Table 1 Average of $\log_2$ ratios for the regions showing DNA copy number alterations in three types of HeLa cells

Region	Average of $\log_2$ ratio					
	Chr. 1	3p11.1–p14.2	9p13.1–p24.3	13q12.11–q21.1	Chr. 21	Chr. 22
HeLa-A (ATCC)	0.120	−0.114	0.581	−0.248	−0.180	−0.145
HeLa-B (P122)	0.080	−0.154	**0.337**	−0.245	−0.170	−0.105
HeLa-C (P150)	**−0.126**	**−0.635**	**0.126**	**−0.511**	*−0.146*	*−0.029*
Number of clones	299	34	63	50	68	100

Chr. indicates whole region of the chromosome; P122 and P150, analysis after 122 and 150 passages, respectively; number of clones, the number of BAC clones in the corresponding region; bold type, the value of the $\log_2$ ratio decreased in comparison to that for HeLa-A; italic type, the value of the $\log_2$ ratio increased in comparison to that for HeLa-A

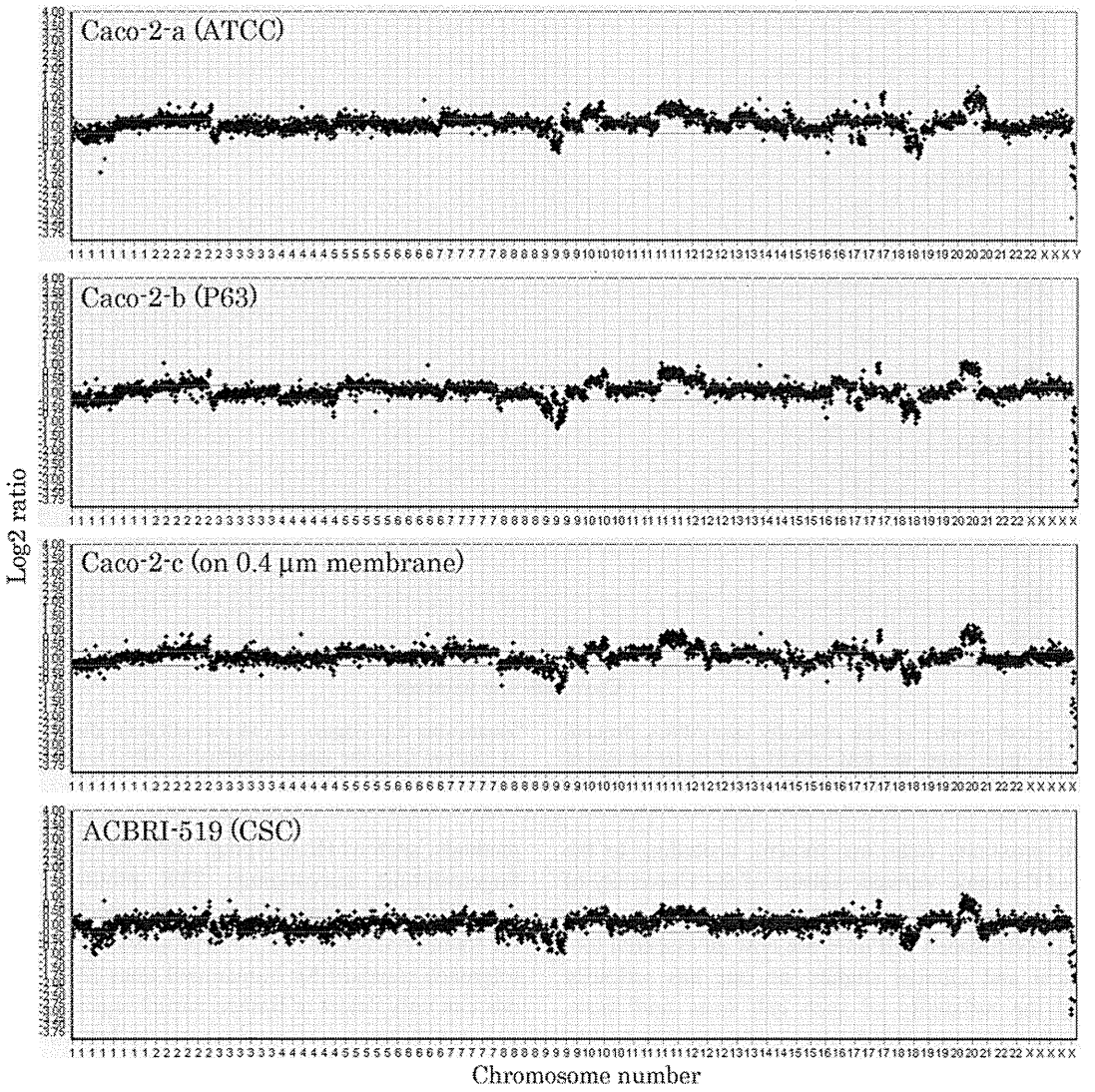

Fig. 2 BAC aCGH profiles of 3 Caco-2 cells and ACBRI-519 cells. Sequentially from the upper panel are the BAC aCGH profile of Caco-2-a (ATCC), -b (after 63 passages), -c (cultured on 0.4-μm diameter microporous membrane), and ACBRI-519 (Cell System Corp.)

safety of these MSCs at the genome level, especially DNA copy number change, which correlates well with tumorigenesis. In Fig. 3, several BAC clones that were outside the normal range (considered as normal from −0.3 to 0.3, see "Materials and methods") were possible copy number variants (CNV). These clones were confirmed as CNV loci

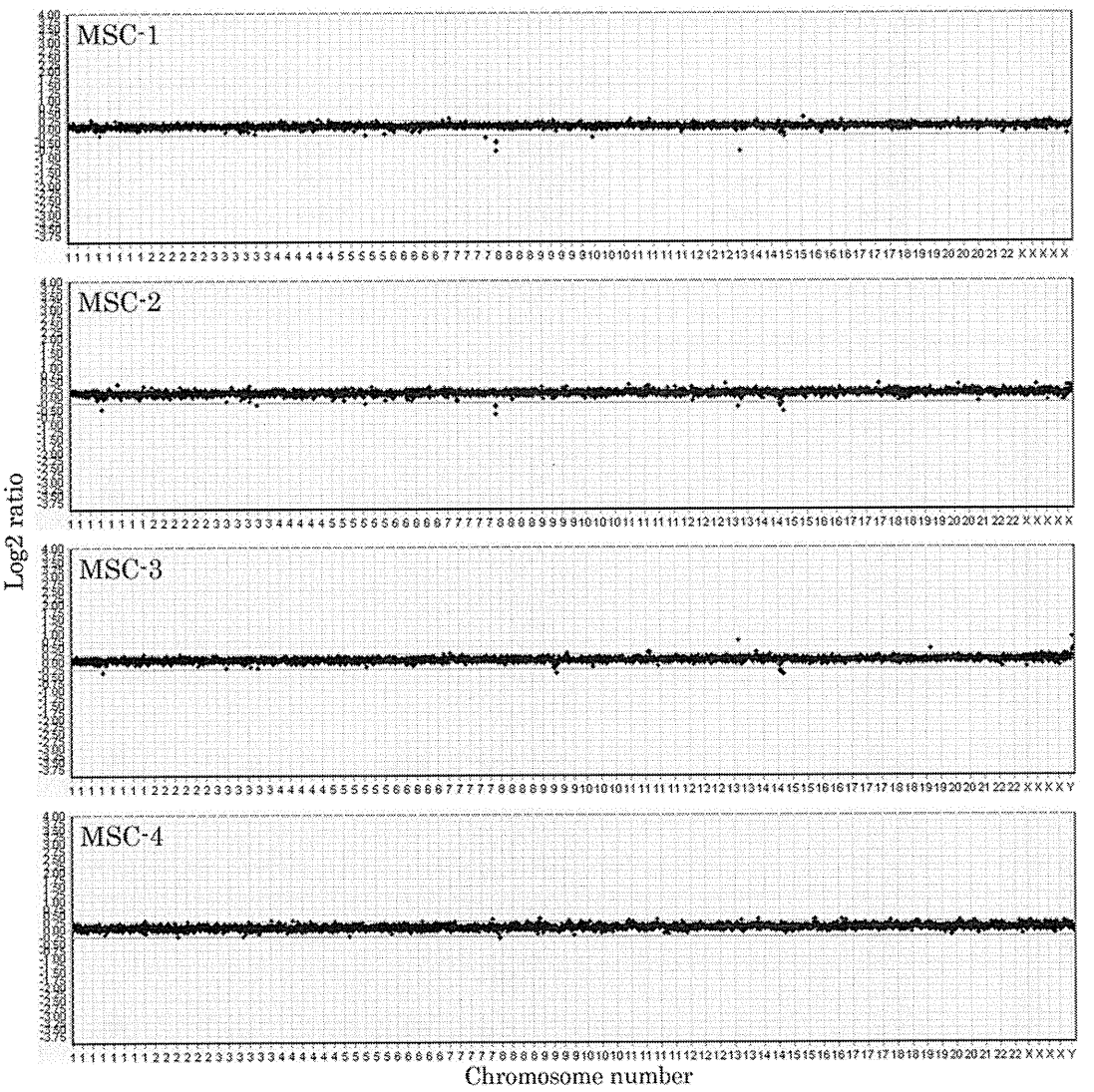

Fig. 3 BAC aCGH profiles of four clinically used MSCs. Sequentially from the upper panel the BAC aCGH profiles are shown of MSC-1 (69-year-old female after three passages), -2 (16-year-old female after 3 passages), -3 (34-year-old male after 4 passages), and -4 (derived from the same individual as MSC-3, after 7 passages)

or in their proximity (data not shown), according to the Database of Genomic Variants website of the University of Toronto (http://projects.tcag.ca/variation/).

Although established cell lines are now an essential tool in biological and clinical studies, no one has seriously questioned the reliability of such cell lines until now. Actually, most investigators have noted morphological and/or biological alterations of cell lines during long-term culture. For coping with such alterations, these cell lines have been discarded and renewed from frozen stocks after a certain number of passages. However, the substantial alterations caused by long-term culture have not been seriously considered. Validation to detect cross contamination of cell lines has been made by using various methods, such as HLA typing, DNA polymorphism, DNA fingerprinting, karyotyping, STR profiling, and SNPs [24–29]. In particular, STR profiling, which was developed for forensic sciences [30], was proposed as an international reference standard for human cell lines [28]. These methods can only detect at limited partial regions of the human genome. However, tumorigenesis is known to correlate with various chromosomal instabilities including DNA copy number changes throughout the entire human genome. Therefore, to avoid overlooking the possibility of tumorigenesis, it is necessary to validate cells by using BAC aCGH, as it can analyze all regions in the entire human genome. In this study, we investigated chromosomal stability and instability of established cell lines,

HeLa cell, Caco-2 cells, and MSCs derived from normal human bone marrow by performing BAC aCGH. Our results indicate that BAC aCGH is a suitable tool for validation of cell lines. Thus, we propose that BAC aCGH is a superior method for evaluation of the genomic stability of established cell lines as well as various kinds of cells and suggest that all kinds of cellular investigations should include validation of chromosomal stability by performing BAC aCGH.

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Growth factor-defined culture medium for human mesenchymal stem cells

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

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

Introduction

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

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

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

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

Results

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

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

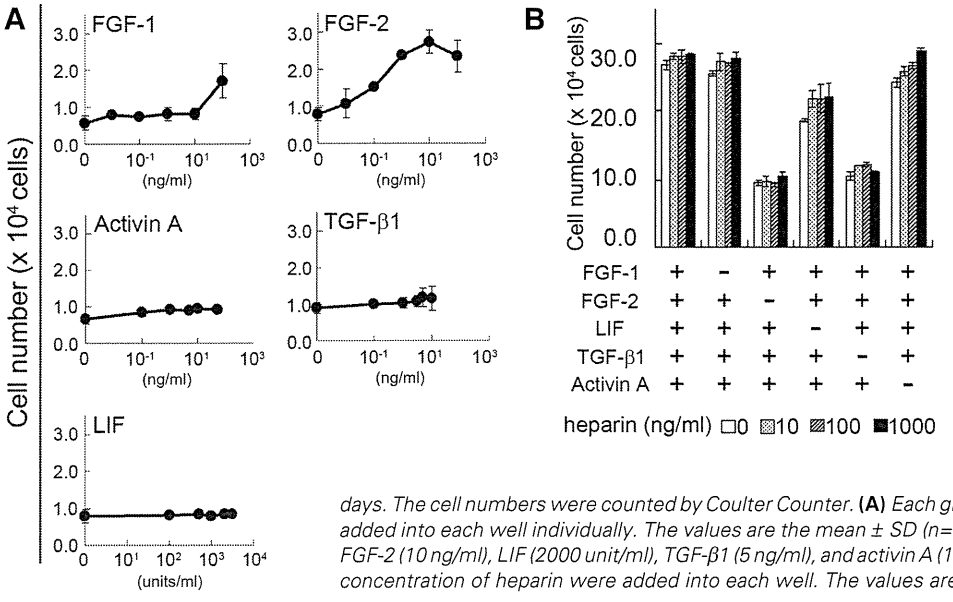

Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. *After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-)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

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

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

We subsequently examined the properties of UE7T-13 cells serially passaged in D-hESF10 medium. The morphology of serum-free expanded UE7T-13 cell populations was comparably small, spindle-shaped cells compared with that in conventional medium (Fig. 5A). The expression of hMSC and hES cell pluripotency markers were determined by real-time PCR analysis (Fig. 5B) in UE7T-13 cells cultured for 4 passages in D-hESF10 medium. The expression of hMSC markers, *CD105*, *THY1*, and *integrin β1* (*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

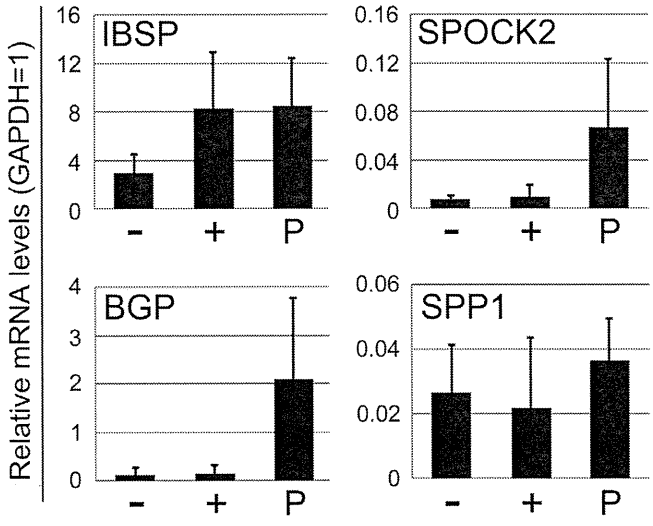

Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. *The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression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

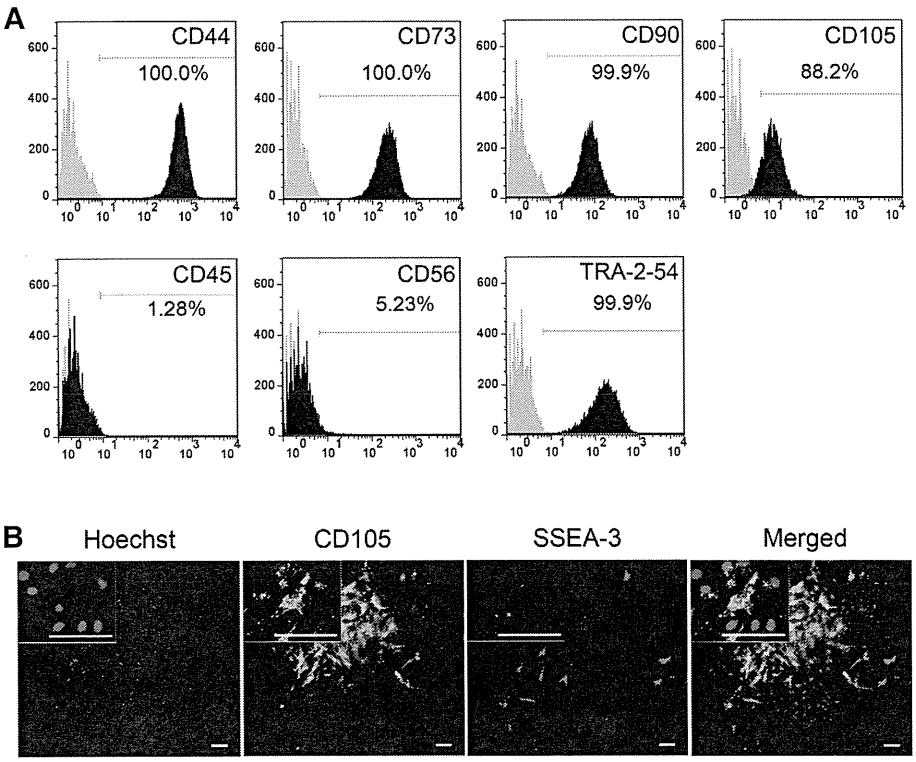

Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) *Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal 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.*

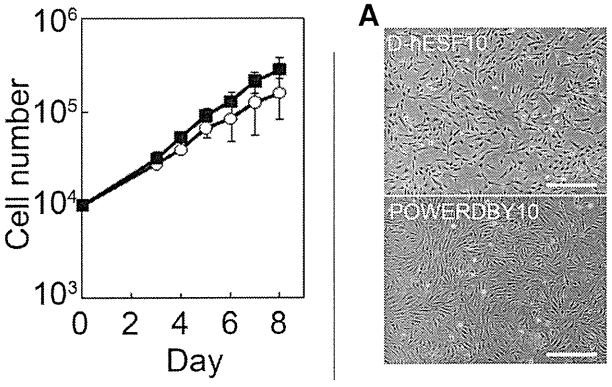

Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. *The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density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

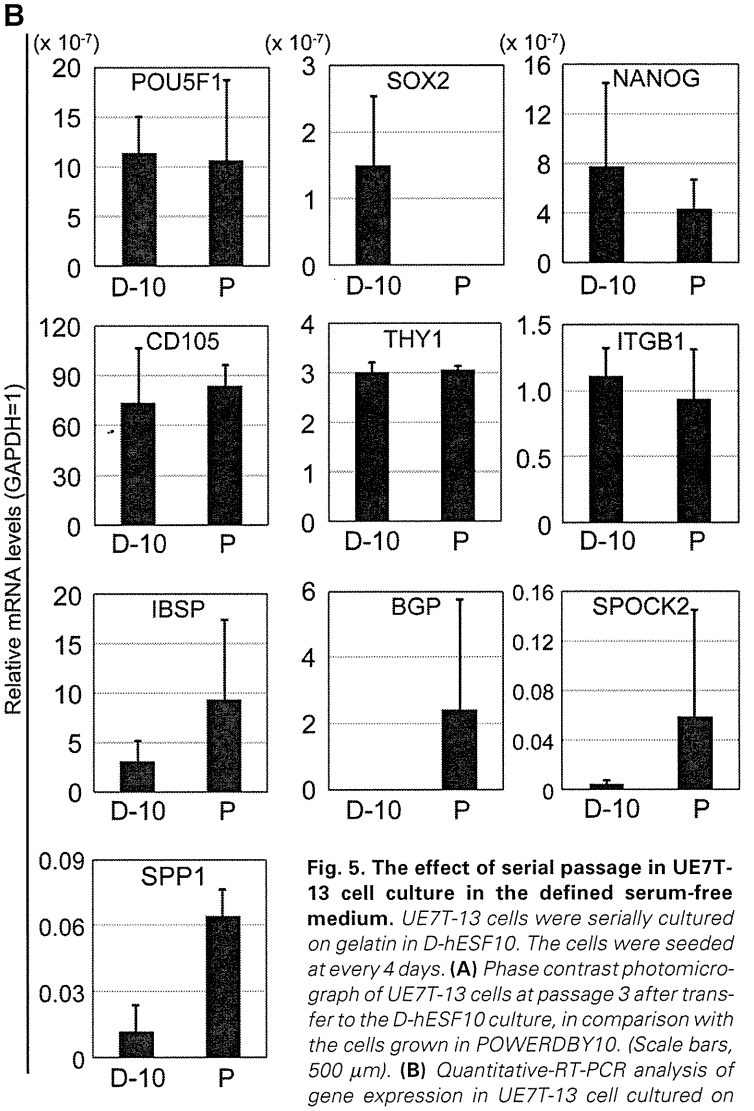

Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. *UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days.* **(A)** *Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture, 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.

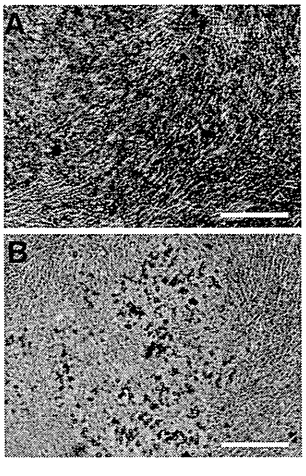

Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. *The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium.* **(A)** *Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were 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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投稿論文

ヒト多能性幹細胞の命名法の国際統一規格案について

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summary

In a few years, thousands of human embryonic stem (ES) / induced pluripotent stem (iPS) cell lines have been established in laboratories around the world. To date, confusions have arisen due to duplicate or redundant naming of cell lines. In addition, not all the important information such as provenance, derivation method and characterization are provided by researchers. To address these issues, a convention for naming and reporting human ES/iPS cell lines is urgently called. Recently Stem Cell Banks and researchers in the US, UK, China, Australia and the other countries proposed a new nomenclature system and a minimum set of criteria for reporting newly generated human ES/iPS cell lines. In this review, we have introduced their recommendations for developing a rule for naming and reporting of human ES/iPS cell lines.

はじめに

1998年にヒト胚性幹細胞(embryonic stem cell：ES細胞)[1)]が樹立され，2007年には，ヒト人工多能性幹細胞(induced pluripotent stem cell：iPS細胞)[2)]が開発された。これらの多能性幹細胞は，発生や疾患メカニズム解明など基礎研究のみならず，再生医療や創薬，毒性評価，ワクチン作製などへ応用の期待が高まっている。ヒトES/iPS細胞の株数は急ピッチで増加しており，すでに数千株にも及ぶ。実用化への研究を進めるために，国際幹細胞バンキングイニシアティブ(International Stem Cell Banking Initiative：ISCBI)では各国の細胞バンクや樹立機関が協力して世界中の研究者が相互に利用できる環境の整備を推進している。ところが，ヒトES/iPS細胞株の命名法について整備されておらず，混乱が生じている。このような現状から，2011年4月に，米国，英国，オーストラリア，中国などの幹細胞バンクや幹細胞研究者らから，「ヒトES/iPS細胞株の命名法および発表に関する標準化」[3)]が提案され，さらにその提案に対する意見[4)5)]が寄せられた。ISCBIや国際細胞バンク・ワーキンググループに参加する筆者らが，その内容を概説したい。

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ヒトES/iPS細胞の国際的な相互利用に向けて

2003年に設置された日本を含む22ヵ国からなる国際幹細胞フォーラム(http://www.stem-cell-forum.net/ISCF/)からの助成を受けて，2005年から英国シェフィールド大学Andrews教授が中心となって推進しているInternational Stem Cell Initiatives (ISCI)プロジェクトでは，日本(京都大学再生医科学研究所・中辻憲夫教授)を含めた世界11ヵ国のヒトES細胞樹立研究者らが連携して，ヒトES細胞株を登録し，樹立の方法，未分化/分化マーカーの発現などの解析方法とその結果を公表し[6)7)]，ヒトES細胞研究の標準化を進めてきた(http://www.stem-cell-forum.net/ISCF/initiatives/)。ISCIワークショップには筆者らも加わり標準化についての議論を行った。2008年からは，ヒトiPS細胞も含めて検討されている。さらに，ヒトES/iPS細胞株を各国間で相互に利用する体制を構築する必要があるとの認識のもとに，2007年から英国UK Stem cell Bankをはじめとする世界各国の細胞バンクが連携し，ISCBIプロジェクトが開始され，筆者らが参加している。このプロジェクトにおいては，ヒトES細胞のドナーの情報管理，資源化，品質管理法や分譲について，国際的にコンセンサスを図ってヒトES細胞を資源化するためのガイドラインを作成している[8)9)](和訳は，京都大学再生医科学研究所・細胞プロセシング・高田らより本誌Vol.10 No.4，p79-96，2011に掲載されているので参照されたい)。さらに，相互利用するためには不可欠な細胞登録における「細胞株の命名法」に関しても統一規定を設けることが現在の重要課題であり，国際的に活発な議論が展開されている。

これまでの現状

国内において細胞バンクが整備される1984年以前は，日本組織培養学会が細胞株を認定してJTCの番号を付与して登録する事業を実施していた。現在は，細胞バンクが整備され，JCRB(医薬基盤研究所細胞バンク，旧国立医薬品食品衛生研究所細胞バンク)，RCB(理化学研究所バイオリソースセンター細胞バンク)に，研究者が細胞株を寄託し，バンクの略称とともに登録番号で管理され，データベース上で公開されている。海外においても，米国のATCC，国立がん研究所(National Cancer Institute：NCI)，欧州細胞培養コレクション(European Collection of Animal Cell Cultures：ECACC)などの細胞バンクが各機関の略称や独自の登録番号(カタログ番号)を用いて管理し，情報を公開している。このように整備されていても，細胞株の情報や原著論文を検索するときに不都合が起こる。たとえば，“3T3 Swiss Albino”“3T3-Swiss albino”“Swiss-3T3”は同種の細胞株名であるが，データベースや論文での記載方法は他にも何通りも存在する。3T3と入力して検索すると，“3T3 (+3)”，“3T3-L1”，“3T3-SV40”など，別種の細胞株やサブクローンも検索にかかる。

まだ歴史の浅いヒトES/iPS細胞株においても，異なる研究機関で樹立された別個の細胞に全く同じ名前がつくといった問題がすでに生じている。たとえば，全く別の患者から採取した羊水(amniotic fluid：AF)に由来する2つのiPS細胞株の両方ともが“AF-iPS”と命名されたり[10)11)]，ジストロフィン遺伝子に異なる箇所に変異をもつ2人のデュシェンヌ型筋ジストロフィー(Duchenne muscular dystrophy：DMD)患者から樹立した全く別のiPS細胞であるにも関わらず，両方ともが“DMD-iPS1”と命名されたりしている[12)13)]。“iPS-1”や“iPS-WT”といった名称は汎用され，その名称のみから細胞株を特定することはできない[4)]。また，“KhES-1”“KhES-3”“HES-3”など，ヒトES細胞の名称に汎用される“HES”は，ヒト胎児皮膚(human embryonic skin：HES)由来線維芽細胞の株名“HES 5”[14)]などとも同じ表記であるため混同されやすい。細胞株を混同してしまえば，研究成果の妥当性，重要性

を正当に評価できなくなる。このように細胞命名法の国際的な統一規定がなかったことがデータベースの管理・利用を不便なものにしている。

ヒトES/iPS細胞の命名法の提案

2010年の国際幹細胞学会(International Society for Stem Cell Research：ISSCR, 2010年 7 月15日開催), およびISCI(2010年 9 月15日開催)のワークショップで議論された内容に準拠して，米国マサチューセッツ医科大学ヒト幹細胞バンクのInternational stem cell registry(ISCR)が代表として提案する「ヒトES/iPS細胞株の命名法および細胞登録に関する統一規定の案」が米国科学誌「Cell Stem Cell」2011年 4 月 8 日号[3]に掲載された。これに対し，京都大学iPS細胞研究所(CiRA)山中伸弥所長らの意見[4]と米国細胞バンクAmerican Type Culture Collection(ATCC) Brian Pollok所長らの意見[5]が同誌の 6 月 3 日号に掲載された。両者ともISCRの提案に大筋で同意した上で，幹細胞研究の将来展望をもとに想定される問題を提起し，改善案を提示した。

ヒトES/iPS細胞の命名法についての統一規定案

ISCRによる命名法の統一規定案(図)[3]は，特に次に示す 5 点に配慮したものである。①独自の識別方法(樹立機関IDと細胞株シリアル番号)を採用し，細胞株間で混同しないようにすること。②細胞株に関する情報が直感的に認識できること。③既存の細胞株名の表記方法(例：KhES-1, KhES3, CT4, B124-2)と同じフォーマットを採用すること。④異なる系統の細胞株であること(例：TSRI68iとSHEF4e-ALS)や，同じ系統の細胞株であること(例：SHEF3とSHEF5)を容易に認識できること。⑤柔軟性のあるルールにすること。

その細胞の名称の表記方法は図[3]に示すような 4 つの構成要素からなるものであり，(a)細胞株の樹立機関

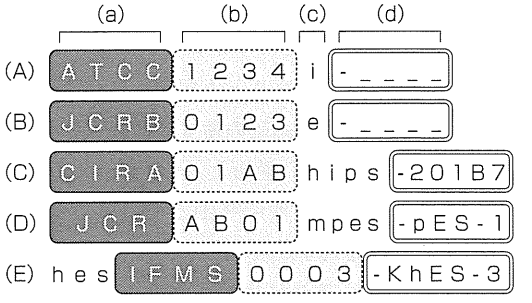

図1　ヒトES/iPS細胞株の命名法の案

(A)(B) ISCR[3]，(C)(D)山中教授ら[4]，(E)筆者らの案による細胞株名表記の例

(a)細胞株を樹立した研究機関(研究室または研究所)のID

(b)細胞株のID

(c)細胞種や由来を識別する記号。ISCRの提案[3]によると“iPS細胞”を“i”，“ES細胞”を“e”で表す。

(d)“-(ハイフン)”とその後に続くアルファベットまたは数字で細胞株の特徴やクローン番号等を表す。ISCR[3]は，(c)と(d)の部分は任意とし，(a)と(b)のみで細胞名を表すことも考慮している。それぞれの要素を表す部分に使用する文字数と数字の桁数に自由度をもたせることも可能だが，データベース管理及び検索の便宜上，①スペースを含まない，②アルファベットの大文字や小文字の表記法，数字の桁数，ハイフンの位置なども統一し，③全体で14文字に限定したものが望ましいとしている。細胞株名の表記法については，さらに議論が必要である。　(文献 3 より引用改変)

のID，(b)細胞株のシリアル番号(ID)，(c) ES細胞またはiPS細胞を区別する略号，および(d)細胞の特徴を示す情報を記載する。さらに，データベース上で処理するため，規定された場所にハイフンを使用する，文字数と数字の桁数を規定する，スペースを使用しない，ハイフンを含めて14桁に統一することを提案[3]している。

ヒトES/iPS細胞サブクローン株の数への対応

iPS細胞は 1 種類のドナー細胞から100種類以上のクローンを作製することもある。山中所長らは，細胞株を識別するためのIDの表記(図(b)の部分)は増大する株数に対応し得る方式でなければならないと提言[4]

している。また，サブクローンを作製した場合，オリジナルのクローン番号とサブクローン番号の両方を含むよう命名するべきであるとも提言している。現在，世界中の研究室でiPS細胞の樹立が進められており，その株数は数年のうちに数万という値に達することが予想される。シリアル番号やIDをつけていくとしたら，このような莫大な細胞株数に対応できるものでなければならない。アラビア数字のみではなく，アルファベットなどの文字や記号とアラビア数字を組み合わせてIDを表記すれば，細胞株数が膨大になっても対応できるのではないかと山中所長ら[4]は提案している。

ヒトES/iPS細胞の既存の細胞株について

山中所長ら[4]は，すでに世界中に広く知られている細胞株(例：201B7, hFIB2-iPS2)に新規にシリアル番号等で設定し直す場合にも，オリジナルの名称およびクローンIDを継承することができるような柔軟性をもたせるべきであると提言している。オリジナルの名称から細胞株の情報や原著論文を簡単に収集できるなど，研究者にとって都合が良い点が多いとしている[4]。すでに独自の方式で命名し，細胞株を管理している研究機関は多いため，すべての研究機関の樹立細胞株に対して公平にIDを分配するには多くの困難が予想される。しかし，集積された細胞情報や研究成果を活用するためにも，国際規格のIDを公平に付与できるよう整備し，細胞株のデータベース化を推進していく必要があるのではないだろうか。

すべての多能性幹細胞へ適応

ISCRの提案は，図の(c)の部分には，“i”あるいは“e”を表記することによりその細胞株がヒトiPS細胞株とヒトES細胞株のいずれかであることを識別できるようにするというものである[3]。この点に関して，山中所長らとPollok所長ら両者ともに，マウスiPS細胞，体細胞核移植ES細胞(NT-ESC)，単為発生胚由来ES細胞(parthenogenetic-ESC)，胚性腫瘍細胞(embryonal carcinoma cell：ECC)，胚性生殖系細胞(embryonal germ cell：EGC)，エピブラスト幹細胞(epiblast stem cell：EpiSC)などの多能性細胞をすべてこの命名法規定の対象に含めるべきであり，これらを正確に識別できるよう動物種や由来細胞を表すコードを図の(c)に表記することを提案している[4][5]。しかし，筆者らは，細胞種を識別するコードを頭につけたほうが分類しやすいのではないかと考える(図)。

ヒトES/iPS細胞において特定の病名を表すのは適当ではない

名称に病名を含めることに関して，Pollok所長ら[5]は懸念を抱いている。多くのヒトiPS細胞は，新生児表皮線維芽細胞(neonatal foreskin fibroblasts)から人工的に誘導され，“正常(non-diseased)”な指標細胞としても使用されている。しかしながら，組織を採取する段階でドナーの異常を検出できることは難しく，その匿名性からドナーの病歴の追跡は不可能である。現段階では，ヒトES/iPS細胞や分化させた細胞の病気に対する感受性を明らかにすること(どのような病気になりやすいかを予測すること)も不可能である。細胞登録の際にはドナーの病歴などに関する情報もわかっている範囲で報告すべきであるが，遺伝子の変異や欠失などの確定された情報の表記を提案している。

ヒトES/iPS細胞を培養する現場での作業

山中所長ら[4]とPollok所長ら[5]は両者とも，細胞株の名称に使用する文字数はできるだけ短くするよう主張している。データベース上で管理する際の利便性も重要だが，現場での作業も考慮するべきである。培養デッシュや凍結チューブにグローブをした手で書きやすく，読み取りやすくすることが重要である。ATCCで1.5mLチューブを用いる場合，細胞株の名称が10文字以下であることが理想的であるとPollok所長ら[5]は述べている。山中所長ら[4]も，ISCRの提案した14

表1　海外の細胞登録サイト

	機関	アドレス
Stem Cell Registry	ISCI	http://www.stem-cell-forum.net/ISCF/initiatives/isci/stem-cell-registry/
ISCR	UMass	http://www.umassmed.edu/iscr/index.aspx
hESCreg	EU連携	http://www.hescreg.eu/
NIH Human Embryonic Stem Cell Registry	NIH	http://grants.nih.gov/stem_cells/registry/current.htm

ISCI：The International Stem Cell Initiative
ISCR：The International Stem Cell Registry
UMass：The University of Massachusetts Medical School, Human Stem Cell Bank and Registry
hESCreg：European Human Embryonic Stem Cell Registry

文字[3]は不便を感じる長さであり，簡略化した名称を使用し始めるようになることを危惧する。簡略化した名称の使用は，細胞の混同のリスクにつながる。医薬基盤研JCRB細胞バンクや理化学研究所バイオリソースセンター細胞バンクでは，場合によってバーコードラベルを用いて管理している。最近は安価なバーコードリーダーもあり，研究室レベルにおいても利用が可能ではないだろうか。

これまでに命名法が規定され，広く活用されている例がある[3]。分化抗原群は，“CD42a”“CD42b”のようにCD番号で表記され，個別の抗原が認識される。また，遺伝子や蛋白質などについてはさまざまな名称が使用されるが，データベースに登録されたアクセッション番号によって識別され，容易にその原著論文まで確認できる。利便性の高い命名法の策定とデータベースの構築を行い，現場のニーズに対応する鍵となるのが，やはり細胞登録システムの整備である。細胞株の名称やIDとともに細胞情報を登録し，管理していくことが必要であろう。

ヒトES/iPS細胞の登録

ヒトES/iPS細胞を樹立した際，具体的な報告方法に関する国際的な統一規定はなく，新規の細胞の樹立を含めた研究成果の報告項目などは研究者やジャーナルの査読者に任されている。今後，産業応用される可能性があることから，各国の倫理規定を尊重して共有できるよう倫理的妥当性および科学的合理性を将来にわたって確保することが肝要である。**表1**の記載の通り，海外のヒトES細胞については，ISCBI，EUヒトES細胞登録(European Human Embryonic Stem Cell Registry：hESCreg)が連携して，それぞれのホームページで公開をしている。また，NIHヒトES細胞登録(NIH Human Embryonic Stem Cell Registry)では，NIHの研究費を使用して研究が可能な細胞が掲載されている。細胞登録に必要な情報として，**表2**に示す5つの項目が提案されている[3][8]。このようなヒトES/iPS細胞の情報整備は，新規細胞株の樹立に必要な基本データ作成とその情報公開の推進につながると思われる。

おわりに

幹細胞研究者の方々には，細胞樹立の際に前記の問題をご一考いただければ幸いである。一方で，幹細胞研究者らの声をさらに集約し，想定される問題を回避し，かつ利便性の高い命名法を早期に確立することが望まれる。ES/iPS細胞株を含む多能性幹細胞の命名法および発表に関するルールを設け，情報をデータベース化し，世界中で共有することは，幹細胞研究の推進につながる。本総説が日本の幹細胞研究推進の一助となれば幸いである。

表2　ES/iPS細胞の登録や研究成果の報告の際に必要な情報

細胞株の由来（source） 細胞のタイプ，由来組織，継代数など ドナーから採取された場合：ドナーの年齢，性別，人種（自己報告または解析結果）* 細胞バンクや民間企業から入手した場合：細胞株のアクセッション番号
樹立方法（derivation method） 細胞の株化までの方法，培地および添加物，培養期間，継代数など詳細な培養方法 ES細胞の場合：胚の取り扱い方法，胚盤胞を得るための透明帯除去方法，胚盤胞からの内部細胞塊の単離方法 iPS細胞の場合：リプログラミングに用いたベクターシステム，低分子，蛋白質，mRNAやmiRNAとその導入・誘導方法
細胞特性（characterization） 未分化状態の確認（免疫染色，フローサイトメトリー，遺伝子発現プロファイリングなど） 多能性の確認（*in vitro*分化，テラトーマ形成，遺伝子発現プロファイリングなど） 核型，SNP（一塩基多型）によるゲノム解析結果*
細胞同一確認（genetic identity）と無菌性（sterility） STR（short tandem repeat）やSNP解析による細胞認証試験結果* 無菌試験結果およびマイコプラズマ否定試験結果
細胞の来歴（provenance） ドナーに対する説明および同意（インフォームド・コンセント），利益相反についての確認

研究者から提供される細胞株の情報を名称・登録番号と合わせて管理していくべきだが，特に個人を特定できる情報（*）は，各国の倫理規定を尊重し，慎重に管理されなければならない。

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Expression of gangliosides, GD1a, and sialyl paragloboside is regulated by NF-κB-dependent transcriptional control of α2,3-sialyltransferase I, II, and VI in human castration-resistant prostate cancer cells

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Gangliosides are sialic acid–containing glycosphingolipids that are associated with tumor malignancy and progression. Among the enzymes required for the production of gangliosides, sialyltransferases have received much attention in terms of their relationship with cancer. In our previous report, ganglioside GD1a and sialyl paragloboside (SPG), a neolacto-series ganglioside, were much more abundant in PC3 and DU145 cells, castration-resistant prostate cancer cells, as compared with hormone-sensitive prostate cancer cells and normal prostate epithelium. GD1a is synthesized from GM1 by α2,3 sialyltransferase (ST3Gal) I and mainly by ST3Gal II. The enzyme to synthesize SPG is ST3Gal VI. The high production of GD1a and SPG in castration-resistant prostate cancer cells was correlated with the high expression of ST3Gal II and VI, respectively. The expression of ST3Gal I and II was mildly induced by phorbol-12-myristate-13-acetate (PMA), and PMA-induced expression of ST3Gal I and ST3Gal II was inhibited by NF-κB decoy oligodeoxynucleotides (ODN) but not by AP-1 decoy ODN. Among the five mammalian homologs of the NF-κB family, RelB RNAi most effectively inhibited the expression of ST3Gal I and ST3Gal II. The expression of ST3Gal VI was also most effectively inhibited by RelB RNAi. The amount of GD1a and SPG was significantly reduced by RelB siRNA treatment in PC3 cells. Thus, the production of GD1a and SPG in castration-resistant prostate cancer cells was indirectly controlled by NF-κB, mainly by RelB, through the transcriptional regulation of ST3Gal I, II, and VI.

Cancer Cell Biology

Prostate cancer is one of the most commonly diagnosed malignant tumors.[1] In the United States, prostate cancer is the second leading cause of cancer-related death in men, and it is a growing problem worldwide.[1] Prostate cancer progresses from prostatic intraepithelial neoplasia through locally invasive adenocarcinoma to castration-resistant metastatic carcinoma.[2] Because prostate cancer exerts androgen-dependent growth, androgen ablation or anti-androgen therapies are very effective. Although most patients initially respond to androgen ablation, in many cases, castration-resistant prostate cancers develop within a couple of years.[3] However, effective therapies have not yet been established against castration-resistant prostate cancers.

Against castration-resistant prostate cancers, several gene therapy approaches have been clinically tested.[4] Oncolytic viruses have been developed to selectively augment antitumor effects, and some viruses such as adenovirus and herpes simplex virus are also used for prostate cancers.[5,6] Recently, we reported that inactivated Sendai virus particles selectively induced apoptosis in some human cancer cells by RIG-I–mediated gene expression and the induction of multiple antitumor immunities.[7,8] We also found that Sendai virus very efficiently infects castration-resistant human prostate cancer cell lines, PC3 and DU145, because of the high production of viral receptor gangliosides such as GD1a and sialyl paragloboside (SPG).[7] Both of these gangliosides bear an *N*-acetylneuraminic acid attached to the terminal galactose residue by α2-3 linkages and are recognized by the HN protein of Sendai virus.[9] GD1a is the most prevalent ganglioside among castration-resistant prostate cancer cell lines (including PC3 and DU145), while it is barely detectable in hormone-sensitive prostate cancer LNCap cells and normal prostate epithelium.[7,10,11] Endogenous immune response to GD1a was observed in patients with prostate cancer but not in healthy

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controls.[11] We were the first group to identify the increased level of SPG, a neolacto-series ganglioside, in castration-resistant prostate cancer cells.[7]

Gangliosides have crucial regulatory roles in normal physiological process, such as embryogenesis,[12] as well as in pathological conditions, including tumor onset and progression.[13] GD1a has several biological actions that promote cancer progression. Highly metastatic cancer cells have abundant GD1a, and GD1a is involved in cancer cell adhesion to endothelial cells during metastasis.[14] GD1a shed by tumor cells in the tumor microenvironment promotes angiogenesis and enhances EGFR signaling by an increase in dimerization.[15–17] Thus, GD1a may be involved in cancer cell proliferation and metastasis. However, the function of SPG remains unknown, although it is enriched in cultured human brain microvascular endothelial cells.[18]

Among the enzymes required for the production of gangliosides, sialyltransferases have received much attention in terms of their relationship with cancer. GD1a is synthesized from GM1 by α2,3 sialyltransferase (ST3Gal) I and II.[19] The K_m value of ST3Gal II for GM1 is smaller than that of ST3Gal I[20]; thus, ST3Gal II preferentially contributes to the synthesis of GD1a.[20–22] SPG is synthesized from neolactotetraosylceramide by ST3Gal VI.[19,23] ST3Gal I or II is up-regulated in human cancer tissues such as bladder cancer or renal cell carcinoma.[24,25] ST3Gal VI is up-regulated in melanoma cells.[23] Therefore, these sialyltransferases may be involved in tumorigenesis and/or tumor progression. However, the mechanism by which the gene expression of sialyltransferases is modified is totally unknown.

In this study, we have investigated the regulatory mechanism of the expression of ST3Gal I, II, and VI in castration-resistant prostate cancer cells to elucidate why GD1a and SPG are so rich in castration-resistant prostate cancers.

Material and Methods

Cell culture

Castration-resistant human prostate cancer cell lines, PC3 and DU145, and a hormone-sensitive human prostate cancer cell, LNCap clone FGC, were purchased from American Type Culture Collection (Rockville, MD). A normal human prostatic epithelial cell, PNT2, was purchased from the European Collection of Animal Cell Cultures (Porton Down, UK). PC3 cells were maintained in Dulbecco's modified Eagle F12 medium (Nakarai Tesque, Kyoto, Japan), and DU145, LNCap, and PNT2 cells were maintained in RPMI 1640 medium (Nakarai Tesque, Kyoto, Japan). All media was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Reagents and antibodies

Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma Chemical (St. Louis, MO). Anti-human c-Jun (60A8) was purchased from Cell Signaling (Danvers, MA). Anti-human c-Fos (sc-253), RelA (sc-8008), and RelB (sc-226) were purchased from Santa Cruz (Santa Cruz, CA). Anti-human β-actin (AC-15) was purchased from Abcam (Cambridge, UK).

Real-time quantitative RT-PCR

Total RNA was isolated using an RNeasy RNA isolation kit (Qiagen, Valencia, CA). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed with an Applied Biosystems 7900 HT Fast Real-Time PCR system under the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Mixtures of probes and primer pairs specific for human ST3Gal I (Hs00161688_m1), ST3Gal II (Hs00199480_m1), ST3Gal VI (Hs00196086_m1), MMP9 (Hs00957562_m1), RelA (Hs00153294_m1), RelB (Hs00232399_m1), c-Rel (Hs00231279_m1), NF-κB1 (Hs00765730_m1), NF-κB2 (Hs00174517_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were purchased from Applied Biosystems. The relative expression levels were calculated from a standard curve obtained using log dilutions of cDNA containing the gene of interest, and values were normalized to an internal GAPDH control.

Western blot analysis

Cells were harvested in RIPA lysis buffer. The nuclear extracts and cytoplasmic extracts were prepared using NE-PER extraction reagents (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions. Protein samples (10 μg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins were transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk and then incubated overnight at 4°C with anti-c-Jun (1:500), anti-c-Fos (1:500), anti-β-actin (1:5000), anti-RelA (1:200), or anti-RelB (1:200) antibodies. The membranes were washed and labeled with a 1:2000 dilution of horseradish peroxidase–conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK) at room temperature for approximately 1 hour. Detection by chemiluminescence was performed according to the ECL user's guide (Amersham, Buckinghamshire, UK).

Evaluation by reporter gene

Genes were transfected into cells along with a luciferase gene driven by the AP-1 binding site (AP-1 luciferase reporter gene; BD Bioscience Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen, Tokyo, Japan). The luciferase activity was measured with the dual-luciferase assay system (Promega, Madison, WI) after the transfected cells were incubated with serum-free medium for 48 h.

Decoy oligodeoxynucleotides experiments

The following phosphorothioated and double-stranded decoy oligodeoxynucleotides (ODN) were purchased from Gene Design (Osaka, Japan): AP-1 decoy ODN, 5′-CGCTTGATGA

CTCAGCCGGAA-3′, 3′-GCGAACTACTGAGTCGGCCTT-5′; corresponding mutant decoy ODN, 5′-CGCTTGATTACTTAG CCGGAA-3′, 3′-GCGAACTAATGAATCGGCCTT-5′[26]; NF-κB decoy ODN, 5′-CCTTGAAGGGATTTCCCTCC-3′, 3′-GGA ACTTCCCTAAAGGGAGG-5′; corresponding scramble decoy ODN, 5′-TTGCCGTACCTGACTTAGCC-3′, 3′-AACGGCATG GACTGAATCGG-5′.[27] Cells were transfected with 1.0 μM decoy ODN with Lipofectamine 2000 (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. Transfected cells were used for experiments 48 h after transfection.

RNA interference experiments

The following double-strand stealth small interfering RNA (siRNA) oligonucleotides and scramble RNA were purchased from Invitrogen: siRNA oligonucleotides against c-Jun, (sense) 5′-UGGAAACGACCUUCUAUGA-3′ and (antisense) 5′-UCA UAGAAGGUCGUUUCCA - 3′; siRNA oligonucleotides against RelA, (sense) 5′-UUUACGUUUCUCCUCAAUCCGGUGA-3′ and (antisense) 5′-UCACCGGAUUGAGGAGAAACGUA AA-3′; siRNA oligonucleotides against RelB, (sense) 5′-UGC UGAACACCACUGAUAUGUCCUC-3′ and (antisense) 5′-GAGGACAUAUCAGUGGUGUUCAGCA-3′; siRNA oligonucleotides against c-Rel, (sense) 5′-UUUACACGACAAAU CCUUAAUUCUG-3′ and (antisense) 5′-CAGAAUUAAGGA UUUGUCGUGUAAA-3′; siRNA oligonucleotides against NF-κB1, (sense) 5′-UAAAUCAUCAGAUGUAAACUCUGGC-3′ and (antisense) 5′-GCCAGAGUUUACAUCUGAUGAUUUA-3′; and siRNA oligonucleotides against NF-κB2, (sense) 5′-CAAUGGAGGAGUUCAAUUUGAAAUC-3′ and (antisense) 5′-GAUUUCAAAUUGAACUCCUCCAUUG-3′. Transfections were performed with lipofectamine RNAiMAX (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions.

Analysis of acidic glycosphingolipids

The structures of acidic glycosphingolipids (GSLs) were analyzed by the enzymatic release of carbohydrate moieties, fluorescent labeling with aminopyridine and two-dimensional mapping and mass spectrometry. The majority of experimental procedures have been reported previously.[28] In brief, the acidic GSLs were extracted from cells and digested with recombinant endoglycoceramidase II from *Rhodococcus* sp. (Takara Bio, Shiga, Japan). The released oligosaccharides were labeled with 2-aminopyridine (2-AP) and separated on a Shimadzu LC-20A HPLC system equipped with a Waters 2475 fluorescence detector. Normal-phase HPLC was performed on a TSK gel Amide-80 column (0.2 × 25 cm, Tosoh, Tokyo, Japan). The molecular size of each PA-oligosaccharide is given in glucose units (Gu) based on the elution times of PA-isomaltooligosaccharides. Reversed-phase HPLC was performed on a TSK gel ODS-80Ts column (0.2 × 15 cm, Tosoh). The retention time of each PA-oligosaccharide is given in glucose units based on the elution times of PA-isomaltooligosaccharides. Thus, a given compound on these two columns provides a unique set of Gu (amide) and Gu (ODS) values, which correspond to coordinates of the 2-D map. PA-oligosaccharides were analyzed by LC/ESI MS/MS. Standard PA-oligosaccharides, PA-GM1 and PA-GD1a, were purchased from Takara Bio, and PA-LST-a and PA-SPG were obtained from our previous study.[28]

Statistics

Results are reported as the mean ± standard error (S.E.). The two-tailed unpaired Student's *t*-test was used to determine statistical significance between two groups. Probability values of $p < 0.05$ were considered statistically significant. The statistical analysis was performed with StatView 5.0 software (SAS Institute, Cary, NC).

Results

Expression of ST3Gal I and ST3Gal II in prostate cancer cells and normal prostate epithelium

In our previous report, the constitutive expression levels of gangliosides were analyzed in castration-resistant prostate cancer cells (PC3 and DU145), hormone-sensitive prostate cancer cells (LNCap) and normal prostate epithelial cells (PNT2) using HPLC.[7] Castration-resistant prostate cancer cells (PC3 and DU145) produced higher amounts of GD1a than LNCap and PNT2 cells.[7] In LNCap cells, the expression of acidic GSLs was barely detectable, including GM1 (from which GD1a is synthesized).[7] The expression of SPG was also up-regulated in castration-resistant prostate cancer cells.[7]

GD1a is synthesized from GM1 by ST3Gal I and II,[19] with ST3Gal II preferentially contributing to the synthesis of GD1a.[20–22] We determined the constitutive expression level of ST3Gal I and II in prostate cancer cells and normal prostate epithelium using quantitative real-time PCR (Fig. 1*a*). Castration-resistant prostate cancer cells (PC3 and DU145) produced greater amounts of ST3Gal II than LNCap and PNT2 cells. Thus, the high expression level of GD1a in castration-resistant prostate cancer cells was correlated with the high expression level of ST3Gal II. Although LNCap cells had a high expression level of ST3Gal I (Fig. 1*a*), their expression of acidic GSLs (including GM1, a precursor of GD1a) was barely detectable.[7]

PMA is a promoter of tumorigenesis and can activate AP-1 and NF-κB transcription factors.[29] We examined whether the expression of ST3Gal I and II is induced by PMA. MMP-9 was used as a positive control because it plays an important role in the invasion and metastasis of prostate cancer cells,[30] and its expression is induced by PMA stimulation.[31] PC3 cells were starved for 24 h in serum-free medium and then treated with 50 nM PMA for 24 h. Quantitative real-time PCR analyses showed that the expression of ST3Gal I and II was mildly induced by PMA (Fig. 1*b*). The expression of both ST3Gal I and II reached to the peak within 12 h, although the induction was much smaller than that of MMP-9.

AP-1 and NF-κB are constitutively activated in castration-resistant prostate cancer cells

The PMA-induced increase in the expression of ST3Gal I and II suggested that AP1 and/or NF-κB transcription factors may be involved in the expression of these two enzymes. AP-