scientists is, at this time, preparing a consensus standard on the authentication of human cells using short tandem repeat (STR) profiling. This standard, which will be submitted for review and approval as an American National Standard by the American National Standards Institute, will provide investigators guidance on the use of STR profiling for authenticating human cell lines. Such guidance will include methodological detail on the preparation of the DNA sample, the appropriate numbers and types of loci to be evaluated, and the interpretation and quality control of the results. Associated with the standard itself will be the establishment and maintenance of a public STR profile database under the auspices of the National Center for Biotechnology Information. The consensus standard is anticipated to be adopted by granting agencies and scientific journals as appropriate methodology for authenticating human cell lines, stem cells, and tissues.

Keywords Cell authentication · STR profiling · Consensus standard · Quality control

Introduction

Animal and human primary cell cultures, continuous (immortalized) cell lines, and tissues are of overwhelming importance to the biopharmaceutical industry and to biomedical research as reagents, therapeutic modalities, and as proxy materials for the study of more complex physiological systems. Cell cultures have, from the beginning, been at risk for misidentification due to labeling errors, incorrect classification by pathologists, and cross-contamination with other cell types. Continuous cell lines are potentially jeopardized due to the extended time these are in culture and the frequent manipulations involved in the course of feeding and subculturing. Human stem cell preparations which are propagated in the presence of non-human feeder cell layers are at risk of cross-contamination with the feeder cells. Tumor cells propagated by xenografting onto host animals are at risk of cross-contamination with the host cells.

We know the risks involved in establishing and maintaining cell cultures. We know that periodic identity testing (authentication) is the only way to prove that the cell we are studying is the cell that we believe it to be, and not a contaminating tumor cell line such as HeLa. Why then are many investigators blindly assuming that they are using correctly identified cells? Recent publications appear to indicate that the problem of cell misidentification is not going away. For instance, Berglind et al. (2008) evaluated the p53 status of 1,211 cell lines published between 1989 and 2007 and found discrepancies in the p53 status for 23% of the cell lines. Schweppe et al. (2008) evaluated 40 human thyroid cancer cell lines and found that only 23 of these actually had unique genetic profiles, as determined using short tandem repeat (STR) profiling and single nucleotide polymorphism analysis. Certain of the presumed thyroid cancer cell lines were found to have profiles matching colon cancer or melanoma cells. Another recent revelation was that of Boonstra et al. (2010) indicating that three widely used esophageal cancer cell lines are, in fact, derived from other tumor types. Dittmar et al. (2010) have reported two new cases of misidentification of supposed human cells. Their work clearly demonstrates that phenotypic evaluation alone cannot provide adequate assurance of the authenticity of a cell line. More extensive lists of misidentified cells are available from a number of sources (e.g., ATCC SDO Workgroup ASN-0002 2010; Capes-Davis et al. 2010).

Within the highly regulated biopharmaceutical industry, cell lines used as production substrates must be characterized for identity through phenotypic analysis and confirmation of

M. Nardone
Bio-Trac Program, The Foundation for the Advanced Education in the Sciences at the National Institutes of Health,
Bethesda, MD 20892, USA

R. M. Nardone
Catholic University of America, Cell and Molecular Biology,
620 Michigan Ave NE,
Washington, DC 20064, USA

R. W. Nims
RMC Pharmaceutical Solutions, Inc.,
2150 Miller Drive, Suite A,
Longmont, CO 80501, USA

P. J. Price
D-Finitive Cell Technology, Room B-33,
1023 Wappoo Rd,
Charleston, SC 29407, USA

A. F. Steuer
BioReliance,
14920 Broschart Road,
Rockville, MD 20850, USA

D. R. Storts
Nucleic Acid Technologies, Promega Corporation,
2800 Woods Hollow Road,
Madison, WI 53711, USA

Z. Taraporewala
Division of Cellular and Gene Therapies, Office of Cellular, Tissue, and Gene Therapies,
FDA/Center for Biologics Evaluation and Research,
1401 Rockville Pike, Room 200N,
Rockville, MD 20852, USA

Y. A. Reid · G. Sykes · C. Alston-Roberts · L. Kerrigan
American Type Culture Collection,
10801 University Blvd.,
Manassas, VA 20110, USA

animal species of origin (US FDA 1993). This, together with implementation of current good manufacturing practices, is believed to have contributed to the relatively low frequency of cell line misidentification reported in this industry (Nims and Herbstritt 2005).

Remediation of the problem of cell line misidentification within the biomedical research community may eventually need to be driven by requirements for authentication from granting agencies and journal editors. An international group of scientists is now preparing a consensus standard which will provide investigators with guidance on the appropriate methodology for authenticating human cells. In this article, we describe the rationale for and the process involved in preparing this standard.

Efforts to Remediate the Problem of Cell Misidentification

The earliest efforts toward tackling the problem of cell misidentification centered on disclosure of the issue through conference presentations and publications. Gartler (e.g., Gartler 1967) and Nelson-Rees (e.g., Nelson-Rees et al. 1974) were among the first and most vocal of those attempting to convince the scientific community of the seriousness of the issue. They hoped that such disclosures would motivate scientists to voluntarily take actions to remediate the problem.

More recently, Roland Nardone championed a series of efforts intended to reemphasize the seriousness of the cell misidentification problem and take any required steps to begin remediating the various causes for the continuing issue. His efforts began with the authoring of a white paper, entitled "Eradication of Cross-Contaminated Cell Lines: A Call for Action" (Nardone 2007). This paper presented recommendations for strict compliance measures in addition to continuing efforts to educate scientists. Nardone believed the time had come for granting agencies to demand cell line authentication as a condition for the receipt of funds and for journals to add a similar requirement to their instructions for authors for manuscripts submitted for publication.

As part of his efforts to convince granting agencies of the need for their participation in his overall remediation strategy, Nardone and a group of prominent cell scientists composed and signed an open letter to Michael O. Leavitt, Secretary of Health and Human Resources (Nardone et al. 2007), beseeching the NIH to take appropriate actions. On November 28, 2007, the NIH published an addition to their Guidelines for Research—Notice Regarding Authentication of Cultured Cell Lines (National Institutes of Health 2007) calling for diligence and more careful peer review.

Communications between Nardone (and others) and journal editors have achieved the desired result as slowly and surely, journals are beginning to add the requirement for cell authentication to their instructions for authors (e.g., *Cell Biochemistry and Biophysics*, *In Vitro Cellular & Developmental Biology—Animal*, *International Journal of Cancer*, and the journals of the American Association for Cancer Research).

Attempts to educate scientists in general of the need for cell authentication must go beyond simply raising the level of awareness of the problem. In his white paper, Nardone also stressed the need for training in cell authentication to be added to conference agendas. He recommended that societies sponsor conferences, workshops, and/or training activities to facilitate the adoption of cell line authentication standards (Nardone 2007).

As the requirement for cell authentication is adopted by granting agencies and scientific journals, the need for standardized methods and expectations regarding authentication itself to be defined becomes more critical. Recognizing this, an effort to prepare a consensus standard on authentication of human cells was initiated.

The Concept of the Consensus Standard

The idea of the consensus standard is to allow a greater input from the overall international biomedical community into standards. The derivation of a standard through the consensus process improves the chance of universal voluntary acceptance. In turn, that acceptance will foster reproducibility and comparability of research employing human cells. Such a consensus-driven standard, if universally adopted, should ultimately lead to a marked decrease in the misidentification of human cells used by the biomedical community.

The ATCC® Standards Development Organization

The mission of the ATCC® Standards Development Organization (SDO) is to develop best practices (standards) for use in the life science industry and to promote their global use, using a consensus-driven process that balances the viewpoints of the stakeholder community. Membership is free and open to all stakeholders in the biomedical community, including those involved in the development, production, application, and regulation of life science products. Stakeholders include, but are not limited to, members from academia, government, regulatory, and industry. All members are participants in the consensus review, comment, and voting process.

In 2007, the SDO became the first biological resource organization to become an American National Standards Institute (ANSI)-accredited standards development organi-

zation. ANSI accreditation ensures that procedures used by standards developers meet requirements for openness, balance, consensus, and due process.

The standard development process employed by the ATCC® SDO is shown in Fig. 1.

ATCC SDO Workgroup ASN-0002

ATCC® SDO workgroup ASN-0002 "Development of a consensus standard for the authentication of human cells: standardization of STR profiling" was formally assembled in early 2009 as a result of a proposal submitted in 2008 by John Masters and Roland Nardone. The workgroup constitutes an international group of concerned and experienced scientists. Chaired by Masters, the workgroup includes individuals with relevant and current experience in DNA profiling technologies, as well as "stakeholders" or representatives from major cell repositories, industry, academia, and government agencies.

Preparation of the Standard. Working under the auspices of the ATCC SDO, the ASN-0002 workgroup has met monthly since early 2009. The overall effort was divided between two subgroups which have met independently at monthly or more frequent intervals.

The first subgroup is charged with drafting the introduction to the Standard, defining what is meant by "human cell line authentication," describing the historical aspects, from early discovery of cell line misidentification through to the present efforts encouraging remediation of the problem. The subgroup also is delineating the causes of cell line misidentification, surveying the existing technologies for cell line authentication, and providing the rationale for selection of STR profiling for the Standard. The subgroup is chaired by Raymond Nims.

The second subgroup, chaired by Yvonne Reid, is fleshing out the procedural details of the general protocol to be recommended for STR profiling. This subgroup is also responsible for determining the format and structure of an associated public database of STR profiles of

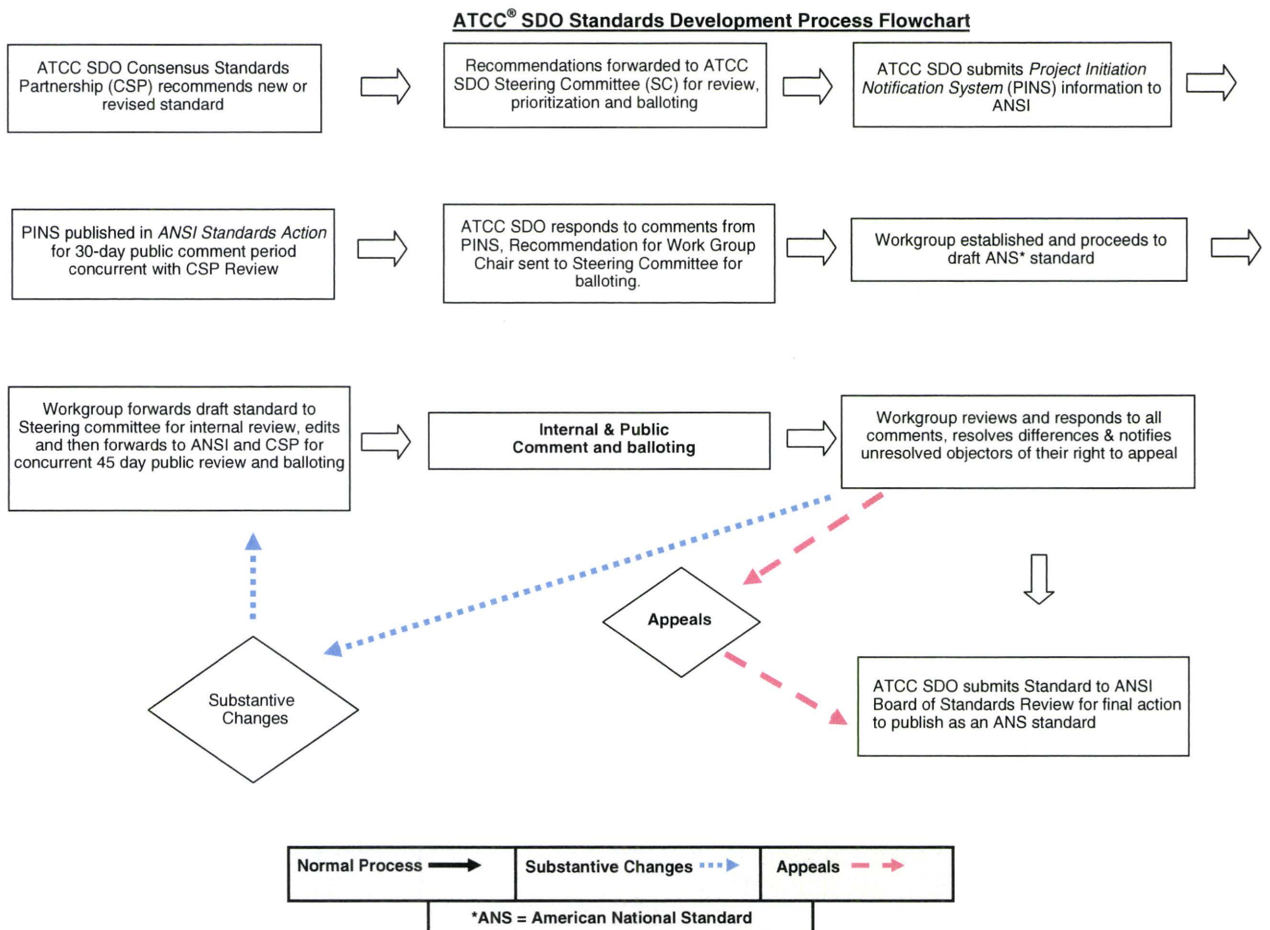

Figure 1. ATCC SDO standards development process flowchart.

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

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

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

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

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

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

Anticipated Flow and Impact

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

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

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

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

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Growth factor-defined culture medium for human mesenchymal stem cells

SUMIYO MIMURA[1,2,#], NAOHIRO KIMURA[3,#], MITSUHI HIRATA[1], DAIKI TATEYAMA[1], MIDORI HAYASHIDA[1], AKIHIRO UMEZAWA[4], ARIHIRO KOHARA[1], HIROKI NIKAWA[2], TETSUJI OKAMOTO[3] and MIHO K. FURUE[*,1]

[1]JCRB Cell Bank, Laboratory of Cell Cultures, Department of Disease Bioresources, National Institute of Biomedical Innovation, Osaka, [2]Department of Oral Biology and Engineering, Division of Oral Health Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, [3]Department of Molecular Oral Medicine and Maxillofacial Surgery, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima and [4]Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, Tokyo, Japan

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

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

Introduction

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

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

***Address correspondence to:** Miho Kusuda Furue. Laboratory of Cell Cultures, Department of Disease Bioresources, National Institute of Biomedical Innovation, 7-6-8 Saito Asagi, Ibaraki, Osaka, 567-0085, Japan. Fax: +81-72-641-9851. e-mail: mkfurue@nibio.go.jp **#Note:** These authors contributed equally to this work.

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

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

Results

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

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

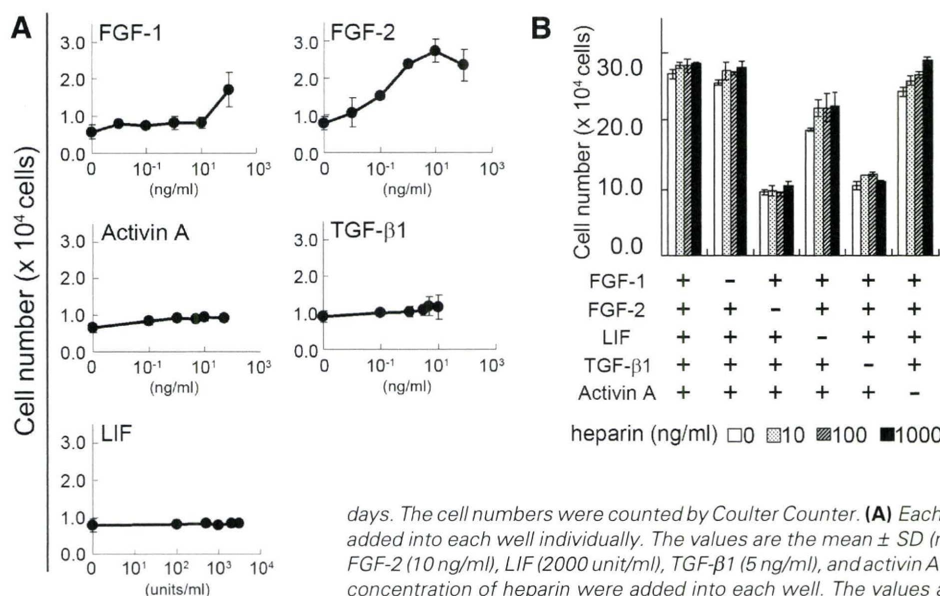

Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. *After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-) at 1 x10^4 cells per well and cultured for 6 days. The cell numbers were counted by Coulter Counter.* **(A)** *Each growth factor at indicated concentration was added into each well individually. The values are the mean ± SD (n=3).* **(B)** *All five factors of FGF-1 (100 ng/ml), FGF-2 (10 ng/ml), LIF (2000 unit/ml), TGF-β1 (5 ng/ml), and activin A (10 ng/ml) or four factors of them with varying concentration of heparin were added into each well. The values are the mean ± SD (n=3).*

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

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

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

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

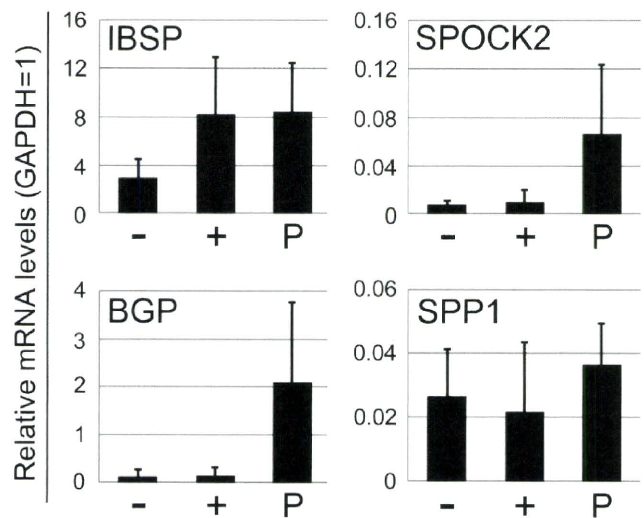

Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. *The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression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

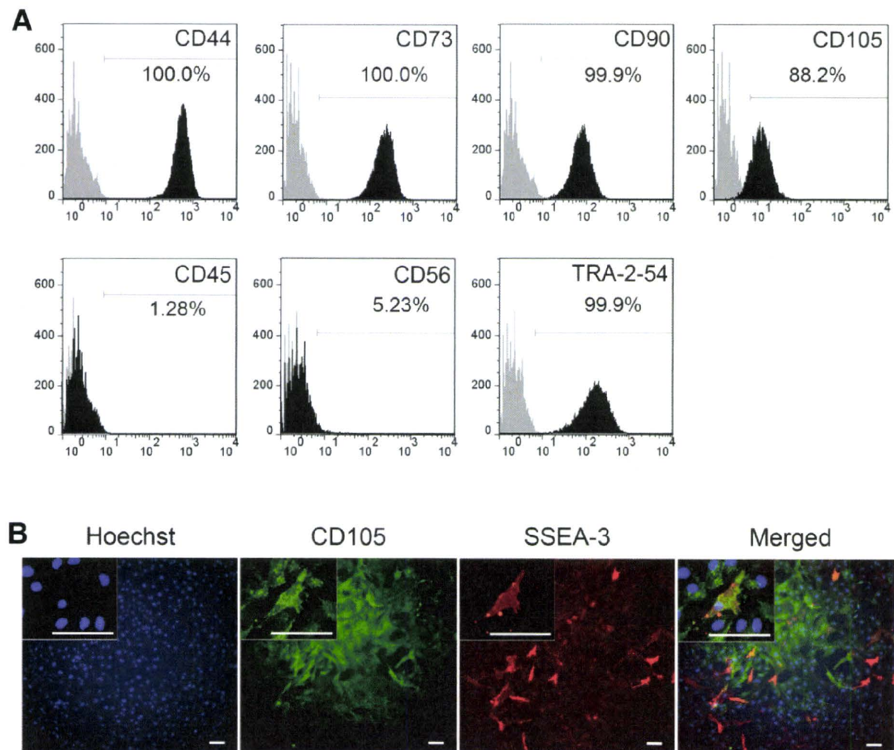

Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) *Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal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

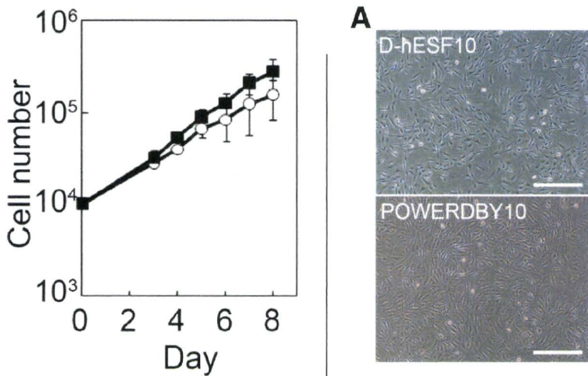

Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. *The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density of 1x10⁴ cells per well. Cell numbers were counted every day. The values are the mean ± SD (n=3).*

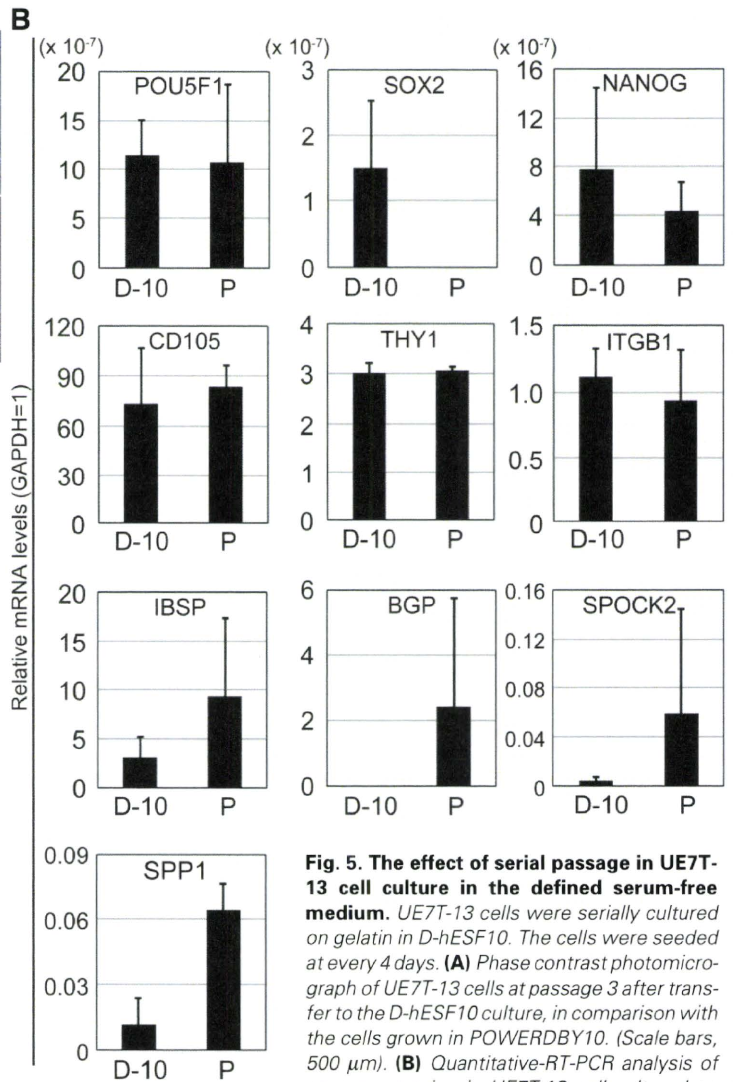

Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. *UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days.* **(A)** *Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture,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

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

Discussion

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

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

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

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

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

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

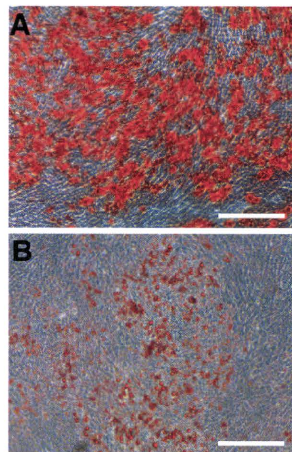

Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. *The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium.* **(A)** *Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were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

Materials and Methods

Cell Cultures

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

Serum-free Cell Culture Media

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

Cell proliferation

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

Gene expression

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

Antigen expression

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

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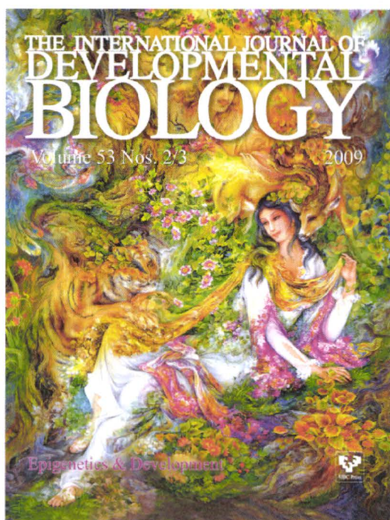

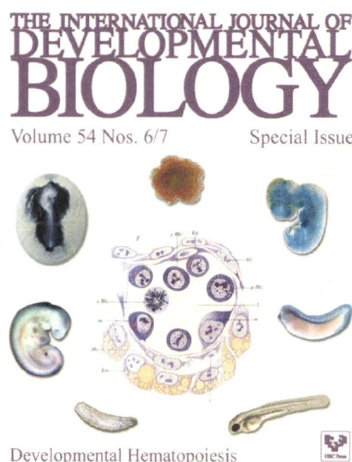

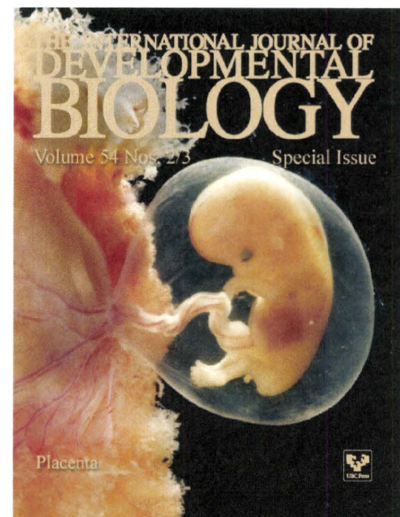

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Therapeutics, Targets, and Chemical Biology

Cancer Research

Cell Surface Tetraspanin CD9 Mediates Chemoresistance in Small Cell Lung Cancer

Satoshi Kohmo[1], Takashi Kijima[1], Yasushi Otani[1], Masahide Mori[2], Toshiyuki Minami[1], Ryo Takahashi[1], Izumi Nagatomo[1], Yoshito Takeda[1], Hiroshi Kida[1], Sho Goya[1], Mitsuhiro Yoshida[1], Toru Kumagai[1,3], Isao Tachibana[1], Soichiro Yokota[2], and Ichiro Kawase[1]

Abstract

Small cell lung cancer (SCLC) is an aggressive malignancy with extremely high mortality due to the appearance of widespread metastases early in its clinical course and rapid acquisition of chemoresistance after initial therapy. A theory of cell adhesion–mediated drug resistance is thought to be a principal mechanism in which extracellular matrix proteins provide a survival advantage against cytotoxic drug-induced apoptosis. We found that the tetraspanin family member CD9 was expressed preferentially in SCLC tumors and metastases from three of seven relapsed patients, whereas chemonaïve primary tumors from 16 patients were CD9 negative with only one exception. Additionally, CD9 was highly expressed on SCLC cell lines rendered resistant to cisplatin or etoposide, and was upregulated in parental chemosensitive cells within 48 hours after exposure to either of these compounds. CD9-expressing chemoresistant SCLC cells adhered more tightly to fibronectin via β1 integrin, but they were less motile than the respective chemosensitive parental lines. Notably, treatment of the chemoresistant cells with chemokine CXCL12 downregulated CD9 and transiently restored motility. Moreover, selective targeting of CD9 by treatment with specific monoclonal antibody ALB6 or a small interfering RNA triggered apoptosis in the chemoresistant cells. Taken together, our findings implicate CD9 in the cell adhesion–mediated drug resistance mechanism, highlighting CD9 as an attractive therapeutic target to improve therapeutic outcomes in SCLC. *Cancer Res; 70(20); 8025–35. ©2010 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Approximately 15% of all histologic types consist of small cell lung cancer (SCLC) that ultimately bears poor outcome. The extreme aggressiveness of SCLC is due to its unique biological characteristic; namely it metastasizes microscopically to systemic organs even in the early stages and often recurs with a multidrug resistance (MDR) phenotype shortly after dramatic response to initial treatments, which is achieved in approximately 80% of cases (1, 2). It is noticeable for several molecular target drugs including gefitinib, erlotinib, cetuximab, and bevacizumab to have shown survival benefit in non-SCLC (3–7). However, no favorable therapeutic strategy has been established in recurrent SCLC to date. Development of novel drugs that overcome MDR and bring about significant survival benefit is urgently desired in SCLC.

Two well-characterized drug efflux pumps, P-glycoprotein and MDR-related protein, have been thought to be crucial for MDR of SCLC, but the importance of this mechanism is still controversial because ectopic overexpression of these pumps could induce partial resistance compared with the level achieved by long-term drug selection (8, 9). Clinical studies also revealed that expression of these pumps was not common and did not necessarily correlate with outcome in SCLC (10, 11). Therefore, these molecules cannot completely account for MDR of SCLC and substitutable mechanisms must be involved in the process.

SCLC cells exist in an extracellular matrix (ECM)–rich environment *in vivo*, and an interaction between the tumor and the host microenvironment is central for their survival and proliferation. Sethi and colleagues proposed the concept of cell adhesion–mediated drug resistance (CAM-DR) as a principal mechanism for SCLC cells to acquire the MDR phenotype. They showed that β1 integrin–mediated adhesion of SCLC cells to ECM protects them from chemotherapy-induced apoptosis through activation of several intracellular proteins (12, 13). Kraus and colleagues showed that chemoresistance of SCLC correlates with adhesion to ECM and constitutive activation of Akt and mitogen-activated protein kinase pathways (14). We also reported that adhesion

Authors' Affiliations: [1]Department of Respiratory Medicine, Allergy, and Rheumatic Diseases, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; [2]Department of Respiratory Medicine, National Hospital Organization, Toneyama National Hospital, Toyonaka, Osaka, Japan; and [3]Department of Respiratory Medicine, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Osaka, Japan

Note: S. Kohmo and T. Kijima contributed equally to this work.

Corresponding Author: Takashi Kijima, Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Phone: 81-66879-3833; Fax: 81-66879-3839; E-mail: tkijima@imed3.med.osaka-u.ac.jp.

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of SCLC cells to fibronectin enhanced their viability and cytoskeletal function mainly by activating phosphatidylinositol-3-kinase and p125 focal adhesion kinase (15). Tsurutani and colleagues showed that laminin-mediated activation of the phosphatidylinositol-3-kinase/Akt pathway also enabled SCLC cells to escape from imatinib-induced apoptosis (16). Furthermore, Hodkinson and colleagues elucidated that interaction with ECM prevented SCLC cells from etoposide-induced G_2-M cell cycle arrest by overriding the upregulation of $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, and the downregulation of cyclins E, A, and B (17). The importance of the CAM-DR mechanism has been also reported in various malignancies other than SCLC (18–22).

Tetraspanin is the generic term for the glycoprotein family containing four transmembrane domains. Members of this family form multimeric complexes with one another and other cell surface proteins including integrins, leukocyte antigens, and signaling molecules at specialized tetraspanin-enriched microdomains. As organizer, stabilizer, and facilitator of these molecular networks termed the "tetraspanin web," tetraspanins regulate cellular morphology, motility, fusion, and intracellular signals (23, 24). Among more than 30 members in mammals, CD9 was identified as a molecule that suppresses cellular motility and metastatic potential in a human lung adenocarcinoma cell line (25, 26). Clinicopathologic findings indicated that CD9 is a predictor for better prognosis in adenocarcinoma of the lung (27, 28). However, the biological relevance of CD9 in SCLC has not been well understood thus far. We reported that CD9 was absent in chemonaïve tumors at the primary site in SCLC patients as well as in the majority of SCLC cell lines, and that ectopic overexpression of CD9 suppressed their motility (29). This evidence prompted us to investigate whether CD9 was involved in the advance of the disease and the CAM-DR mechanism and whether CD9 could then be a therapeutic target to overcome MDR in SCLC.

Materials and Methods

Cell lines and cell culture

SCLC cell lines SBC-3 (30), SBC-3/CDDP (31), and SBC-3/ETP (32) were kindly provided by Dr. K. Kiura, Okayama University, Okayama, Japan. NCI-H69 (H69) was purchased from American Type Culture Collection. H69/CDDP (33) and H69/VP (34) were obtained from National Cancer Center, Tokyo, Japan. H69/CD9 and SBC-3/CD9, which stably overexpress ectopic CD9, were established as described previously (29). All cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL).

Antibodies

Monoclonal antibodies (mAb) against CD9, clones 72F6 (Novocastra Laboratories), MM2/57 (Biosource International), and ALB6 (Immunotech), were used as indicated in each experiment. The mAbs for β1 integrin (4B4), β-actin (C4), and poly(ADP)-ribose polymerase (PARP; 46D11) were purchased from Beckman Coulter, MP Biomedicals, and Cell Signaling Technology, respectively. Rabbit polyclonal antibodies against phosphorylated or regular extracellular signal–regulated kinase 1/2 (ERK1/2), c-*Jun* NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 mitogen-activated protein kinase, and Akt were available from Cell Signaling Technology. Normal mouse IgG_1 (Thermo Scientific) was used as a control in several experiments.

Reagents

Nippon Kayaku Co. (Tokyo, Japan) supplied us with cisplatin (CDDP) and etoposide (VP-16). SN-38, the active form of irinotecan, and amrubicin were provided by Yakult Co. (Tokyo, Japan) and Dainippon Sumitomo Pharma Co. (Osaka, Japan), respectively. Recombinant human CXCL12 was purchased from R&D Systems.

Immunohistochemistry

Immunoperoxidase procedures were carried out as described elsewhere (29). After antigen retrieval, inactivation of endogenous peroxidase, and blockade of any nonspecific reaction, the sections were subjected to immunoreaction with primary anti-CD9 mAb 72F6 (diluted 1:400) overnight at 4°C and subsequently incubated with goat anti-mouse IgG biotinylated secondary antibody (diluted 1:300; Dako) for 10 minutes at room temperature and then labeled with streptavidin-peroxidase complex. They were developed and counterstained with Mayer's hematoxylin.

Flow cytometry

Cells (1×10^4) were incubated with 10 μg of primary mAb or normal mouse IgG_1 for 30 minutes at 4°C followed by labeling with FITC-conjugated goat anti-mouse IgG (Biosource). Stained cells were analyzed by FACScan (Becton Dickinson).

Reverse transcription-PCR

Total RNA was isolated from cells by the Isogen method (Nippon Gene), 1 μg of which was reverse-transcribed using the cDNA Synthesis Kit (Invitrogen). Generated cDNAs were amplified with the following forward and reverse primer sets: *CD9* (5′-TGCATCTGTATCCAGCGCCA-3′ and 5′-CTCAGGGATGTAAGCTGACT-3′) and *β-actin* (5′-TGAACCCTAAGGCCAACCGTG-3′ and 5′-GCTCA TAGCTCTTCTCCAGGG-3′). Using TaKaRa Ex Taq Hot Start Version (Takara Bio), the reaction mixtures were subjected to 30 amplification cycles with 30 seconds of denaturing at 94°C, 30 seconds of annealing at 60°C (CD9) and 61°C (β-actin), and a 60-second extension at 72°C. These primer pairs for *CD9* and *β-actin* amplified 799 bp and 397 bp fragments, respectively. Amplicons were electrophoresed on a 1% agarose gel and visualized with ethidium bromide.

Immunoblotting

Whole cell lysates were separated in a 10% to 20% gradient gel (Wako) by SDS-PAGE under nonreducing conditions, transferred to Immobilon-P membrane (Millipore) as described previously (35). Proteins were immunoblotted with proper primary antibodies (diluted 1:500–1,000) followed by

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appropriate horseradish peroxidase–conjugated secondary antibodies (diluted 1:1,000, donkey anti-rabbit or sheep anti-mouse IgG; Amersham) for 1 hour at room temperature. Immunoreactive bands were visualized using a chemiluminescent technique with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Regulation of CD9 expression

Cells (1×10^6) were treated with CDDP or VP-16 at various concentrations (≈ IC_{50} and ≈2 × IC_{50} for respective parental clone) for 48 hours (Fig. 1D) or incubated in the presence (100 ng/mL) or absence of CXCL12 for 6 hours (Fig. 4B) in serum-containing (10% fetal bovine serum)

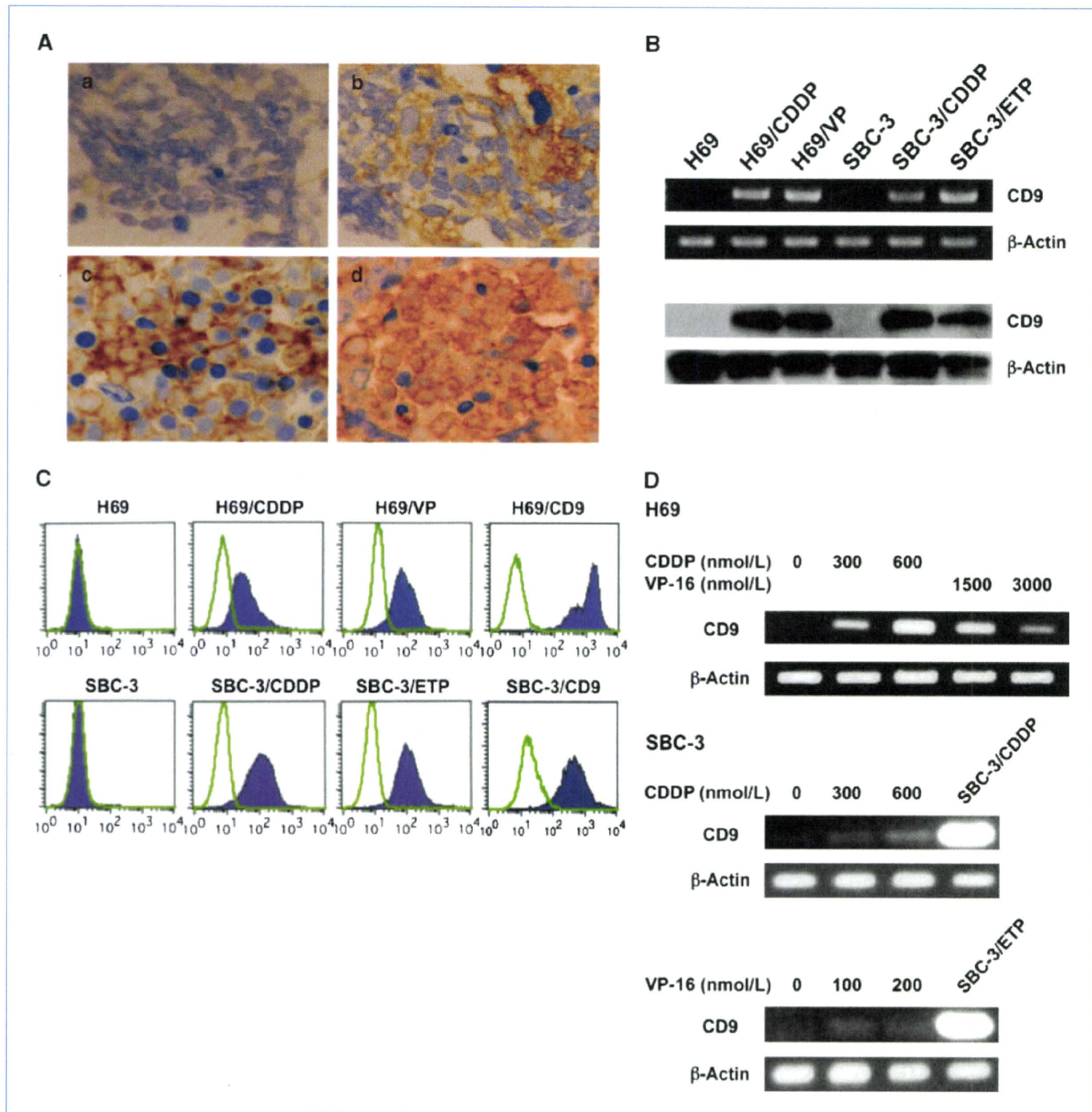

Figure 1. CD9 is expressed in recurred and metastasized tissues and upregulated by exposure to cytotoxic drugs in SCLC. A, tissues stained with mAb 72F6 from a representative SCLC patient. No immunoreactive CD9 is detectable in pretreated specimens from the primary site (a). In pathologically dissected tissues, CD9 is positive in the relapsed primary tumor (b), metastasized mediastinal lymph nodes (c), and the liver (d). B, CD9 is upregulated exclusively in chemoresistant cells at transcriptional (top) and protein (bottom) levels. C, FACS analysis confirms that CD9 is properly expressed on the cell surface of chemoresistant clones and CD9 transfectants. D, exposure of H69 and SBC-3 to various concentrations (≈ IC_{50} and ≈2 × IC_{50}) of CDDP or VP-16 for 48 h induces transcriptional upregulation of *CD9*.

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medium. Total RNAs were isolated and subjected to reverse transcription-PCR.

Drug sensitivity assays

Cells (5×10^3/well) were plated onto 96-well tissue culture–treated plates (Sumitomo Bakelite) and treated with serially diluted cytotoxic compounds in serum-containing medium for 120 hours. The relative number of viable cells was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (15, 36).

Adhesion assay

Cellular adhesive potential was evaluated as mentioned in our previous reports (15, 29, 36). Briefly, the wells of a 96-well plate (Linbro) were precoated with 10 μg/mL of human plasma fibronectin (Sigma-Aldrich) overnight at 4°C. After blocking nonspecific binding sites with RPMI 1640 containing 0.1% (w/v) bovine serum albumin, cells (5×10^4/well) were allowed to adhere with 1 μg/mL of 4B4 or control IgG_1 for 6 hours (H69 series) or 1 hour (SBC-3 series) at 37°C. The relative number of attached cells was determined by MTT assay.

Cell motility analysis by timelapse video microscopy

Cells (2×10^5) plated in fibronectin-coated 35-mm dishes (Corning) in serum-containing medium were untreated or treated with 100 ng/mL of CXCL12. After 6 hours of preincubation to urge cells to adhere, dishes were placed into a temperature- and humidity-controlled chamber (IX-IBM, Olympus). Cells were recorded for another 8 hours by timelapse video microscopy (TLVM) using an inverted microscope (IX70, Olympus) equipped with a charge-coupled device camera (Cool-Snap HQ cooled 12-bit; Roper Scientific). Digital video images were saved every 5 minutes and cellular movement was analyzed with MetaMorph software (Universal Imaging, Co.) and then plotted to show the trace of movement. The mean distance that a cell traversed for each 5-minute period was also calculated.

ALB6 treatment

Cells (1×10^4/well) were placed into 96-well plates (Sumitomo Bakelite) and then cultured either untreated or treated with 25 μg/mL of antibodies (ALB6 or IgG_1) in serum-containing medium. After 72 hours of treatment, cells were subjected to cell death detection assay to determine the biological toxicity of antibodies. The values of nucleosome enrichment that represent the increase of apoptosis were measured using Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics). In parallel, a 100 times larger scale (1×10^6 cells) treatment with antibodies or cytotoxic drugs was also carried out for immunoblotting.

Small interfering RNA treatment

Cells were transfected with either 40 nmol/L of small interfering RNA (siRNA) against human *CD9* (SHF27A-0631) or control cocktail RNAs (S30C-0126; B-Bridge International) using LipofectAMINE RNAiMAX (Life Technologies). After 48 hours of incubation, cells were replated in serum-containing medium (1×10^4/well of a 96-well plate for MTT assay or 1×10^6/60-mm dish for immunoblotting and FACS) then cultured for another 72 hours.

Statistical analysis

All the studies for statistical evaluation were performed in triplicate in each experiment and repeated at least three times. Mean ± SD values were calculated and differences were evaluated by two-sided Welch's *t* test using Statcel-12 software (OMS). $P < 0.05$ values were considered statistically significant.

Results

CD9 is preferentially expressed in recurred and metastasized SCLC tissues

To address CD9 expression in recurred and metastasized SCLC tissues, immunohistochemistry was carried out in matched pre/posttreatment samples from seven patients who had undergone pathologic dissection. None of the pretreated primary tumors from these subjects showed immunoreactive CD9. Additional pretreatment samples from nine other patients were also negative for CD9 (with only one exception). However, relapsed primary tumor, mediastinal lymph nodes, and metastasized tumors in the liver were all positive for CD9 staining in two patients, tissues from one of whom are shown in Fig. 1A, and another patient also had CD9-positive tumors in the liver. These findings suggested that CD9 is involved in the disease progression and spread of SCLC.

CD9 is upregulated by exposure to cytotoxic drugs in SCLC cells

We next investigated CD9 expression in chemoresistant SCLC clones established by long-term exposure to cytotoxic agents. CD9 was upregulated at the transcriptional level (Fig. 1B) and abundantly expressed on the cell surface (Fig. 1C) in all resistant clones, but not in parental cells, even without chemotherapeutic drugs. To clarify if cytotoxic agents induce CD9 expression in chemosensitive SCLC cells, transcripts for CD9 were analyzed by reverse transcription-PCR after 48 hours of exposure to CDDP or VP-16 at approximately equal and double the concentrations of IC_{50}. CD9 was upregulated in both H69 and SBC-3 cells (Fig. 1D) but this effect was transient and abrogated by drug withdrawal (data not shown), in which the precise mechanism is unknown. These results suggested that chemotherapy upregulates CD9 in primarily sensitive SCLC cells rather than clonally selecting intrinsically resistant CD9-positive cells during treatment.

CD9 is involved in but not essential for acquisition of chemoresistance in SCLC cells

As SCLC often recurs with the MDR phenotype shortly after successful initial treatment, we carried out an MTT drug sensitivity assay. Proliferation rates were not significantly different among all clones including CD9 transfectants in both H69 and SBC-3 series (data not shown). H69/VP and SBC-3/ETP lost sensitivity to amrubicin compared with parental lines with IC_{50} (nmol/L) values of 286 ± 65.1 (versus 42.3 ± 5.50 for H69, $P < 0.03$) and 310 ± 35.1 (versus 18.7 ± 12.6 for SBC-3, $P < 0.01$), respectively. SBC-3/CDDP showed

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cross-resistance towards all of VP-16 (IC_{50}: 2,333 ± 416 versus 107 ± 11.6 for SBC-3, $P < 0.01$), amrubicin (IC_{50}: 70.7 ± 13.0 versus 18.7 ± 12.6 for SBC-3, $P < 0.01$), and SN-38 (IC_{50}: 23.3 ± 5.80 versus 1.13 ± 0.71 for SBC-3, $P < 0.03$). On the other hand, CD9 transfectants did not become resistant to any drug (Fig. 2). These data suggested that CD9 upregulation by chemotherapy might not be essential for the acquisition of drug resistance but could be a result of cellular adaptation.

CD9 enhanced β1 integrin–mediated adhesion to fibronectin in SCLC cells

Integrins are essential cell surface receptors for ECM from which intercellular survival signals go downstream. Because almost all SCLC cell lines express β1 integrin subunit (37), and because fibronectin is one of the major ECM components and common substrates for integrins, adhesion assays were performed to evaluate adhesive potential of SCLC clones onto this ECM (Fig. 3). H69 cells usually grow as floating aggregates and a majority of them still kept floating even in the presence of fibronectin with only 8.15 ± 3.25% attached cells. In contrast, approximately half of both H69/CDDP (48.2 ± 2.39%, $P < 0.01$) and H69/VP (50.0 ± 4.69%, $P < 0.01$) adhered. Moreover, SBC-3 cells which usually grow in an anchorage-dependent fashion significantly increased adhesive faculty when they became resistant to CDDP and VP-16 with attachment ratios ranging from 55.0 ± 9.16% to 83.6 ± 3.26% ($P < 0.01$) and 86.6 ± 4.47% ($P < 0.01$), respectively. Treatment with function-blocking anti-β1 integrin mAb (4B4) completely canceled this phenomenon in H69/CDDP (7.25 ± 3.23%, $P < 0.01$) and H69/VP (7.47 ± 4.19%, $P < 0.01$) to the level of H69 (8.64 ± 1.35%) and also markedly decreased the adhesive potential in all SBC-3/CDDP (28.7 ± 11.2%, $P < 0.01$), SBC-3/ETP (33.0 ± 14.0%, $P < 0.03$), and SBC-3 (12.4 ± 9.08%, $P < 0.01$). Ectopic overexpression of CD9 extremely enhanced adhesion in both H69/CD9 (85.8 ± 2.75%, $P < 0.01$) and SBC-3/CD9 (78.6 ± 5.08%, $P < 0.01$), which was abrogated by 4B4 (7.63 ± 0.70%, $P < 0.01$ and 25.4 ± 10.7%, $P < 0.01$, respectively). These results showed that CD9 is one of the key molecules in β1 integrin–mediated cell adhesion.

CD9 and CXCL12 participate in the CAM-DR mechanism regulating cell motility in SCLC

The tighter the cells attach to ECM, the less motile they become in general. We compared the cellular motility of SBC-3/CDDP, SBC-3/ETP, and SBC-3/CD9 with SBC-3 on fibronectin using TLVM. Movement distances (μm/5 min) of 10 randomly selected cells are shown in Fig. 4A. Not only SBC-3/CDDP (0.65 ± 0.28, $P < 0.005$) and SBC-3/ETP (0.98 ± 0.47, $P < 0.03$) but also SBC-3/CD9 (0.63 ± 0.41, $P < 0.005$) were significantly less motile than SBC-3 (2.12 ± 1.23). We previously reported that CXCR4, the unique receptor for a chemokine CXCL12, was commonly expressed in SCLC cell lines and CXCL12 enhanced their motility (36). As all the clones used here express similar amounts of CXCR4 (data not shown), we tested if CXCL12 could restore the motility of these resistant clones. Compared with the unstimulated

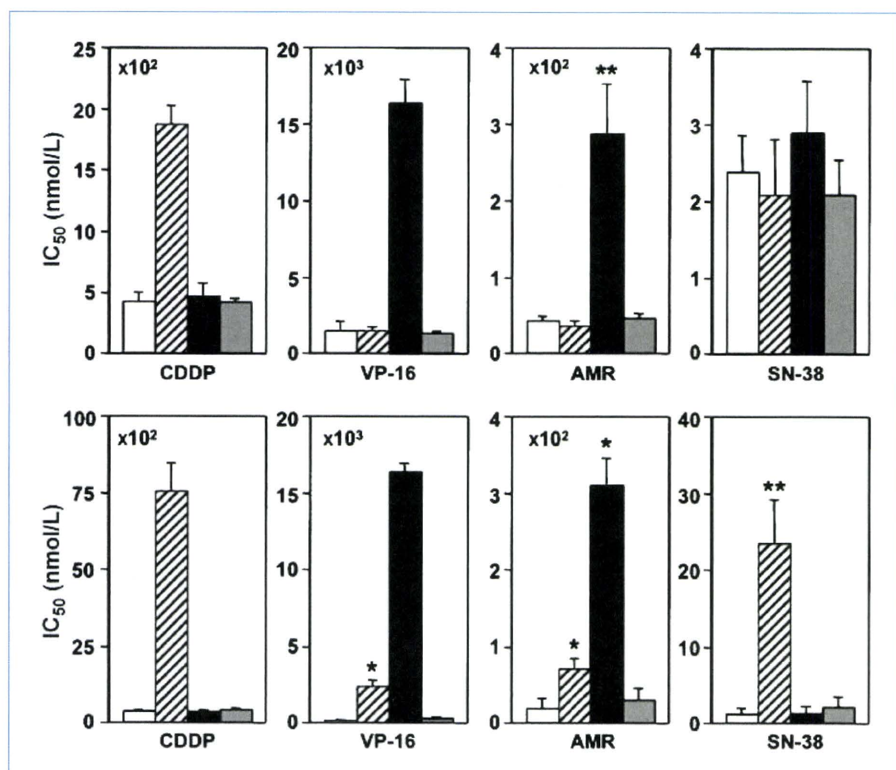

Figure 2. CD9 is involved but is not essential for acquired chemoresistance in SCLC cells. Cells of H69 series (top) and SBC-3 series (bottom) were treated with serially diluted drugs for 120 h. Relative number of viable cells was quantified by MTT assay. Columns, the mean IC_{50} (nmol/L) values for parental (white), cisplatin-resistant (striped), and etoposide-resistant (black) clones or CD9 transfectants (gray) from three independent experiments; bars, SD. *, $P < 0.01$ and **, $P < 0.03$ versus respective parental clone AMR, amrubicin.

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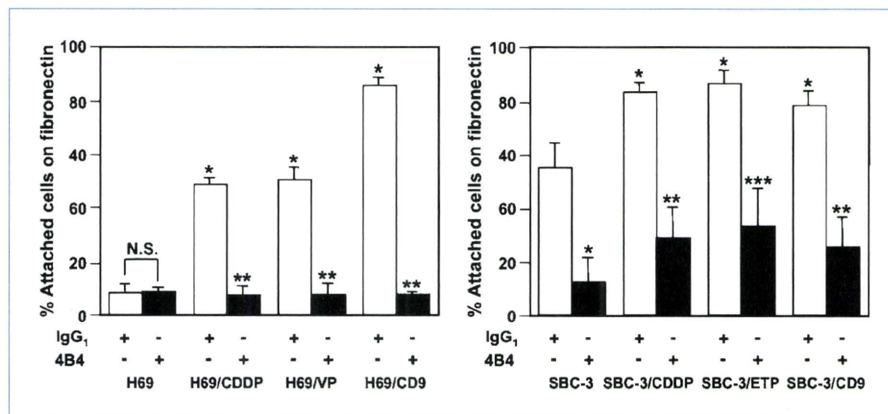

Figure 3. CD9 enhances β1 integrin–mediated adhesion to fibronectin in SCLC cells. Cells were allowed to adhere to the fibronectin-coated surface for 6 h (H69 series) or 1 h (SBC-3 series) in the presence of 1 μg/mL of control IgG_1 (white) or 4B4 (black). The relative number of attached cells was determined by MTT assay. Columns, mean relative ratios to unwashed well; bars, SD. *, $P < 0.01$ versus IgG_1-treated parental clone; **, $P < 0.01$ versus IgG_1-treated resistant clone; and ***, $P < 0.03$ versus IgG_1-treated SBC-3/ETP.

state, movement of SBC-3/CDDP (1.95 ± 1.23, $P < 0.01$) and SBC-3/ETP (2.18 ± 1.01, $P < 0.005$) significantly increased to the level of SBC-3 in response to CXCL12. On the other hand, SBC-3 (3.35 ± 1.48, $P = 0.059$) and SBC-3/CD9 (0.83 ± 0.37, $P = 0.26$) did not respond to CXCL12, probably due to extremely low or high expression of CD9, respectively. Of interest, transcripts for CD9 were markedly reduced by CXCL12 within 6 hours in SBC-3/CDDP and SBC-3/ETP with movement recovery, but were marginal in SBC-3/CD9 (Fig. 4B). This effect did not last so long and CD9 transcriptional levels returned to baseline after 24 hours (data not shown). We also examined if CXCL12 treatment could affect the chemosensitivity of these cells. SBC-3/CDDP and SBC-3/ETP cells were not resensitized even in the presence of CXCL12, probably because its effect was transient (Fig. 4C).

These results could strongly support the CAM-DR theory that motility of cancer cells increases up to a peak then goes down again to the baseline, which forms a bell-shaped curve, along with continuously increasing cell-ECM adhesion and chemoresistance (38, 39). Upregulation of endogenous CD9 probably plays some important roles in CAM-DR mechanism and exogenous CXCL12 may transiently, but dynamically, regulate cellular motility by controlling CD9 expression in SCLC (Fig. 4D).

Selective inhibition of CD9 induced the apoptosis of chemoresistant SCLC cells

Because ALB6, a mAb against CD9, was reported to induce apoptosis of CD9-expressing malignant cells (40), we also examined if selective targeting of CD9 by ALB6 or siRNA could induce the apoptosis of chemoresistant SCLC cells. ALB6, but not control IgG_1, led to apoptotic cell death exclusively in CD9-expressing cells coupled with cleavage of PARP. When the extent of apoptosis was evaluated using an increase of nucleosome enrichment index by cell death detection assay, values for ALB6 and control IgG_1 were 0.50 ± 0.32 versus 0.02 ± 0.04 ($P < 0.05$) in H69/CDDP, 0.52 ± 0.16 versus 0.00 ± 0.05 ($P < 0.001$) in H69/VP and −0.09 ± 0.18 versus −0.02 ± 0.02 in H69 (N.S.), 0.79 ± 0.15 versus 0.04 ± 0.09 ($P < 0.005$) in SBC-3/CDDP, 0.71 ± 0.27 versus 0.02 ± 0.04 ($P < 0.03$) in SBC-3/ETP, and −0.02 ± 0.19 versus 0.03 ± 0.08 (N.S.) in SBC-3, respectively (Fig. 5A). In terms of intracellular signaling, ALB6 induced the phosphorylation of JNK/SAPK and p38 but not Akt and ERK1/2 in SBC-3/CDDP cells within 10 minutes but the effect was transient and disappeared by 30 minutes, which was consistent with the previous report (ref. 40; Fig. 5B).

We next examined the effect of siRNA-induced CD9 depletion on survival of chemoresistant clones. Immunoblot and FACS analyses confirmed that CD9 was successfully ejected from the cell surface at least from day 2 to day 5 (Fig. 6A). Similar to ALB6, siRNA treatment also induced apoptosis exclusively in chemoresistant clones in parallel with cleavage of PARP. When evaluated by MTT assay, the percentage of viable cells treated with targeted versus scramble RNAs were 21.1 ± 9.3 versus 92.2 ± 6.7 ($P < 0.0001$) in H69/CDDP, 42.5 ± 1.9 versus 90.8 ± 4.3 ($P < 0.0001$) in H69/VP, and 91.5 ± 3.7 versus 92.7 ± 5.3 in H69 (not significant), 12.5 ± 3.2 versus 94.9 ± 3.0 ($P < 0.0001$) in SBC-3/CDDP, 15.4 ± 3.0 versus 94.7 ± 9.9 ($P < 0.0001$) in SBC-3/ETP, and 93.5 ± 6.8 versus 94.8 ± 5.6 (not significant) in SBC-3, respectively (Fig. 6B). These findings showed the possibility of using CD9 as a therapeutic target to overcome the drug resistance of SCLC.

Discussion

CD9 is a tetraspanin that is well characterized by its close association with β1 integrin and its ability to reinforce its diverse biological actions on cell adhesion, migration, motility, and survival via interaction with ECM (23, 24). We found that CD9 was preferentially expressed on SCLC cells at relapsed primary tumors and metastasized organs in patients who had received prior chemotherapy. Therefore, we hypothesized that CD9 upregulated by chemotherapy could participate in the progression and spread of SCLC.

An immunohistochemical study revealed high levels of immunoreactive ECM proteins in SCLC tissues. In tumor foci, collagen IV and fibronectin are deposited in adjacent host connective tissue; moreover, fibronectin and laminin are also visible in SCLC cells, suggesting that they might directly synthesize some components of the local ECM (12, 41). The production of fibronectin was also observed in all SCLC cell lines used in our experiments (data not shown). Thus, SCLC cells

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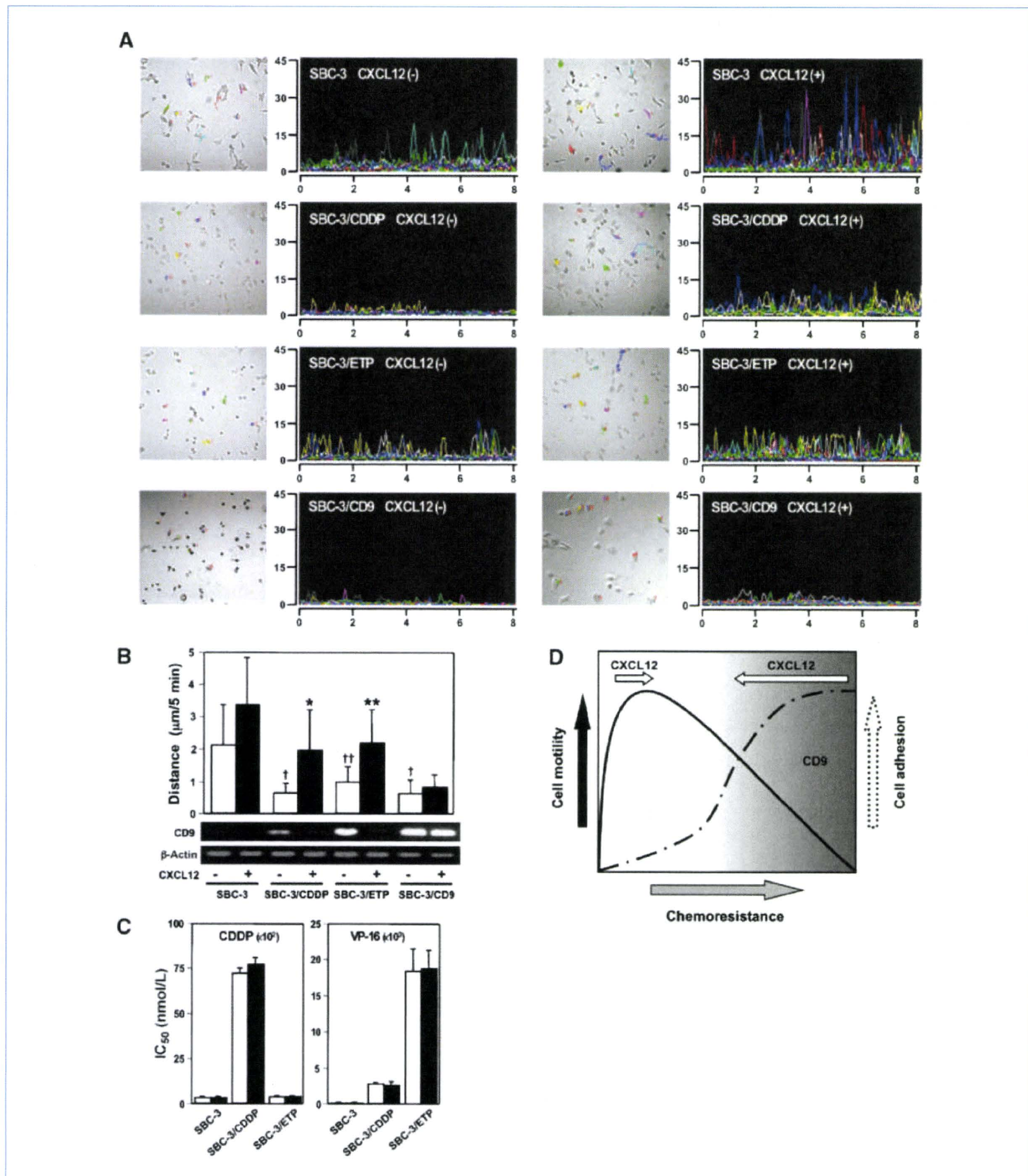

Figure 4. CD9 and CXCL12 participate in the CAM-DR mechanism regulating cell motility in SCLC. A, cells plated on fibronectin were untreated or treated with 100 ng/mL of CXCL12. After 6 h of preincubation, cells were recorded every 5 min for another 8 h by TLVM. Traces from 10 randomly selected cells are shown at ×80 magnification. Cellular movement is also presented in graphs with variables of time (h) as the *X*-axis and the distance that a cell traversed every 5 min (µm/5 min) as the *Y*-axis. B (top), columns, mean distances with (black) or without (white) CXCL12; bars, SD. *, $P < 0.01$ and **, $P < 0.005$ versus untreated resistant clone. †, $P < 0.005$ and ††, $P < 0.03$ versus untreated SBC-3; (bottom), reverse transcription-PCR analysis shows that CXCL12 downregulates CD9 in SBC-3–derived chemoresistant clones within 6 h. C, drug sensitivity in the presence (black) or absence (white) of CXCL12 was tested and shown as in Fig. 2. D, schema of CAM-DR mechanism with hypothetical involvement of CD9 and CXCL12 is shown. Motility (solid line and arrow) of cancer cells increases up to a peak then goes down again to the baseline level in company with continuous increase in chemoresistance (gray arrow) and adhesive potential (dashed line and arrow). Upregulation of endogenous CD9 is probably involved in CAM-DR mechanism (gradient gray zone) and exogenous CXCL12 may dynamically regulate cellular motility controlling CD9 expression (open arrows).

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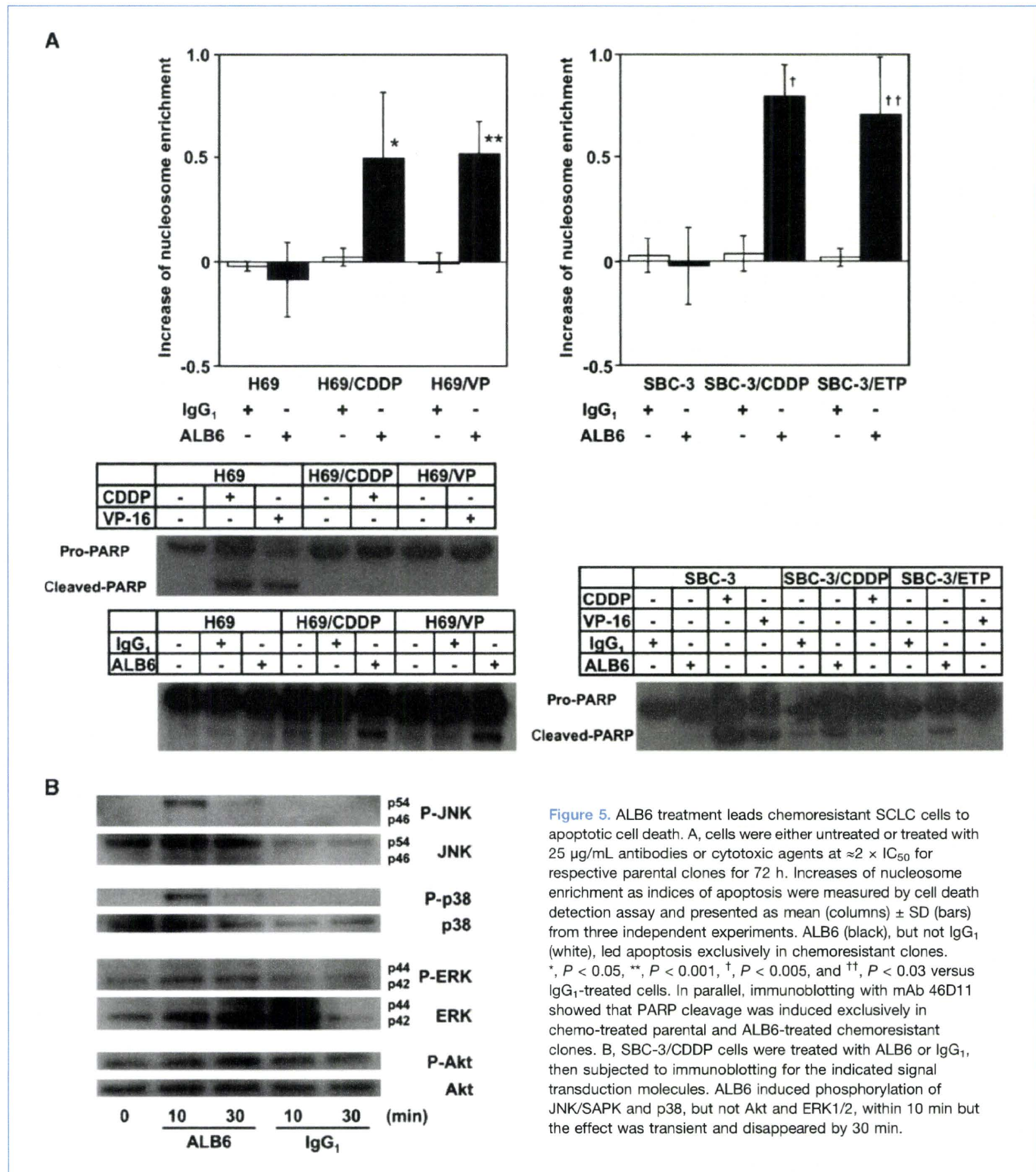

Figure 5. ALB6 treatment leads chemoresistant SCLC cells to apoptotic cell death. A, cells were either untreated or treated with 25 μg/mL antibodies or cytotoxic agents at ≈2 × IC_{50} for respective parental clones for 72 h. Increases of nucleosome enrichment as indices of apoptosis were measured by cell death detection assay and presented as mean (columns) ± SD (bars) from three independent experiments. ALB6 (black), but not IgG_1 (white), led apoptosis exclusively in chemoresistant clones. *, $P < 0.05$, **, $P < 0.001$, †, $P < 0.005$, and ††, $P < 0.03$ versus IgG_1-treated cells. In parallel, immunoblotting with mAb 46D11 showed that PARP cleavage was induced exclusively in chemo-treated parental and ALB6-treated chemoresistant clones. B, SBC-3/CDDP cells were treated with ALB6 or IgG_1, then subjected to immunoblotting for the indicated signal transduction molecules. ALB6 induced phosphorylation of JNK/SAPK and p38, but not Akt and ERK1/2, within 10 min but the effect was transient and disappeared by 30 min.

exist in an ECM-rich environment and ECM proteins produced by adjacent stroma and tumor cells probably offer them a suitable microenvironment to support their proliferation and survival.

This evidence prompted us to study if CD9 is involved in the CAM-DR mechanism and how SCLC cells use CD9 to intensify this mechanism to circumvent chemotherapy-induced apoptosis. According to our findings, CD9 expression would consistently increase in parallel with augmentation of chemoresistance from the initial escape from chemotherapy-mediated apoptosis to the ultimately resistant stage (Fig. 1). Although CD9 might not be essential for the acquisition of drug resistance but a result of cellular adaptation to protect cells from chemotherapy-driven stress (Fig. 2), chemoresistant

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