination (Supplementary information S4 (table)).

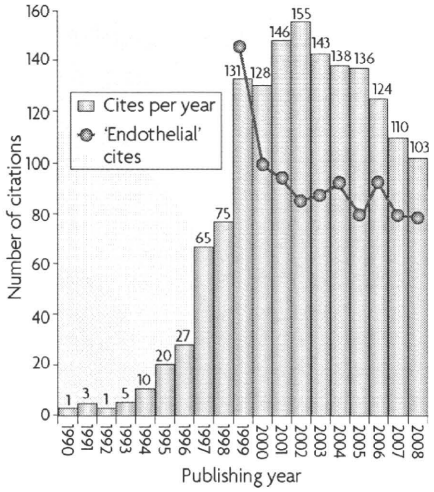


Figure 2 | **Citations of T24 bladder cancer cells referred to as normal endothelial cells.** The demonstration that ECV304 cells are not endothelial cells had little effect on its use as a model for endothelial cells in publications, as shown by the graph. Data generated courtesy of R.A.F. MacLeod, National Institute of Standards and Technology.

A major limitation of STR profiling is that it will not detect contaminating cells of another species, although if human cells are overgrown by cells of another species, the DNA will not amplify using human or higher primate-specific STR primers. PCR using species-specific primers can be used to detect contaminating cells from other species. If STR profiles have been established for the other species (currently restricted to a few commercially important species), STR can be carried out to definitively identify the contaminating cells.

For most of the established cell lines, donor tissue is not available and many originators of widely used cell lines are retired or deceased. In these cases, an assumption has to be made, based on the oldest possible cell stocks in repositories. These profiles will need to be labelled as provisional to indicate the absence of authentication back to the original donor tissue.

Until the database described below is available, there are limited resources available for comparing STR profiles. The ATCC and DSMZ cell bank websites and Cell Line Integrated Molecular Authentication (CLIMA; see Further information for links) database[37] provide some information, and at least two series of STR profiles have been published[21,34]. Currently, one of the most useful resources is the list of misidentified cell lines collected by Amanda Capes-Davis and Ian Freshney (supplementary table in REF. 38), which can also be seen on the European Collection of Cell Cultures (ECACC; see Further information for a link) website. All scientists should check the names of the cell lines they are using against this list.

The interactive database

It is proposed that a database will be established to exploit available STR data to validate the identity of human cell lines. The interactive database will be accessible to everyone, but only the database administrators can make changes or additions. The database will provide DNA profiles and will allow laboratories to compare the STR profiles of their lines, thereby facilitating the validation of experimental data.

Universal criteria are needed for what constitutes a good database. The standard for cell line authentication will establish an interactive database of validated DNA profiles for each unique cell line and will also put in place requirements for carrying out and interpreting the STR assays. The members of the standard committee in conjunction with the National Center for Biotechnology Information (NCBI) will develop the requirements for the database and the database will be maintained by NCBI. The database will initially contain around 500 validated cell lines frequently used by scientists and banked in major cell repositories. The profile of each cell line will be validated before it is submitted to the database.

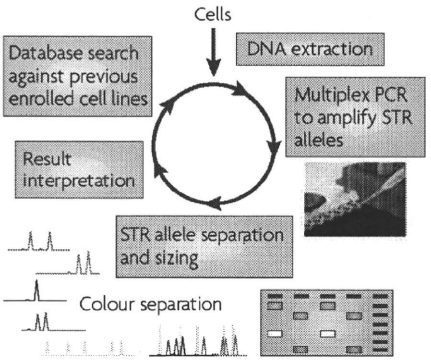


Figure 3 | **Short tandem repeat profiling methodology.** Short tandem repeat (STR) loci consist of repetitive DNA sequences with varying numbers of repeats. Each STR locus can be polymerase chain reaction (PCR) amplified and the amplified products labelled with fluorophores of different colours, making the products easy to distinguish by size and colour. Images courtesy of J. Butler, National Institute of Standards and Technology.

The most effective database to compare cell line STR-profiling data would consist of a common set of markers. However, not all data have been collected for the same STR loci or using the same generation of sequencing instruments. The use of different primer sets for the same STR markers is a common practice for the forensic and human identity community, which in the United States uses a core set of 13 STR markers for data input into the Federal Bureau of Investigation-maintained Combined DNA Index System (CODIS). To maintain the integrity of the data entered into CODIS, laboratories must use CODIS-approved STR-typing kits and instrumentation, and follow strict quality assurance standards[39]. Approved CODIS STR kits have undergone extensive validation studies that include concordance studies designed to elucidate STR-typing differences that may be seen with the use of different primer sets. Similar protocols will be needed for STR profiling of cell lines.

The future

Cell line verification by STR profiling will have a substantial effect on scientific research in terms of increased data credibility and less time, money and effort spent studying misidentified cell lines. Accurate identification of

cell lines is crucial during the development of cell-based medical products to avoid the risks of exposing human subjects to misidentified cells. Although such misidentification can largely be avoided by adherence to quality-control measures, such as proper labelling and tracking schemes during manufacture of a cell-based product, the availability of a standardized method for unambiguous cell and tissue identification could contribute to safety assurance when used to confirm that a cell product came from the intended donor and was not inadvertently mixed with cells from other donors. This issue is of great importance to personalized medicine and the application of stem cell-based technologies, including induced pluripotent stem cells.

No single method is available that provides all the information needed to authenticate a human cell line. STR profiling represents the optimal candidate at this time. Consequently, the standard is intended to evolve as new information becomes available. The interactive, searchable database openly available to everyone will largely eradicate the use of misidentified cell lines. Funding bodies and journals are encouraged to adopt a policy of zero tolerance and demand proof that all cell lines are as claimed.

For members of the ATCC Standards Development Organization (SDO) Workgroup ASN-0002 see BOX 1

John R. W. Masters is at University College London, 67 Riding House Street, London W1W 7EJ, UK.
Correspondence to J.R.W.M.
e-mail: j.masters@ucl.ac.uk

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Competing interests statement
The authors declare competing financial interests; see Web version for details.

DATABASES
CLIMA database: http://bioinformatics.istge.it/clima
PubMed: http://www.ncbi.nlm.nih.gov/pubmed

FURTHER INFORMATION
ATCC SDO homepage: http://www.atccsdo.org
ATCC cell bank: http://www.atcc.org
DSMZ cell culture collection: http://www.dsmz.de/human_and_animal_cell_lines
ECACC cell culture collection: http://www.hpacultures.org.uk/collections/ecacc.jsp

SUPPLEMENTARY INFORMATION
See online article: S1 (table) | S2 (box) | S3 (box) | S4 (table)

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Author biographies
The members of the American Type Culture Collection Standards Development Organization Workgroup ASN-0002 are Christine Alston-Roberts, Rita Barallon, Steven R. Bauer, John Butler, Amanda Capes-Davis, Wilhelm G. Dirks, Eugene Elmore, Manohar Furtado, Liz Kerrigan, Margaret C. Kline, Arihiro Kohara, Georgyi V. Los, Roderick A. F. MacLeod, John R. W. Masters, Mark Nardone, Roland M. Nardone, Raymond W. Nims, Paul J. Price, Yvonne A. Reid, Jaiprakash Shewale, Anton F. Steuer, Douglas R. Storts, Gregory Sykes, Zenobia Taraporewala and Jim Thomson. This workgroup is currently developing a standard for human cell line authentication and the standard development process is outlined in Box 2 of the article.

TOC:

000 **Cell line misidentification: the beginning of the end**

American Type Culture Collection Standards Development Organization Workgroup ASN-0002: Christine Alston-Roberts, Rita Barallon, John Butler, Amanda Capes-Davis, Wilhelm G. Dirks, Eugene Elmore, Manohar Furtado, Liz Kerrigan, Margaret C. Kline, Arihiro Kohara, Georgyi V. Los, Roderick A. F. MacLeod, John R. W. Masters, Mark Nardone, Roland M. Nardone, Raymond W. Nims, Paul J. Price, Yvonne A. Reid, Jaiprakash Shewale, Anton F. Steuer, Douglas R. Storts, Gregory Sykes and Jim Thomson

That a substantial proportion of cell lines is mislabelled or replaced by cells derived from a different individual, tissue or species has been a long known, but largely ignored problem. The history of cell line misidentification and recent efforts to develop a standard for the authentication of human cell lines using short tandem repeat profiling is discussed in this article.

CFI statement
Rita Barallon (LGC), Manohar Furtado (Applied Biosystems), Jaiprakash Shewale (Life Technologies) Douglas Storts (Promega Corporation) and Jim Thomson (LGC) work for organizations that provide reagents for or undertake cell line authentication using STR profiling. Manohar Furtado is a shareholder in Applied Biosystems.

Box 3
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Efficient Generation of Hepatoblasts From Human ES Cells and iPS Cells by Transient Overexpression of Homeobox Gene *HEX*

Mitsuru Inamura[1,2], Kenji Kawabata[2,3], Kazuo Takayama[1,2], Katsuhisa Tashiro[2], Fuminori Sakurai[2], Kazufumi Katayama[1,2], Masashi Toyoda[4], Hidenori Akutsu[4], Yoshitaka Miyagawa[5], Hajime Okita[5], Nobutaka Kiyokawa[5], Akihiro Umezawa[4], Takao Hayakawa[6,7], Miho K Furue[8,9] and Hiroyuki Mizuguchi[1,2]

[1]Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [2]Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Osaka, Japan; [3]Department of Biomedical Innovation, Graduate School of Pharmaceutical Science, Osaka University, Osaka, Japan; [4]Department of Reproductive Biology, National Institute for Child Health and Development, Tokyo, Japan; [5]Department of Developmental Biology and Pathology, National Institute for Child Health and Development, Tokyo, Japan; [6]Pharmaceutics and Medical Devices Agency, Tokyo, Japan; [7]Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan; [8]JCRB Cell Bank/Laboratory of Cell Culture, Department of Disease Bioresource, National Institute of Biomedical Innovation, Osaka, Japan; [9]Laboratory of Cell Processing, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the potential to differentiate into all cell lineages, including hepatocytes, *in vitro*. Induced hepatocytes have a wide range of potential application in biomedical research, drug discovery, and the treatment of liver disease. However, the existing protocols for hepatic differentiation of PSCs are not very efficient. In this study, we developed an efficient method to induce hepatoblasts, which are progenitors of hepatocytes, from human ESCs and iPSCs by overexpression of the *HEX* gene, which is a homeotic gene and also essential for hepatic differentiation, using a HEX-expressing adenovirus (Ad) vector under serum/feeder cell-free chemically defined conditions. Ad-HEX-transduced cells expressed α-fetoprotein (AFP) at day 9 and then expressed albumin (ALB) at day 12. Furthermore, the Ad-HEX-transduced cells derived from human iPSCs also produced several cytochrome P450 (CYP) isozymes, and these P450 isozymes were capable of converting the substrates to metabolites and responding to the chemical stimulation. Our differentiation protocol using Ad vector-mediated transient HEX transduction under chemically defined conditions efficiently generates hepatoblasts from human ESCs and iPSCs. Thus, our methods would be useful for not only drug screening but also therapeutic applications.

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INTRODUCTION

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body,[1–4] and thereby have the potential to provide an unlimited source of cells for a variety of applications.[5] Hepatocytes are useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly applicable to drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models, because the liver is the main detoxification organ in the body.[6] For these applications, it is necessary to prepare a large number of functional hepatocytes from human ESCs and iPSCs. Many of the existing methods for cell differentiation of human ESCs and iPSCs into hepatocytes employ undefined, serum-containing medium and feeder cells.[7–9] Preparation of human ESC- and iPSC-derived hepatocytes for therapeutic applications and drug toxicity testing in humans should be done in nonxenogenic culture systems to avoid potential contamination with pathogens. Furthermore, the efficiency of the differentiation of the human ESCs and iPSCs into hepatocytes is not particularly high using these methods.[9–14]

In vertebrate development, the liver is derived from the primitive gut tube, which is formed by a flat sheet of cells called the definitive endoderm.[5,15] Shortly afterwards, the definitive endoderm is separated into endoderm derivatives containing the liver bud, the cells of which are referred to as hepatoblasts. The hepatoblasts have the potential to proliferate and differentiate into both hepatocytes and cholangiocytes. In the process of hepatic differentiation, the maturation is characterized by the expression of liver- and stage-specific genes. For example, α-fetoprotein (AFP) is an early hepatic marker, which is expressed in hepatoblasts in the liver bud until birth, and its expression is dramatically reduced after birth.[16] In contrast, albumin (ALB), which is the most abundant protein synthesized by hepatocytes, is initially expressed at lower levels in early fetal hepatocytes, but its expression level is increased as the hepatocytes mature, reaching a maximum in adult hepatocytes.[17] Furthermore, isoforms of cytochrome P450 (CYP) proteins also exhibit differential expression levels according to the developmental stages

Correspondence: *Hiroyuki Mizuguchi, Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: mizuguch@phs.osaka-u.ac.jp*

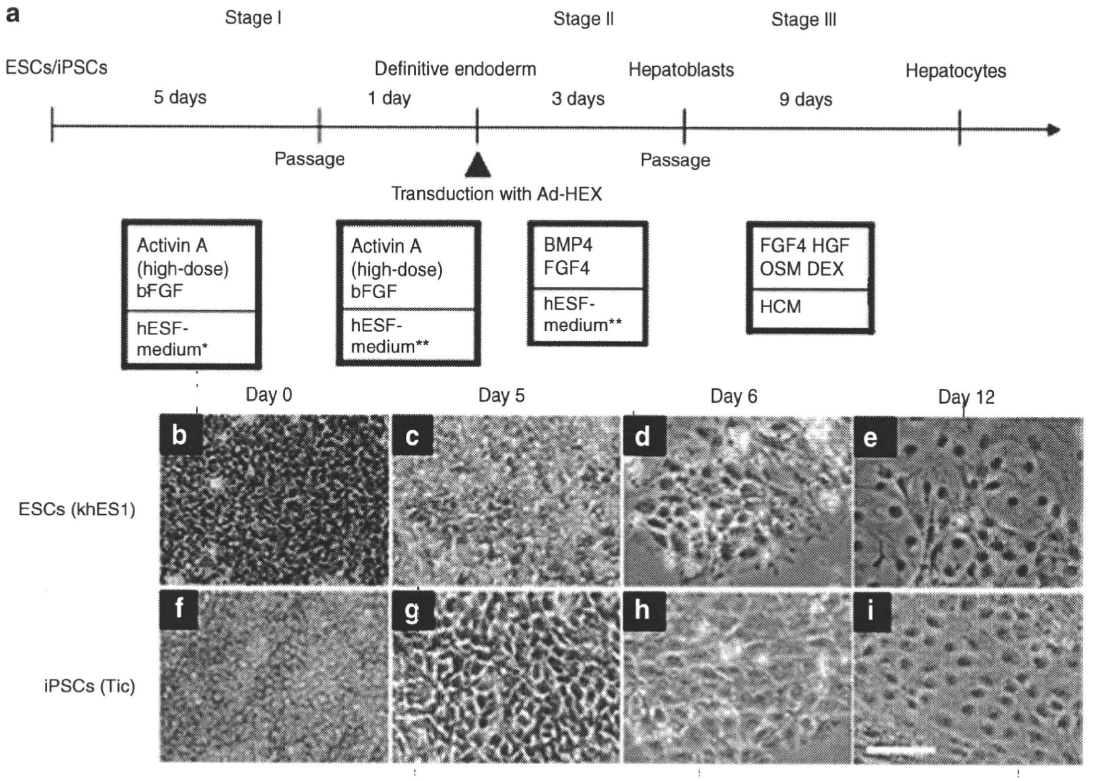

Figure 1 A strategy of differentiation of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to hepatoblasts and hepatocytes. (**a**) Schematic representation illustrating the procedure for differentiation of human ESCs (khES1) and iPSCs (Tic) to hepatocytes. (**b–i**) Phase contrast microscopy showing sequential morphological changes (day 0–12) from (**b–e**) human ESCs (khES1) and (**f–i**) iPSCs (Tic) to hepatoblasts via the definitive endoderm. Bar = 50 µm. bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; DEX, dexamethasone; FGF4, fibroblast growth factor 4; HGF, hepatocyte growth factor; OSM, Oncostatin M; HCM, hepatocytes culture medium; *, hESF-GRO medium that was supplemented with 10 µg/ml human recombinant insulin, 5 µg/ml human apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml fatty acid free BSA; **, hESF-DIF medium that was supplemented with 10 µg/ml insulin, 5 µg/ml apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml BSA.

of the liver. Although most CYPs (including CYP3A4, CYP7A1, and CYP2D6) are only slightly expressed or not detected in the fetal liver tissue, the expression levels are dramatically increased after birth.[18]

For the development of hepatoblasts, numerous transcription factors are required, such as hematopoietically expressed homeobox (*HEX*), GATA-binding protein 6, prospero homeobox 1, and hepatocyte nuclear factor 4A.[15,19] Among them, *HEX* is suggested to function at the earliest stage of hepatic linage.[20] HEX is first expressed in the definitive endoderm and becomes restricted to the future hepatoblasts. Targeted deletion of the *HEX* gene in the mouse results in embryonic lethality and a dramatic loss of the fetal liver parenchyma.[19,21,22] The hepatic genes, including *ALB*, prospero homeobox1, and hepatocyte nuclear factor 4A, are transiently expressed in the definitive endoderm of *HEX*-null embryos, and further morphogenesis of the hepatoblasts does not occur.[23] In general, then, *HEX* is essential for the definitive endoderm to adopt a hepatic cell fate.

Adenovirus (Ad) vectors are one of the most efficient gene delivery vehicles and have been widely used in both experimental studies and clinical trials.[24] Ad vectors are attractive vehicles for gene transfer because they are easily constructed, can be prepared in high titers, and provide high transduction efficiency in both dividing and nondividing cells. We have developed efficient methods for Ad vector-mediated transient transduction into mouse ESCs and iPSCs.[25,26] We have also showed that the differentiations of mouse ESCs and iPSCs into adipocytes and osteoblasts were dramatically promoted by Ad vector-mediated peroxisome proliferator activated receptor γ and runt related transcription factor 2 transduction, respectively.[25,26]

In this study, we hypothesized that transient *HEX* transduction could efficiently induce hepatoblasts from human ESCs and iPSCs. A previous study demonstrated that *HEX* regulates the differentiation of hemangioblasts and endothelial cells from mouse ESCs,[27] whereas the role of *HEX* in the differentiation of hepatoblasts from human ESCs and iPSCs remains unknown. We found that differentiation of hepatoblasts from the human ESC- and iPSC-derived definitive endoderms, but not from undifferentiated human ESCs and iPSCs, could be facilitated by Ad vector-mediated transient transduction of a *HEX* gene. Furthermore, the Ad-HEX-transduced cells that were derived from human iPSCs were able to differentiate into functional hepatocytes *in vitro*. All the processes for cellular differentiation were performed under serum/feeder cell- free chemically defined conditions. Our culture systems and differentiation method based on Ad vector-mediated transient transduction under chemically defined conditions would provide a platform for drug screening as well as safe therapies.

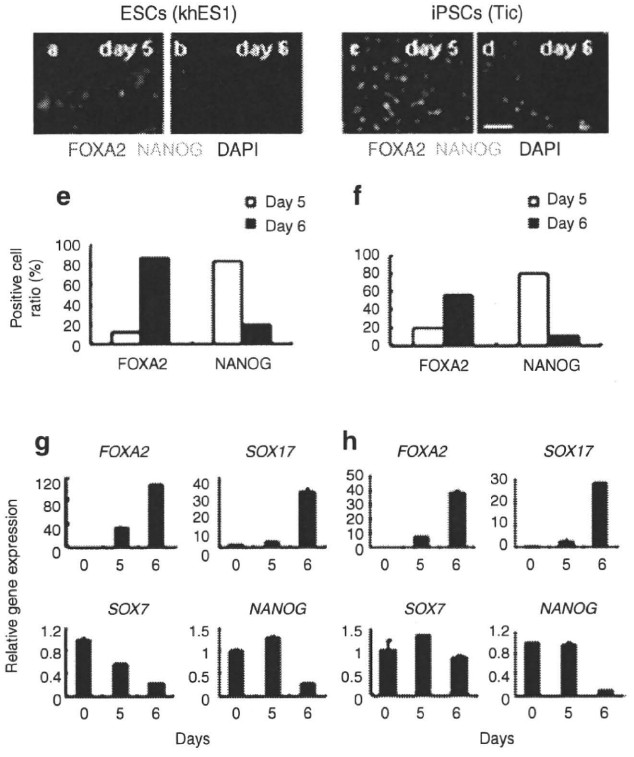

Figure 2 Characterization of the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. (**a–d**) The immunofluorescent staining of the human ESC (khES1)- and iPSC (Tic) derived differentiated cells before (**a** and **c**; day 5) and after passaging (**b** and **d**; day 6). The cells were immunostained with antibodies against FOXA2 and NANOG. Nuclei were stained with DAPI. (**e,f**) Semiquantitative analysis of the immunofluorescent staining in **a–d**. Data are presented as the mean of immunopositive cells counted in eight independent fields. (**g,h**) Real-time RT-PCR analysis of the level of definitive endoderm (*FOXA2* and *SOX17*), pluripotent (*NANOG*), and extra-embryonic endoderm (*SOX7*) gene expression at day 5 and 6. At day 5, the cells were passaged. Therefore, the data at day 5 and 6 show the levels of gene expression before (at day 5) or after the passage (at day 6). Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level of undifferentiated cells at day 0 was taken as 1. Bar = 50 μm. ESC, embryonic stem cells; iPSC, induced pluripotent stem cells.

RESULTS

Differentiation of human ESC- and iPSC-derived definitive endoderms

Our three-step differentiation protocol is illustrated in Figure 1a. After treatment with 50 ng/ml of Activin A (high-dose) and basic fibroblast growth factor (bFGF) for 5 days on a laminin-coated plate, morphologically, the human ESCs and iPSCs were gradually transformed from typical, defined, tight human ESC, and iPSC colonies (day 0) into less dense, flatter cells containing prominent nuclei (day 5), even though the majority of the cells had a morphology resembling that of undifferentiated cells (Figure 1b,c,f,g). FACS analysis showed that ~46% of human iPSC-derived differentiated cells expressed CXCR4 (expressed in the definitive endoderm but not the primitive endoderm) (**Supplementary Figure S1a**). Human ESC- and iPSC-derived differentiated cells were immunostained with the definitive endoderm marker, FOXA2 (Figure 2a,c). However, the majority of the cells expressed the pluripotent marker NANOG, indicating that undifferentiated cells remain in the induced cultures at day 5. After the cells were passaged with trypsin-EDTA and seeded on a laminin-coated plate a second time, the resultant cells were found to be more homogeneous and flatter at day 6 (Figure 1d,h). Semiquantitative analysis by counting immunopositive cells revealed that the number of FOXA2-positive cells was increased and, in turn, the number of NANOG-positive cells was decreased at day 6 after passaging (Figure 2e,f). Real-time reverse transcriptase (RT)-PCR analysis showed that the definitive endoderm markers *FOXA2* and *SOX17* mRNA were upregulated, whereas the pluripotent marker *NANOG* mRNA was downregulated at day 6 (Figure 2g,h). These results were consistent with the immunofluorescence results (Figure 2a–d). The expression levels of the mesoderm marker *FLK1* mRNA and ectoderm marker *PAX6* mRNA were downregulated or unchanged at day 6 (**Supplementary Figure S1b–e**). Importantly, the expression of *SOX7* mRNA (expressed in the extra-embryonic endoderm but not the definitive endoderm) was downregulated (Figure 2g,h). These results indicate that the definitive endoderm is induced or selected from human ESCs and iPSCs after passaging. We obtained the same results using another human iPSC line (**Supplementary Figure S2a–d**).

HEX induces hepatoblasts from the human ESC- and iPSC-derived definitive endoderms

To investigate whether forced expression of transcription factors could promote hepatic differentiation, the human ESC- and iPSC-derived definitive endoderms were transduced with Ad vectors. We used a fiber-modified Ad vector containing the elongation factor-1α promoter and a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob to examine the transduction efficiency in the human ESC- and iPSC-derived definitive endoderms. The elongation factor-1α promoter was found to be highly active in human ESCs.[28] The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing K7 peptides was shown to be efficient for transduction into many kinds of cells.[29,30] The human ESC- and iPSC-derived definitive endoderms were transduced with a LacZ-expressing Ad vector (Ad-LacZ) at 3,000 vector particle/cell. X-Gal staining showed that the Ad-LacZ-transduced human ESC- and iPSC-derived definitive endoderms successfully expressed LacZ (Figure 3). Nearly 100% of the cells transduced with Ad-LacZ were strongly X-gal positive. The transduction efficiency in the human ESC- and iPSC-derived definitive endoderms transduced with the conventional Ad vector containing the wild-type capsid at 3,000 vector particle/cell was ~80% and X-gal staining was much weaker than that in the cells transduced with fiber-modified Ad vectors (**Supplementary Figure S6**).

Next, the human ESC- and iPSC-derived definitive endoderms were transduced with a HEX-expressing fiber-modified Ad vector (Ad-HEX). Although HEX is known to be a transcription factor that is essential for liver development, it remains unclear what the effect of transient *HEX* overexpression is on differentiation from human ESCs and iPSCs or their derivatives *in vitro*. We confirmed the overexpression of *HEX* in the human ESC- and iPSC-derived definitive endoderms transduced with Ad-HEX (**Supplementary Figure S3a–f**). Gene expression analysis revealed the upregulation of *AFP* mRNA, which was expressed by hepatoblasts or early hepatocytes, in Ad-HEX-transduced cells as

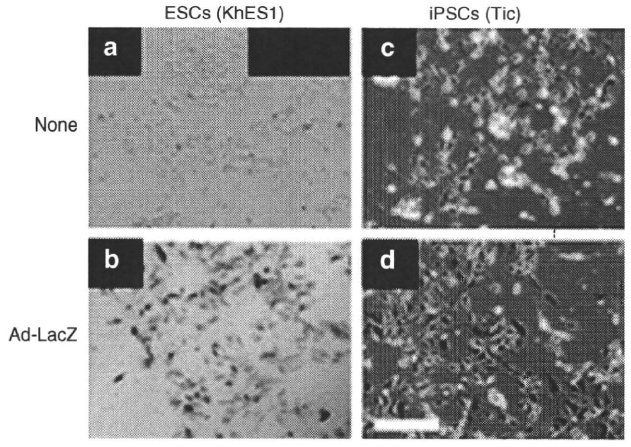

Figure 3 Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1α promoter. (**a,b**) Human ESC (khES1)-derived and (**c,d**) iPSC (Tic) derived definitive endoderms were transduced with 3,000 VP/cell of Ad-LacZ for 1.5 hours. The next day after transduction, X-gal staining was performed as described in the Materials and Methods section. Similar results were obtained in two independent experiments. Scale = 50 μm. Ad, adenovirus; EF-1α, elongation factor-1α; ESC, embryonic stem cells; iPSC, induced pluripotent stem cells; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

compared with nontransduced cells or Ad-LacZ-transduced cells (Figure 4a,c). Expression of *ALB* mRNA, which is the most abundant protein in liver, was also observed in Ad-HEX-transduced cells (Figure 4b,d).

During liver development, both hepatocytes and cholangiocytes were differentiated from the hepatoblasts. We examined the protein expression of AFP, ALB, and the cholangiocyte marker cytokeratin 7 (CK7) in Ad-HEX-transduced cells by immunostaining (Figure 4e–p). The AFP-positive populations were detected in Ad-HEX-transduced cells (Figure 4g,m). ALB-positive cells were also detected, although the detection efficiency was very low (Figure 4j,p). CK7-positive cells were observed among the Ad-HEX-transduced cells, and all CK7-positive cells were found near the AFP- and ALB-positive cells, suggesting that hepatoblasts are generated by the transient overexpression of a *HEX* gene. Semiquantitative RT-PCR analysis showed that the expression levels of the liver-enriched transcription factors hepatocyte nuclear factor 1A, hepatocyte nuclear factor 1B, hepatocyte nuclear factor 4A, and hepatocyte nuclear factor 6 mRNA were upregulated in Ad-HEX-transduced cells (**Supplementary Figure S4a,b**). The expressions of CCAAT/enhancer binding protein α and prospero homeobox 1 mRNA, two transcription factors known to play a pivotal role in the establishment of the hepatoblasts, were also induced in Ad-HEX-transduced cells (**Supplementary Figure S4a, b**). Taken together, these findings indicate that *HEX* enhances the specification of hepatoblasts from the human ESC- and iPSC-derived definitive endoderms. Similar results were obtained with another human iPSC line (**Supplementary Figure S2e–g**).

Time course of differentiation of the definitive endoderm to hepatoblasts

Next, we examined the time course of AFP and CK7 expression during differentiation of human iPSCs to hepatoblasts in Ad-HEX-

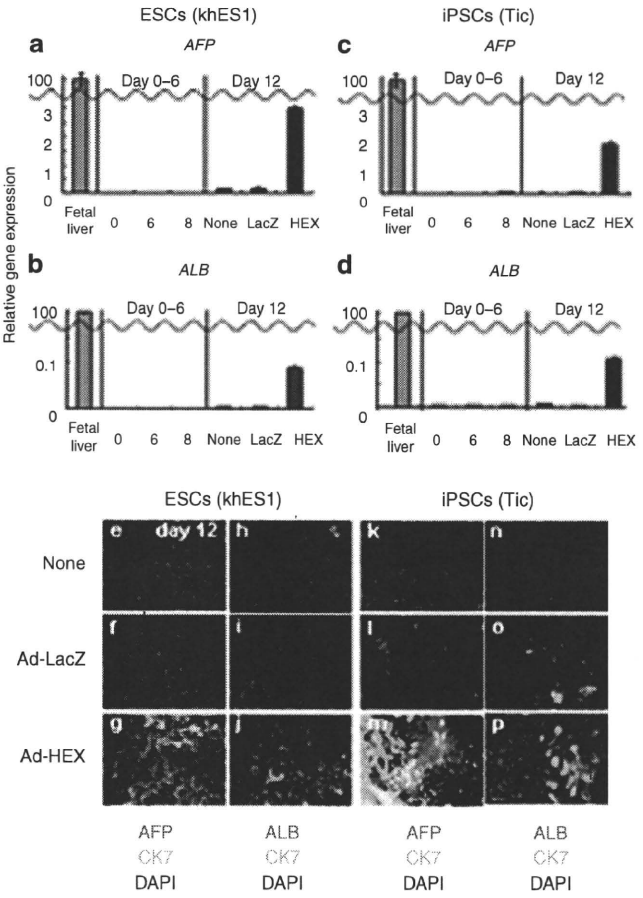

Figure 4 Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by transduction of the *HEX* gene. (**a–d**) Real-time RT-PCR analysis of the level of (**a,c**) *AFP* and (**b,d**) *ALB* expression in nontransduced cells, Ad-LacZ-transduced cells, and Ad-HEX-transduced cells, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms (day 0, 5, 6, and 12). The cells were transduced with Ad-LacZ or Ad-HEX at day 6 as described in **Figure 1a**. The data at day 6 was obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression levels when the level in the fetal liver was taken as 100. (**e–p**) Immunocytochemistry of AFP, ALB, and CK7 expression in nontransduced cells (**e,h,k,** and **n**), Ad-LacZ-transduced cells (**f,i,l,** and **o**), and Ad-HEX-transduced cells (**g,j,m,** and **p**) at day 12, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. Nuclei were stained with DAPI. Bar = 50 μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK7, cytokeratin 7; HEX, Ad-HEX-transduced cells; ESC, embryonic stem cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

transduced cells and nontransduced cells. At day 7 (the day after transduction), the expression of AFP was not detectable in Ad-HEX-transduced or nontransduced cells (**Supplementary Figure S5a,d**). At day 8–9, morphological changes to hepatocyte-like cells were observed in Ad-HEX-transduced cells (**Supplementary Figure S5h,i**). We also observed homogeneous AFP-positive cells at day 9 (**Supplementary Figure S5e**). At day 10, CK7-positive cells appeared, indicating that hepatoblasts started to differentiate into hepatocytes and cholangiocytes at day 9–10 (**Supplementary Figure S5f**). At day 12, ALB-positive cells appeared, indicating that hepatocytes were differentiated from Ad-HEX-transduced cells (Figure 4p). These results showed that *HEX* induces the hepatoblasts from the

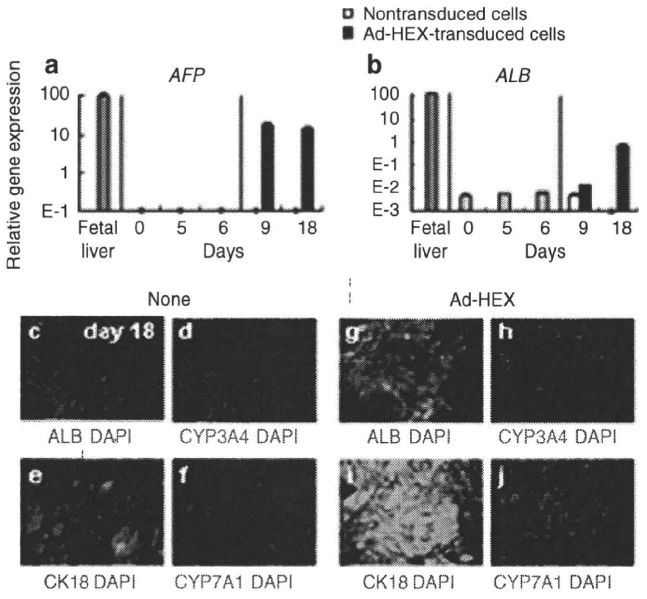

Figure 5 Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes. (**a**,**b**) Real-time RT-PCR analysis of (**a**) *AFP* and (**b**) *ALB* expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPSC (Tic) derived definitive endoderm (day 0, 5, 6, and 12). The cells were transduced with Ad-HEX at day 6 as described in **Figure 1a**. The data at day 6 were obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression level when the level in the fetal liver was taken as 100. (**c**–**j**) Immunocytochemistry of ALB, CYP3A4, CYP7A1, and CK18 expression in (**c**–**f**) nontransduced cells and (**g**–**j**) Ad-HEX-transduced cells, all of which were induced from the human iPSC (Tic) derived definitive endoderm at day 18. Nuclei were stained with DAPI. Bar = 50 μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK18, cytokeratin 18; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; None, nontransduced cells; RT-PCR, reverse transcriptase-PCR.

definitive endoderm, and the Ad-HEX-transduced cells could differentiate into both hepatocytes and cholangiocytes.

Directed hepatic differentiation from hepatoblasts

With the protocol described above, heterogeneous populations containing CK7-positive cholangiocytes were observed at day 12 (Figure 4p). To promote the differentiation of hepatoblasts to hepatocytes, the human iPSC-derived differentiated cells at day 9 (**Supplementary Figure S5e**) were dislodged with trypsin-EDTA and plated on collagen I-coated dishes as previously reported.[11] After 8–11 days in culture with medium containing FGF4, HGF, OSM, and DEX, the Ad-HEX-transduced cells became more flattened (**Supplementary Figure S5m**), whereas the nontransduced cells became fibroblast-like cells (**Supplementary Figure S5i**). Gene expression analysis showed the upregulation of *ALB* mRNA in Ad-HEX-transduced cells under this culture condition, whereas the expression of *ALB* mRNA was reduced in the nontransduced cells at day 18 (Figure 5b). Immunostaining showed that only a small percentage of Ad-HEX-transduced cells expressed ALB at day 12 (Figure 4p), whereas most of the Ad-HEX-transduced cells were ALB-positive at day 18 (Figure 5g). Most of the Ad-HEX-transduced cells also expressed CYP3A4 at day 18 (Figure 5h). More importantly, in the Ad-HEX-transduced cells, CYP7A1 and cytokeratin 18 were detected and these proteins are known

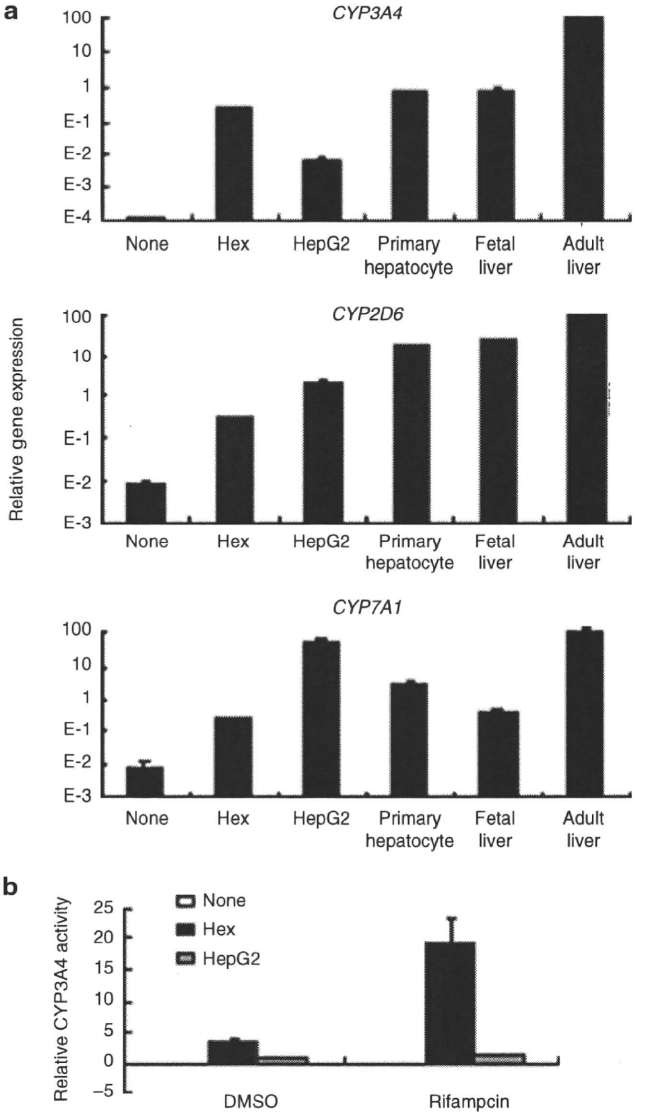

Figure 6 Cytochrome P450 isozymes in human iPSC (Tic) derived hepatocytes. (**a**) Real-time RT-PCR analysis of *CYP3A4*, *CYP7A1*, and *CYP2D6* expression in iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. (**b**) Induction of CYP3A4 by rifampicin in human iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, the HepG2 cell line and primary human hepatocytes, which were cultured 48 hours after plating the cells. Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level in the adult liver was taken as 100. AFP, α-fetoprotein; ALB, albumin; DMSO, dimethyl sulfoxide; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

to be detected in hepatocytes but not in extra-embryonic cells[31,32] (Figure 5i,j). Quantitative analysis showed that ~84, 80, 88, and 92% of Ad-HEX-transduced cells expressed ALB, CYP3A4, CYP7A1, and cytokeratin 18, respectively. These results indicate that Ad-HEX-transduced cells could differentiate to hepatic cells. However, the expression level of *ALB* mRNA in Ad-HEX-transduced cells was lower than that in fetal liver tissue and in turn, the expression of *AFP* mRNA was maintained (Figure 5a). Therefore, Ad-HEX-transduced cells are committed to the hepatic linage, but are not yet mature hepatocytes.

Ad-HEX-transduced cells exhibit hepatic functions

To test the hepatic function in the Ad-HEX-transduced cells, we investigated the liver metabolism, because P450 cytochrome enzymes play a critical role in this function. We examined the expression level of several members of this multigene family, *i.e.*, *CYP3A4*, *CYP7A1*, mRNA and *CYP2D6* in Ad-HEX-transduced cells by real-time RT-PCR. The real-time RT-PCR analysis showed that the mRNAs for *CYP3A4*, *CYP7A1*, and *CYP2D6* were expressed in Ad-HEX-transduced cells, whereas none of these mRNAs were expressed in the nontransduced cells (**Figure 6a**). The expression levels of *CYP3A4* in Ad-HEX-transduced cells were similar to those observed in primary human hepatocytes, which were cultured 48 hours after plating the cells, or fetal liver tissues but lower than those in adult liver. The *CYP2D6* and *CYP7A1* mRNA expressions in Ad-HEX-transduced cells were lower than those in primary hepatocytes or adult tissues. Next, we investigated the metabolism of the P450 3A4 substrates by measuring the activity of P450 isozymes. The metabolites were detected in Ad-HEX-transduced cells, and their activity was 3.4-fold higher than that in the most commonly used human hepatocyte cell line, HepG2 (**Figure 6b**; DMSO column). This result was consistent with the real-time RT-PCR data (**Figure 6a**). We further tested the induction of CYP3A4 upon chemical stimulation, because CYP3A4 is the most prevalent P450 isozyme in the liver and is involved in the metabolism of a significant proportion of the currently available commercial drugs. Because CYP3A4 can be induced with rifampicin, both Ad-HEX-transduced cells and HepG2 cells were treated with rifampicin, followed by treatment with CYP3A4 substrate. Ad-HEX-transduced cells produced 5.4-fold higher levels of metabolites in response to rifampicin treatment (**Figure 6b**; rifampicin column). This result indicates that P450 isozymes are active in Ad-HEX-transduced cells.

DISCUSSION

The object of this study was to develop an efficient method for generating hepatoblasts and hepatocytes from human ESCs and iPSCs for application to drug toxicity screening tests as well as therapeutics such as regenerative medicine. We found that transient HEX transduction in the definitive endoderm together with a culture under chemically defined conditions was useful for this purpose.

It has been reported that a high concentration of Activin A induces differentiation of human ESCs into the definitive endoderm.[8,33,34] On the other hand, undifferentiated human ESCs are maintained by a low concentration of Activin A.[35] Several studies have shown that bFGF promotes the differentiation of ESCs into the definitive endoderm and inhibits the differentiation of ESCs into the extra-embryonic endoderm.[35–38] bFGF has been reported to inhibit the BMP signaling, which can promote the extra-embryonic lineage differentiation.[39] The extra-embryonic endoderm expresses most of the hepatocyte markers, such as AFP.[40] Contamination of the extra-embryonic endoderm makes it difficult to estimate the hepatic differentiation from human ESCs and iPSCs.[11,14,40] In this study, we showed that both Activin A and bFGF induce definitive endoderm populations, while they repress the extra-embryonic endoderm differentiation (**Figure 2g,h**). Interestingly, after the differentiated cells that were cultured on laminin-coated plates with Activin A and bFGF were passaged at day 5, FOXA2-positive cells (definitive endoderm) were enriched in the resultant cells at day 6 (**Figure 2a–f**). This may have been because FOXA2-positive cells efficiently adhered to the laminin-coated plate and/or because trypsinized, single undifferentiated ESCs/iPSCs cannot survive. The passaging of differentiated cells might be attributed to the reduction in the number of not only the extra-embryonic endoderm cells but also the undifferentiated cells. However, the efficiency of the definitive endoderm differentiation in this study was not as efficient as that reported by other groups.[8,33,34] Other cell lineages, such as the mesoderm and extra-embryonic endoderm, might remain at day 6 (**Figure 2g,h** and **Supplementary Figure S1**). Further improvement of the culture conditions will thus be needed in order to enhance the definitive endoderm differentiation.

Hepatoblasts and hepatocytes were differentiated from the human ESC- and iPSC-derived definitive endoderms by transient overexpression of the homeobox gene *HEX*. A fiber-modified Ad vector containing K7 peptides mediated much higher gene expression than conventional Ad vectors in the human ESC- and iPSC-derived definitive endoderms (**Supplementary Figure S6**). This new hepatic differentiation protocol shows that *HEX* induces AFP-positive hepatoblasts at day 9 and ALB-positive hepatocytes at day 12 from human ESCs and iPSCs, whereas the previous protocols require a few weeks or months to induce AFP- and ALB-positive hepatocytes from PSCs.[9–11] Previous studies suggested that *HEX* could regulate liver-enriched transcription factors such as hepatocyte nuclear factor 4A and hepatocyte nuclear factor 6.[19,23] Overexpression of the *HEX* gene under the conditions employed in the present study could activate several transcription factors that are required for hepatic differentiation (**Supplementary Figure S4a,b**). However, the Ad-HEX-transduced cells showed a low level of expression of *ALB* and some CYP450 species, as well as a high level of *AFP* expression, indicating that the cells were still immature. To promote further hepatic differentiation or maturation, it may be effective to culture the hepatic cells in a 3D environment or on feeder cells such as cardiomyocyte- or endothelium-derived cells.[41,42] In addition, the function of our hepatic cells was still limited. Further analysis of the other functions of our hepatic cells, such as glycogen storage, uptake of indocyanine green and organic anion low-density lipoprotein, and transplantation of Ad-HEX-transduced cells into the liver of immunodeficient mice, is clearly needed for the appreciation to drug screening and therapeutic treatment modalities.

During the preparation of this article, Kubo *et al.* have reported that *HEX* could promote hepatoblast differentiation from mouse ESCs.[43] Their report is consistent with our data, suggesting that *HEX* plays a pivotal regulatory role in not only mouse but also human hepatic differentiation. They also showed that the overexpression of *HEX* at the definitive endoderm stage is critical for hepatic specification of the mouse ESCs. We also confirmed that forced expression of *HEX* in the undifferentiated human ESCs and iPSCs did not elevate the expression of *ALB* and *CK7* (**Supplementary Figure S7**), indicating that *HEX* enhances the hepatic differentiation not from the undifferentiated cells but from the definitive endoderm. However, Kubo *et al.* used recombinant mouse ESCs (tet-HEX ESCs), in which the tetracycline-regulated *HEX* expression cassette

is integrated into the host cell genome to induce *HEX* in a stage-specific manner. Their system would not be appropriate for clinical use because the transgene is randomly integrated into the host cell genome and this leads to a risk of mutagenesis.[44] On the other hand, we generated human hepatoblasts by Ad vector-mediated transient *HEX* transduction, method which avoids the integration of exogenous DNA into the host chromosome.

Touboul *et al.* reported that human ESCs and iPSCs can differentiate into functional hepatocytes under chemically defined conditions.[34] In the present study, hepatoblasts were generated in a chemically defined serum-free medium, which minimized exposure to animal cells and proteins, and on a defined extracellular matrix, such as laminin or collagen, which do not contain undefined growth factors. To generate hepatocytes, hepatocyte culture medium, which is serum-free but not defined, was used in the stage III. When defined hESF-medium was used in the stage III, the expression levels of *ALB* and *CYP3A4* mRNA were half the levels seen in the cells cultured with hepatocyte culture medium in the preliminary experiment (data not shown). Human ESCs and iPSCs were also grown for maintaining the undifferentiated state on a feeder layer, which contains xenoantigen such as bovine apolipoprotein B-100. Bovine apolipoprotein B-100 is known to be a dominant xenoantigen for cell-based therapies.[45] Human ESC- and iPSC-derived hepatocytes should be generated and cultured under chemically defined conditions not only to avoid potential contamination with pathogens for the safer therapeutic application, but also to obtain reproducible results using the differentiation protocols.[34,46] Development of differentiation protocols using other genes of transcription factors as well as *HEX* genes based on a chemically defined medium is under way. Overall, our strategy should provide a novel protocol for hepatic differentiation from human ESCs and iPSCs, which could be useful for regenerative medicine and drug screening.

MATERIALS AND METHODS

Ad vectors. Ad vectors were constructed by an improved *in vitro* ligation method.[47,48] The human *HEX* complementary DNA derived from pDNR-LIB-*HEX* (Invitrogen, Carlsbad, CA) was inserted into pHMEF5,[29] which contains the human elongation factor-1α promoter, resulting in pHMEF-HEX. The pHMEF-HEX was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7,[30] resulting in pAd-HEX. Ad-HEX and Ad-LacZ, both of which contain the elongation factor-1α promoter and a stretch of lysine residues (K7) peptides in the C-terminal region of the fiber knob, were generated and purified as described previously.[26,29] The vector particle titer was determined by using a spectrophotometric method.[49]

Human ESCs and iPSCs culture. A human ESC line, khES1, was obtained from Kyoto University (Kyoto, Japan).[50] khES1 was used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan after approval by the review board at Kyoto University. Human ESCs were maintained on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (ICR; ReproCELL Incorporated, Tokyo, Japan) with Dulbecco's modified Eagle's medium/F-12 (Sigma, St Louis, MO) supplemented with 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l nonessential amino acids, 2 mmol/l L-glutamine, 20% GIBCO knockout serum replacement (Invitrogen), and 5 ng/ml bFGF (Sigma) in a humidified atmosphere of 3% CO_2 and 97% air at 37 °C. Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics, Burgess Hill, UK) into small clumps, and subcultured every 5 or 6 days.

Two human iPS clones derived from the embryonic human lung fibroblast cell line MCR5 were provided from JCRB Cell Bank (Tic, JCRB Number: JCRB1331; and Dotcom, JCRB Number: JCRB1327).[3,4] In the present study, we mainly used the Tic cell line, but similar results were obtained using the Dotcom cell line, and these are shown in the supplementary figures. Human iPSCs were maintained on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (Hygro Resistant Strain C57/BL6; Hygro, Millipore, MA) on a gelatin-coated flask in human iPS medium. Human iPS medium consists of knockout Dulbecco's modified Eagle's medium/F12 (Invitrogen), supplemented with 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l nonessential amino acids, 2 mmol/l L-glutamine, 20% knockout serum replacement, and 10 ng/ml bFGF in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Human iPSCs were dissociated with 0.1 mg/ml dispase (Roche) into small clumps and subcultured every 7 or 8 days.

In vitro *differentiation.* Before the initiation of cellular differentiation, the medium of human ESCs and iPSCs was exchanged for a defined serum-free medium hESF9 and cultured in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C.[46] hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with five factors (10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μmol/l 2-mercaptoethanol, 10 μmol/l ethanolamine, 10 μmol/l sodium selenite), oleic acid conjugated with fatty acid free bovine ALB, 10 ng/ml bFGF, and 100 ng/ml heparin (all from Sigma). For induction of definitive endoderm, human ESCs and iPSCs were dissociated into single cells with Accutase (Invitrogen) and cultured for 5 days on a mouse laminin-coated tissue 12-well plate (6.0×10^4 cells/cm^2) in hESF-GRO medium (Cell Science & Technology Institute) supplemented with the five factors, 0.5 mg/ml fatty acid free bovine ALB (BSA) (Sigma), 10 ng/ml bFGF, and 50 ng/ml Activin A (R&D Systems, Minneapolis, MN) in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C. The medium was refreshed every day.

For induction of hepatoblasts, the human ESC- and iPSC-derived definitive endoderms (day 5) were dissociated with 0.0125% trypsin-0.01325 mmol/l EDTA, and then the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). The cells were seeded at 1.2×10^5 cells/cm^2 on a laminin-coated 12-well plate with hESF-DIF (Cell Science & Technology Institute) medium supplemented with the five factors, 0.5 mg/ml BSA, 10 ng/ml bFGF, and 50 ng/ml Activin A in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C. The next day, the cells were transduced with 3,000 vector particle/cell of Ad vectors (Ad-HEX and Ad-LacZ) for 1.5 hours in hESF-DIF medium supplemented with the five factors, BSA, 10 ng/ml FGF4 (R&D Systems) and 10 ng/ml BMP4 (R&D Systems).[10] The medium was refreshed every day.

For induction of hepatocytes, human iPSC-derived hepatoblasts in one well (day 9) were passaged onto two wells with 0.0125% trypsin-0.01325mmol/l EDTA and 0.1% trypsin inhibitor, on type I collagen-coated tissue 12-well plate (15 μg/cm^2) (Nitta Gelatin, Osaka, Japan). The cells were cultured in hepatocyte culture medium supplemented with SingleQuots (Lonza, Walkersville, MD), 10 ng/ml FGF4, 10 ng/ml HGF (R&D Systems), 10 ng/ml Oncostatin M (R&D Systems), and 0.392 ng/ml dexamethasone (Sigma).[11] The medium was refreshed every 2 days.

RNA isolation, RT-PCR, immunostaining, flow cytometry, lacz assay, and assay for cytochrome P4503A4 activity. For details of these procedures, See **Supplementary Materials and Methods, Supplementary Tables S1 and S2.**

SUPPLEMENTARY MATERIAL

Figure S1. Characterization of the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms.

Figure S2. Efficient differentiation of another human iPSC line (Dotcom) into hepatoblasts by overexpression of the *HEX* gene.

Figure S3. Overexpression of *HEX* in the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms.

Figure S4. Characterization of Ad-HEX-transduced hepatoblasts.
Figure S5. Progression of differentiation of the definitive endoderm to hepatoblasts.
Figure S6. X-gal staining of human iPSC (Tic)-derived definitive endoderms transduced with a conventional or a fiber-modified Ad vector containing the EF-1α promoter.
Figure S7. *HEX* promotes the differentiation into the hepatic lineage, not from undifferentiated iPSCs (Tic), but from iPSC (Tic)-derived definitive endoderm.
Table S1. List of Taqman gene expression assays and primers.
Table S2. List of antibodies used.
Materials and Methods.

ACKNOWLEDGMENTS

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Reduction of N-Glycolylneuraminic Acid in Human Induced Pluripotent Stem Cells Generated or Cultured under Feeder- and Serum-Free Defined Conditions

Yohei Hayashi[1☯], Techuan Chan[1☯], Masaki Warashina[2], Masakazu Fukuda[3], Takashi Ariizumi[1], Koji Okabayashi[1], Naoya Takayama[4], Makoto Otsu[4], Koji Eto[4], Miho Kusuda Furue[5], Tatsuo Michiue[1], Kiyoshi Ohnuma[1,6*], Hiromitsu Nakauchi[4], Makoto Asashima[1,7*]

1 Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan, **2** Genome Research Laboratories, Wako Pure Chemical Industries, Ltd., Hyogo, Japan, **3** Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo, Japan, **4** Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **5** Laboratory of Cell Cultures, Department of Disease Bioresources, National Institute of Biomedical Innovation, Osaka, Japan, **6** Top Runner Incubation Center for Academia-Industry Fusion, Nagaoka University of Technology, Nagaoka, Japan, **7** Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Ibaraki, Japan

Abstract

Background: The successful establishment of human induced pluripotent stem cells (hiPSCs) has increased the possible applications of stem cell research in biology and medicine. In particular, hiPSCs are a promising source of cells for regenerative medicine and pharmacology. However, one of the major obstacles to such uses for hiPSCs is the risk of contamination from undefined pathogens in conventional culture conditions that use serum replacement and mouse embryonic fibroblasts as feeder cells.

Methodology/Principal Findings: Here we report a simple method for generating or culturing hiPSCs under feeder- and serum-free defined culture conditions that we developed previously for human embryonic stem cells. The defined culture condition comprises a basal medium with a minimal number of defined components including five highly purified proteins and fibronectin as a substrate. First, hiPSCs, which were generated using Yamanaka's four factors and conventional undefined culture conditions, adapted to the defined culture conditions. These adapted cells retained the property of self renewal as evaluated morphologically, the expression of self-renewal marker proteins, standard growth rates, and pluripotency as evaluated by differentiation into derivatives of all three primary germ layers *in vitro* and *in vivo* (teratoma formation in immunodeficient mice). Moreover, levels of nonhuman N-glycolylneuraminic acid (Neu5Gc), which is a xenoantigenic indicator of pathogen contamination in human iPS cell cultures, were markedly decreased in hiPSCs cultured under the defined conditions. Second, we successfully generated hiPSCs using adult dermal fibroblast under the defined culture conditions from the reprogramming step. For a long therm culture, the generated cells also had the property of self renewal and pluripotency, they carried a normal karyotype, and they were Neu5Gc negative.

Conclusion/Significance: This study suggested that generation or adaption culturing under defined culture conditions can eliminate the risk posed by undefined pathogens. This success in generating hiPSCs using adult fibroblast would be beneficial for clinical application.

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* E-mail: kohnuma@vos.nagaokaut.ac.jp (KO); asashi@bio.c.u-tokyo.ac.jp (MA)

☯ These authors contributed equally to this work.

Introduction

Human induced pluripotent cells (hiPSCs) generated by the introduction of defined factors from somatic cells exhibit pluripotency similar to human embryonic stem cells (hESCs) [1,2]. The broad developmental potential of hiPSCs makes them a possible source of cells for the regenerative medical transplantation of various tissues. However, before hiPSC-derived cells can be used in human transplantation, a number of safety concerns need to be overcome. One such concern is the risk of contamination by undefined pathogens or immunoreactive materials from undefined components used in the culturing of hiPSCs [3]. N-Glycolylneur-

aminic acid (Neu5Gc) has been identified as an immunoreactive material that contaminates cells in culture. Neu5Gc, a sialic acid found on the cell surface, is considered a xenoantigen for humans because human cells cannot produce Neu5Gc genetically [4], although it can be taken up from the culture environment [5,6]. Furthermore, most humans have circulating antibodies specific for Neu5Gc. Contamination of hESCs by Neu5Gc was confirmed following culturing under conventional conditions with mouse embryonic fibroblast (MEF)-derived feeder cells and knockout serum replacement (KSR)-supplemented medium [7,8]. Neu5Gc could therefore be a useful indicator of pathogen contamination in pluripotent stem cell cultures.

Defined culture conditions are therefore required when using hiPSC to avoid contamination from undefined pathogens or immunoreactive materials [7]. KSR-supplemented medium is not defined and thus may contain a variety of contaminating factors [9,10,11]. Based on previous findings indicating that the phenotypes of hiPSCs are similar to those of hESCs [1,2], we hypothesized that hESC culture conditions could also be used for hiPSCs. Previously, we developed a defined serum-free medium, namely hESF9, for culturing hESCs on a type I collagen substrate without feeders [12]. Although several defined culture conditions without feeders for hESCs have been reported, difficulties remain in propagating the undifferentiated hESCs [13,14,15,16]. Recently, we found that adding activin A to hESF9 medium supports robust propagation of hES cells and enhances the stable attachment of these cells to fibronectin [16]. We modified our medium accordingly and subsequently cultured our hESCs on a fibronectin substrate without feeders. The modified medium (hESF9a) comprises a basal medium supplemented with heparin sulphate and five highly purified proteins: bovine pancreatic insulin, human apotransferrin, fatty acid-free bovine serum albumin conjugated with oleic acid, human recombinant fibroblast growth factor (FGF)-2, and human recombinant activin [16].

In the present study, we generated hiPSCs from skin keratinocytes using conventional culture conditions with KSR and feeder cells [17]. The cells were then moved into defined culture conditions in hESF9a medium on fibronectin without feeders. We confirmed that the hiPSCs cultured under defined conditions were pluripotent stem cells on the basis of cell morphology, growth rate, the expression of self-renewal genes, cell differentiation *in vitro*, and teratoma formation *in vivo*. Furthermore, we observed that levels of Neu5Gc decreased steadily in hiPSCs cultured under defined conditions. Finally, we generated hiPSCs from adult dermal fibroblast under defined conditions. For long-term culture, these hiPSCs maintained their pluripotency and normal karyotype.

Results and Discussion

Generation of the hiPSC line and adaptation to defined culture conditions

The hiPSC cell line was established from neonatal skin keratinocytes by the infection of amphotropic retroviruses carrying the *OCT4*, *SOX2*, *KLF4*, and C-*MYC* genes using conventional culture conditions with KSR medium and mitomycin C-treated MEF feeder cells (KSR-based conditions) [1,17,18]. Under the KSR-based conditions, cells maintained their undifferentiated morphology and expressed markers of pluripotency, namely ALP (Figure 1B), NANOG (Figure 1E), OCT3/4 (Figure 1H), SSEA4 (Figure 1N), and TRA1-60 (Figure 1Q), shown by immunocytochemistry or substrate staining for ALP. However, the cells did not express stage-specific embryonic antigen (SSEA)-1, which is a self-renewal marker of murine ES cells and a differentiation marker of hESCs (Figure 1K). The cell line was designated UTA1.

At passage 18, the UTA1 cells were transferred into the defined culture conditions with hESF9a medium on fibronectin-coated dishes without feeder cells (hESF9a-based conditions) [12,16]. After a further five passages, the UTA1 cells also expressed the self-renewal markers, ALP (Figure 1C), NANOG (Figure 1F), OCT3/4 (Figure 1I), SSEA4 (Figure 1O), and TRA1-60 (Figure 1R); however, there was no detectable expression of SSEA1 under either culture condition (Figure 1L), suggesting that hiPSCs grown in the hESF9a-based conditions maintained their undifferentiated characteristics. The UTA1 cells steadily proliferated under hESF9a-based conditions for a prolonged culture period, as in the conventional KSR-based conditions (Figure S1). We continued to culture the UTA1 cells in hESF9a-based condition up to 27 passages. These results suggested that the UTA1 cells cultured under the hESF9a-based conditions retained the property of self-renewal.

In vitro and in vivo differentiation of hiPSCs under defined culture conditions

Differentiation potential in the UTA1 cells grown under KSR- and hESF9a-based conditions was assessed using *in vitro* differentiation assays involving embryoid body generation. After 24 days in differentiation culture conditions, the embryoid bodies contained various types of differentiated cells characterized by germ-layer markers as follows: MAP2 (Figure 2A, B) and TUJ1 (Figure 2C, D) as ectoderm markers; FLK1 (Figure 2E, F) and vimentin (Figure 2G, H) as mesoderm markers; and PDX1 as an endoderm marker (Figure 2I, J). We also validated the pluripotency of UTA1 cells under KSR-based conditions (17 passages) and hESF9a-based conditions (18 passage in KSR-based condition and 12 passage in ESF9a-based condition) by teratoma formation in the testes of severe combined immunodeficient (SCID) mice injected with hiPSCs. Eight weeks after injection, histological analysis demonstrated that the formed teratomas were derived from all three primary germ layers. Neural tissues (ectoderm), muscle (mesoderm), cartilage (mesoderm), and intestinal epithelia (endoderm) were all identified histologically in the hiPSC-derived teratomas (Figure 3). These results suggested that UTA1 cells remained pluripotent to differentiate into all three germ layers when grown under hESF9a-based conditions.

The level of xenoantigen Neu5Gc in the hiPSC under defined culture conditions

Our data showed that hESF9a culture conditions maintain the pluripotency of hiPSCs. Conventional culture conditions currently use KSR for human ES/iPS cells, and it is accepted that these commercially supplied components may contain undefined animal-derived xenoantigens and pathogens. Because human cells cannot produce Neu5Gc genetically [4], it becomes a useful indicator of xenogenic contamination in human pluripotent stem cells [7]. We therefore examined the expression of Neu5Gc in UTA1 cells grown under KSR- and hESF9a-based conditions by flow cytometry using an antibody against Neu5Gc. The level of Neu5Gc was high in UTA1 cells cultured under the KSR-based conditions (23 passages) and was comparable with that in Chinese hamster ovary (CHO) cells cultured in fetal calf serum (FCS)-containing medium (Figure 4A, B). The Neu5Gc expression decreased almost to negative control levels (with control antibody or no primary antibody) in UTA1 cells cultured under the hESF9a-based conditions (18 passages in KSR-based condition and 27 passages in ESF9a-based condition) (Figure 4C). The conventional culture conditions contain animal-derived compo-

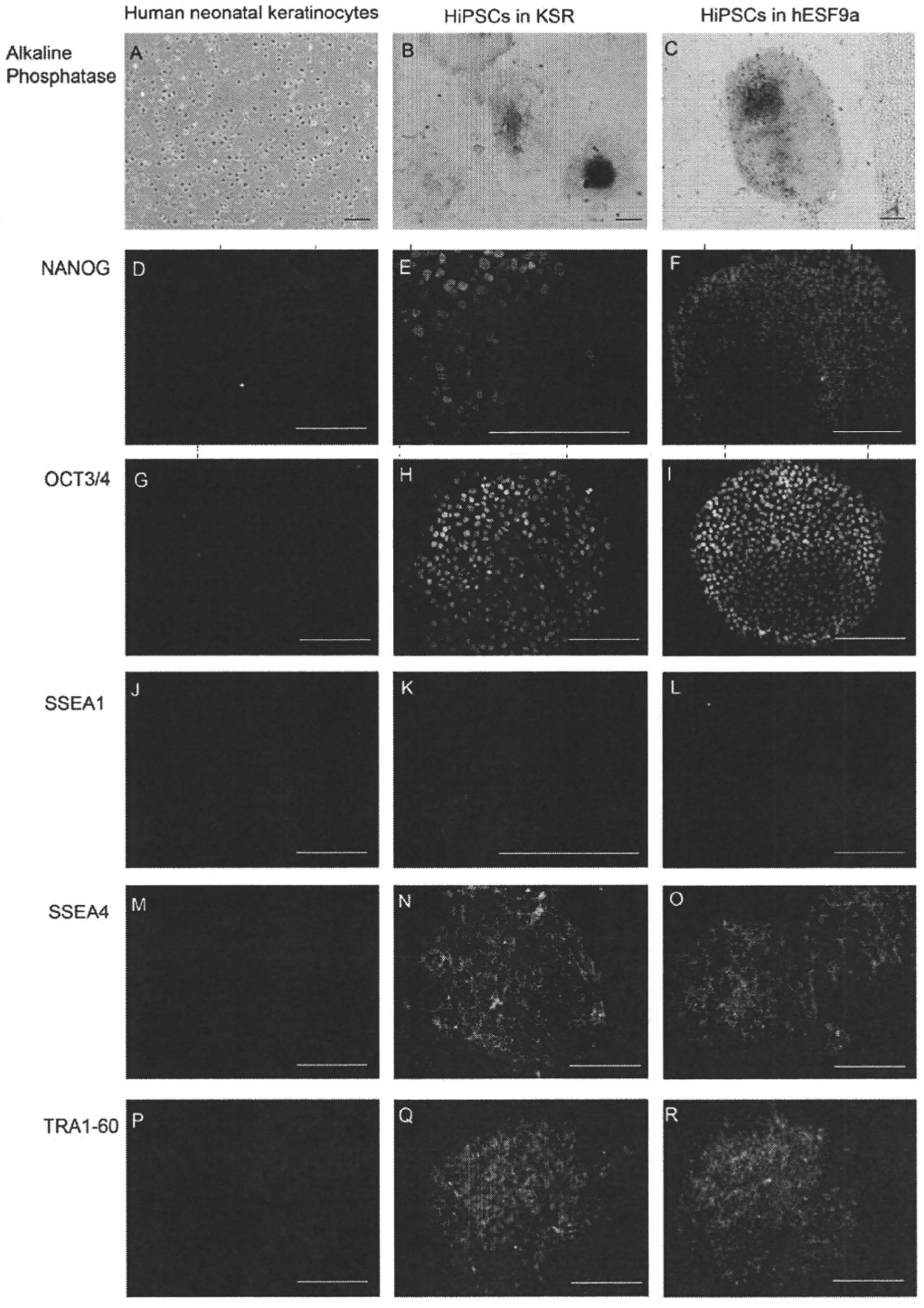

Figure 1. Self-renewal marker expression of pluripotent stem cells in hiPSCs adapted in defined culture conditions. Parental human neonatal keratinocytes (A, D, G, J, M, and P), hiPSC line, UTA1, grown under KSR-based conditions (B, E, H, K, N, and Q), and UTA1 grown under hESF9a-based culture conditions for 5 passages (C, F, I, L, O, and R) were fixed and reacted with antibodies (or stained with alkaline phosphatase substrate, Fast Red). (A–C): Alkaline phosphatase staining. (D–F): Immunocytochemistry of NANOG protein. (G–I): Immunocytochemistry of OCT3/4 protein. (J–L): Immunocytochemistry of SSEA1 antigen. (M–O): Immunocytochemistry of SSEA4 antigen. (P–R): Immunocytochemistry of TRA1-60 antigen. Binding of these antibodies was visualized with AlexaFluor 488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). Scale bars represent 50 μm.
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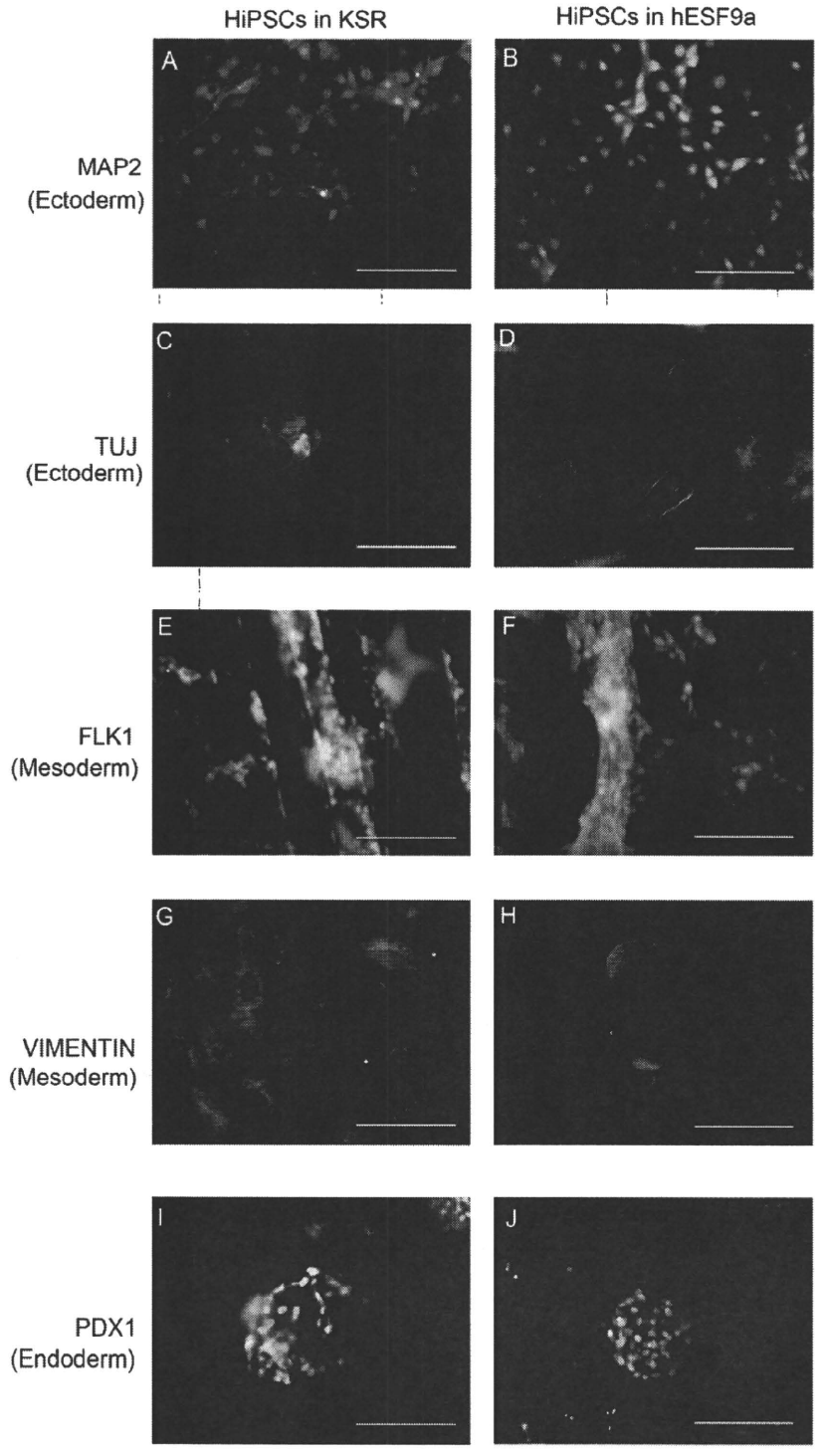

Figure 2. *In vitro* differentiation using embryoid bodies from hiPSCs adapted in defined culture conditions. Immunocytochemistry of MAP2 (A, B), TUJ (C, D), FLK1 (E, F), vimentin (G, H), and PDX1 (I, J) in the differentiated hiPSC line, UTA1, grown under KSR-based conditions (A, C, E, G, I) or hESF9a-based conditions (B, D, F, H, J). Differentiation was performed using embryoid body formation, and the differentiated cells were fixed and reacted with antibodies. Binding of these antibodies was visualized with AlexaFluor 488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). Scale bars represent 50 μm.
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