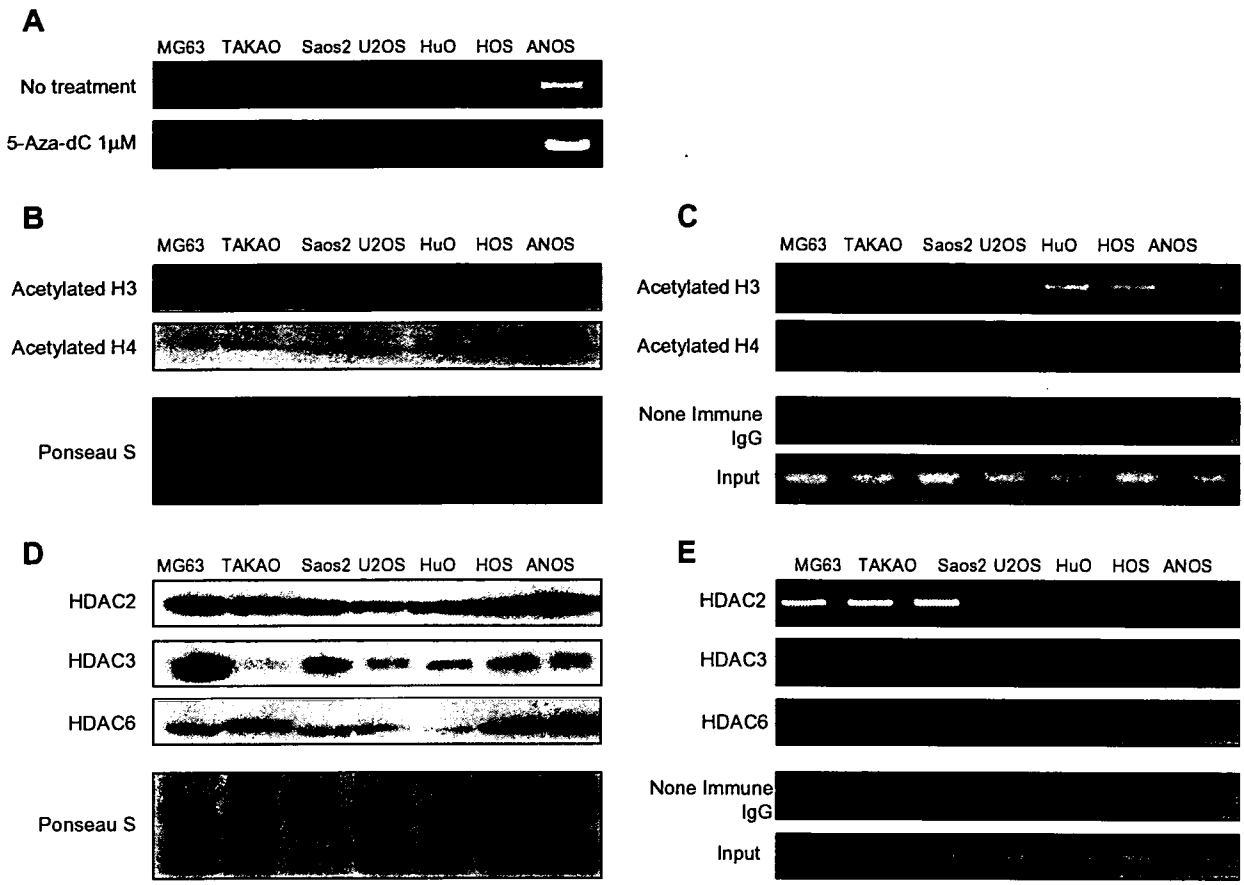

Fig. 1. Histone H3 and H4 tails surrounding the promoter of ChM-I were deacetylated by the binding of HDAC2 in ChM-I-negative OS cells. (A) mRNA expression of ChM-I before and after the treatment with 5-aza-dC (1 μM). (B) Protein expression of acetylated H3 and H4 tail in OS cells. (C) ChIP assay demonstrating the association of acetylated H3 and H4 with the core promoter region of the ChM-I gene in OS cells. (D) Protein expression of HDAC2, HDAC3, and HDAC6. (E) ChIP assay demonstrating the binding of HDACs to the core promoter region of the ChM-I gene in OS cells.

responsible for the deacetylation of histone associated with the promoter region of the ChM-I gene in ChM-I-negative OS cells.

Inhibition of HDAC2 restored the binding of Sp3 and the expression of ChM-I gene

MS-275 is an inhibitor for class I HDACs including HDAC2. The global histone acetylation was promoted by MS-275 in both ChM-I-positive (ANOS) and negative (MG63 and TAKAO) cells (Fig. 2A), whereas the local histone acetylation status caused by the MS-275 treatment differed among the cell lines. Histone H4 was further acetylated in ANOS, and the acetylation of both histone H3 and H4 was induced in MG63. No such induction of histone acetylation was observed in TAKAO (Fig. 2B).

Then we analyzed the binding of Sp3 and HDAC2 to the promoter region of the ChM-I gene after the treatment with MS-275 and/or 5-Aza-dC (Fig. 2C). In ANOS, the binding status of Sp3 or HDAC2 was not changed by any treatment (Fig. 2C). In MG63, the binding of Sp3 was induced in association with the elimination of the binding of HDAC2 by MS-275, but not by 5-Aza-dC treatment (Fig. 2C). In TAKAO, the binding of Sp3 was induced by 5-Aza-dC treatment with no change in the binding of HDAC2, and treatment with MS-275 caused a reduction in the binding of HDAC2, but failed to induce the binding of Sp3 (Fig. 2C). Simultaneous treatment with 5-Aza-dC and MS-275 resulted in a reduction in the binding HDAC2. The expression of the ChM-I gene in three cell lines correlated with the status of Sp3 binding; MS-275 treatment and 5-Aza-dC treatment induced the expression in MG63 and TAKAO, respectively. The binding of Sp1, HDAC3, and HDAC6 was not changed by MS-275 and/or 5-aza-dC treatment in these three cell lines (Fig. 2C). We performed identical experiments using Saos2, another ChM-I-negative OS cell line, and obtained essentially the same results as in TAKAO (data not shown).

CpG methylation of the promoter lesion was demethylated by MS-275 in a DNA replication-dependent manner

The induction of ChM-I gene expression in MG63 by HDACi was an unexpected result because the promoter

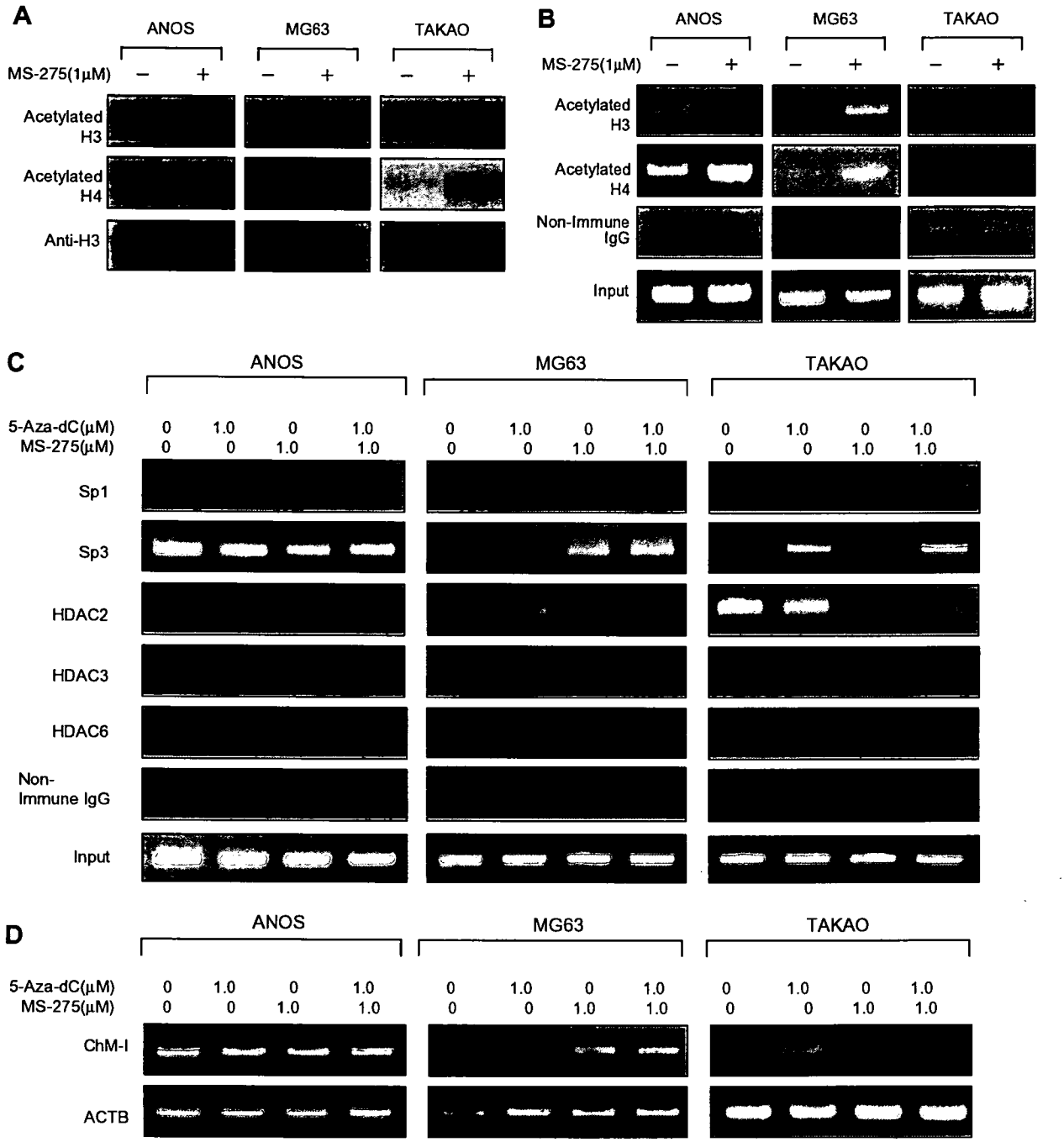

Fig. 2. Histone H3 and H4 tails were acetylated by MS-275, eliminating HDAC2. (A) Protein expression of acetylated H3 and H4 in OS cells with or without treatment with MS-275 (1 μM). (B) ChIP assay demonstrating the association of acetylated H3 and H4 with the core promoter region of the ChM-I gene in OS cells with or without treatment with MS-275 (1 μM). (C) ChIP assay demonstrating the binding of Sp1, Sp3, HDAC2, HDAC3, and HDAC6 with the core promoter region of the ChM-I gene in OS cells. Cells were treated with 5-Aza-dC and MS-275 at the indicated concentration. (D) mRNA expression of the ChM-I gene in OS cells treated with 5-Aza-dC and MS-275 at the indicated concentration.

region was heavily methylated in this cell line [1]. Bisulfite genomic sequencing of the promoter region revealed that the methylation of MG63 was significantly reduced and almost equivalent with that of ANOS (Fig. 3A). Methylation in the promoter region of TAKAO was also reduced but to much less of an extent (Fig. 3A). Conversion between acetylation and methylation of H3-K9 is known to regulate the methylation status of CpG [5]. Notably, the dimethylation of H3-K9 is known to be correlated with methylation of CpG [6]. Therefore, the modification of H3-

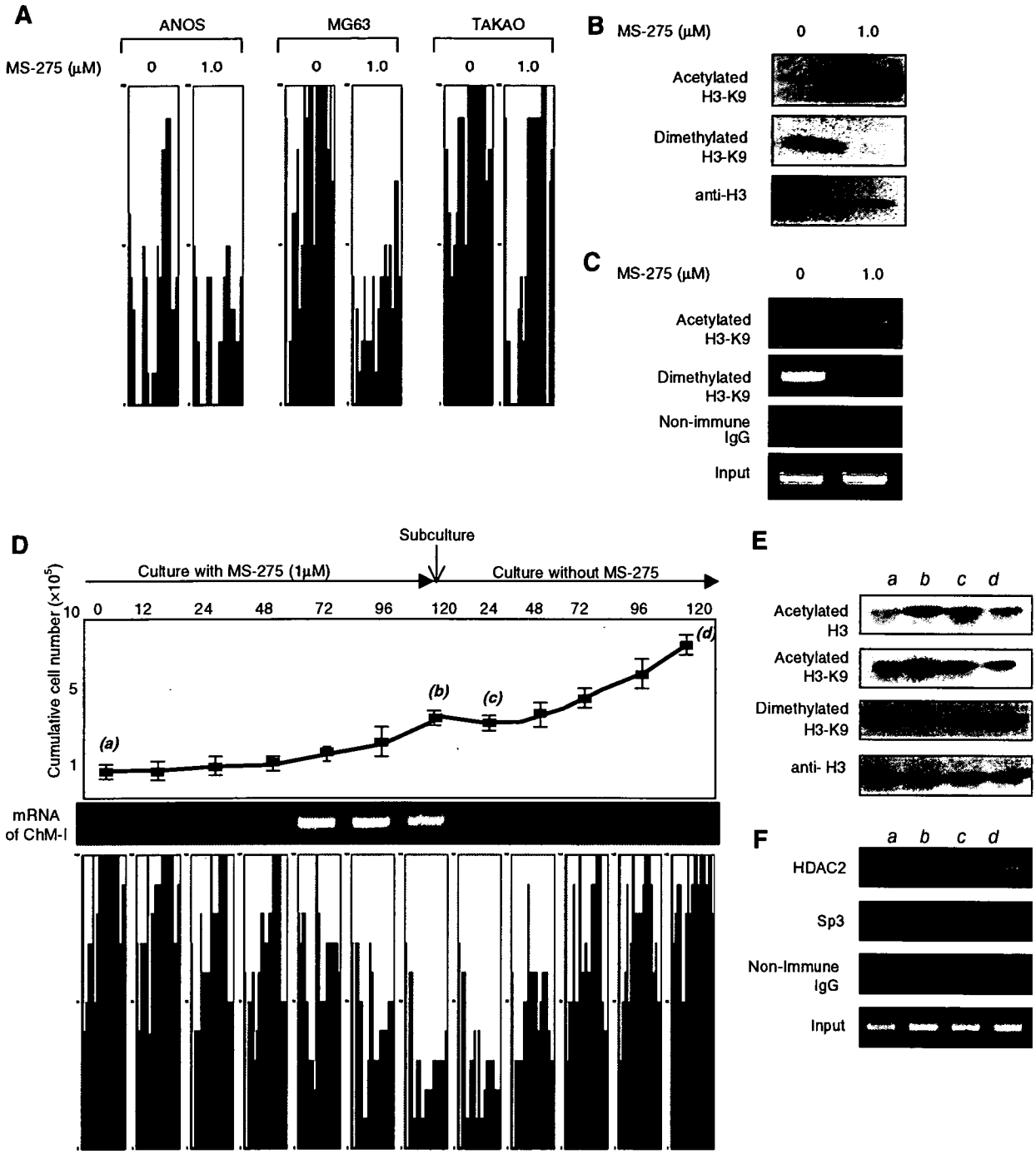

Fig. 3. Modification of the histone tail was associated with the methylation of CpG in the promoter region of the ChM-I gene. (A) Methylation status of the core promoter region of OS cells analyzed by bisulfite sequencing. Cells were treated with the indicated concentration of MS-275 for 96 h. *Y*-axis indicates the number of methylated alleles and *X*-axis indicates the position of each CpG site relative to the transcription start site. (B) Protein expression of acetylated H3-K9 and dimethylated H3-K9 in MG63 treated with the indicated concentration of MS-275. (C) ChIP assay demonstrating the association of acetylated H3-K9 and dimethylated H3-K9 with the core promoter region of ChM-I gene in MG63 cells treated with the indicated concentration of MS-275. (D) Temporal association of the mRNA expression and the methylation status of the core promoter region of the ChM-I gene in MG63 treated with MS275 (1 μM). Cumulative cell number (upper column), mRNA expression (middle column), and methylation status of the core promoter region of the ChM-I gene (lower column) were sequentially analyzed at the indicated time points. (E) Protein expression of acetylated H3, acetylated H3-K9, and dimethylated H3-K9 of MG63 at the time point indicated in (D). (F) ChIP assay demonstrating the binding of HDAC2 and Sp3 to the core promoter region of the ChM-I gene in MG63 at the time points indicated in (D).

K9 was analyzed by Western blotting (Fig. 3B) and ChIP assay (Fig. 3C). The dimethylation was converted to acetylation by MS-275 treatment in both global (Fig. 3B) and local promoter areas (Fig. 3C) in MG63.

To investigate the temporal relationship between the demethylation process and the acetylation process, the methylation status of the promoter region of MG63 was sequentially analyzed after exposure to MS-275 (Fig. 3D). The expression of ChM-I was gradually up-regulated in parallel with the demethylation of the promoter region in a DNA replication-dependent manner (Fig. 3D, a and b). At 120 h after the exposure to MS-275, the modification of H3-K9 was converted from dimethylation to acetylation (Fig. 3E, b), and Sp3 replaced HDAC2 (Fig. 3F, b). The expression of the ChM-I gene completely disappeared 24s hr after the withdrawal of MS-275 from the culture medium (Fig. 3D, c). At this time point, the binding of HDAC2 was restored eliminating the binding of Sp3 (Fig. 3F, c), although the promoter region was still hypomethylated (Fig. 3D, c). The methylation of CpG was gradually increased in a DNA replication-dependent manner and returned to the original status at 120 h after the withdrawal of MS-275 (Fig. 3D, d). These results suggested that MS-275 inhibited the maintenance of CpG methylation after DNA replication by replacing methylated H3-K9 with acetylated H3-K9.

Discussion

Both histone deacetylation and DNA methylation are important mechanisms to silence the transcription of genes unrelated to the biological phenotype of each cell. Acetylation of the histone tail is catalyzed by histone acetyltransferase (HAT) and analyzed using HDAC, each of which consists of a large family [4]. The acetylation status of histone associated with each genomic locus will be determined by the balance of two factors, HAT and HDAC, in each case, and therefore the deacetylation is regarded as a reversible silencing mechanism [4]. On the other hand, because there are no intrinsic factors with demethylase activity, DNA methylation catalyzed by DNA methyltransferase has been considered an irreversible silencing mechanism [3]. Recent reports have shown that these two mechanisms were not independent and moreover, closely related to each other [8–10].

One of the most intriguing results of this study is that the methylation of CpG was reduced by HDACi without further treatment with 5-Aza-dC. The reduction in methylation by HDACi seems to be dependent on DNA-replication (Fig. 3C). Several reports have emphasized the interplay between DNA methylation and histone methylation [11,12], notably, that H3-K9 dimethylation directry and H3-K9 acetylation inversely correlates with DNA methylation [5]. Loss-of-function mutations in H3-K9 methyltransferases of *Neurospora* and *Arabidopsis* were found to reduce overall levels of DNA methylation in vivo [8]. Consistent with these findings in fungi and plants, we here showed that the reduction in level of dimethylated histone H3-K9 was associated a reduction of DNA methylation at a specific residue in the regulatory region of a human gene, presumably due to the increase in acetylated histone H3-K9 caused by HDACi. The demethylating effect of another HDACi has been reported [9,10]. Further analysis of this intriguing matter may provide new insight into the regulation of gene expression by chromatin remodeling factors.

Hypermethylation of CpG dinucleotides in the core promoter region was a common feature of the three ChM-I-negative OS cell lines, which also shared deacetylation of the histone tail associated with the promoter region. The response to DNA-demethylating or histone-acetylating treatment was, however, quite different. In the case of TAKAO and Saos2, HDACi treatment failed to induce histone acetylation and therefore Sp3 binding, whereas 5-Aza-dC treatment induced Sp3 binding without modifying of histone acetylation, suggesting that DNA methylation may be a dominant factor in these cells. In the case of MG63, however, HDACi effectively induced histone acetylation and Sp3 binding in association with DNA demethylation. We have no clear explanation for the difference between these cell lines. MG63 is a unique cell line, in which the expression of osteogenic markers is not remarkable under normal culture conditions [1]. The transcription of the alkaline phosphates (ALP) gene was suppressed by CpG methylation and induced by 5-Aza-dC [13]. In contrast, 5-Aza-dC treatment failed to induce the expression of the osteocalcin and ChM-I genes, even when CpG methylation was eliminated [1,13]. In contrast, TAKAO and Saos2 retained features of osteogenic cells. Saos2 has strong ALP activity without any induction, and produces abundant immature bone tissue, osteoid, in the subcutaneous environment of athymic mice (data not shown). It is intriguing whether the difference in differentiation stage relates to the difference in the plasticity of epigenetic regulation, and the ChM-I gene in OS cells will be a suitable material with which to investigate this important issue.

Acknowledgments

We are grateful for Dr. M. Nakanishi for providing helpful suggestions. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Education, Culture, Sports, Science, and Technology, and from the Ministry of Health, Labor, and Welfare.

References

[1] T. Aoyama, T. Okamoto, S. Nagayama, K. Nishijo, T. Ishibe, K. Yasura, T. Nakayama, T. Nakamura, J. Toguchida, Methylation in the core-promoter region of the chondromodulin-I gene determines the cell-specific expression by regulating the binding of transcriptional activator Sp3, J. Biol. Chem. 279 (2004) 28789–28797.

[2] Y. Hiraki, H. Tanaka, H. Inoue, J. Kondo, A. Kamizono, F. Suzuki, Molecular cloning of a new class of cartilage-specific matrix, chondromodulin-I, which stimulates growth of cultured chondrocytes, Biochem. Biophys. Res. Commun. 175 (1991) 971–977.

[3] M. Esteller, Cancer epigenomics: DNA methylomes and histone-modification maps, Nat. Rev. Genet. 8 (2007) 286–298.

[4] T. Jenuwein, C.D. Allis, Translating the histone code, Science 293 (2001) 1074–1080.
[5] Y. Kondo, L. Shen, J.P. Issa, Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer, Mol. Cell. Biol. 23 (2003) 206–215.
[6] W. Zhao, H. Soejima, K. Higashimoto, T. Nakagawachi, T. Urano, S. Kudo, S. Matsukura, S. Matsuo, K. Joh, T. Mukai, The essential role of histone H3 Lys9 di-methylation and MeCP2 binding in MGMT silencing with poor DNA methylation of the promoter CpG island, J. Biochem. (Tokyo) 137 (2005) 431–440.
[7] Q. Gan, T. Yoshida, O.G. McDonald, G.K. Owens, Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells, Stem Cells 25 (2007) 2–9.
[8] H. Tamaru, E.U. Selker, A histone H3 methyltransferase controls DNA methylation in Neurospora crassa, Nature 414 (2001) 277–283.
[9] N. Cervoni, M. Szyf, Demethylase activity is directed by histone acetylation, J. Biol. Chem. 276 (2001) 40778–40787.
[10] J.N. Ou, J. Torrisani, A. Unterberger, N. Provencal, K. Shikimi, M. Karimi, T.J. Ekstrom, M. Szyf, Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines, Biochem. Pharmacol. 73 (2007) 1297–1307.
[11] L.M. Johnson, X. Cao, S.E. Jacobsen, Inteplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methlyation, Curr. Biol. 12 (2002) 1360–1372.
[12] Y. Zhang, D. Reinberg, Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails, Gene. Dev. 15 (2001) 2343–2360.
[13] R.M. Locklin, R.O. Oreffo, J.T. Triffitt, Modulation of osteogenic differentiation in human skeletal cells in Vitro by 5-azacytidine, Cell Biol. Int. 22 (1998) 207–215.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 365 (2008) 406–412
BBRC
www.elsevier.com/locate/ybbrc

Expression of vascular cell adhesion molecule-1 indicates the differentiation potential of human bone marrow stromal cells

Kenichi Fukiage [a,b], Tomoki Aoyama [a], Kotaro R. Shibata [a,b], Seiji Otsuka [a,c], Moritoshi Furu [a,b], Yoshiki Kohno [a,b], Kinya Ito [a,c], Yonghui Jin [a], Satoshi Fujita [d], Shunsuke Fujibayashi [b], Masashi Neo [b], Tomitaka Nakayama [b], Takashi Nakamura [b], Junya Toguchida [a,*]

[a] *Department of Tissue Regeneration, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*
[b] *Department of Orthopaedic Surgery, Graduate School of Medicine, Kyoto University, Japan*
[c] *Department of Musculoskeletal Medicine, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan*
[d] *Department of Reparative Materials, Institute for Frontier Medical Sciences, Kyoto University, Japan*

Received 11 October 2007
Available online 5 November 2007

Abstract

Bone marrow stromal cells (BMSCs) are a mixture of cells differing in differentiation potential including mesenchymal stem cells, and so far no CD antigens were found to be predictable for the differentiation property of each BMSC. Here we attempted to isolate differentiation-associated CD antigens using 100 immortalized human BMSC (ihBMSC) clones. Among 13 CD antigens analyzed, only CD106/Vascular cell adhesion molecule-1 (VCAM-1) showed a clear correlation with the differentiation potential of each clone; CD106-positive ihBMSC clones were less osteogenic and more adipogenic than CD106-negative clones. This association was confirmed in primary BMSCs sorted by CD106, showing that the CD106-positive fraction contained less osteogenic and more adipogenic cells than the CD106-positive fraction. The evaluation of CD106 fraction of BMSC strains in early passages predicted clearly the osteogenic and adipogenic potential after in vitro induction of differentiation, indicating the usefulness of CD106 as a differentiation-predicting marker of BMSC.

Keywords: Bone marrow stromal cell; Cell surface marker; CD106; Adipogenesis; Osteogenesis; Mesenchymal stem cell

Mesenchymal stem cells (MSCs) are defined as plastic-adherent fibroblastic cells with the potential to differentiate into multiple mesenchymal tissues, including bone, cartilage, and fat [1]. There have been many reports of regeneration therapy using bone marrow stromal cells (BMSCs), which are heterogeneous cell populations including MSCs [2,3]. One long-standing enigma concerning MSCs is whether they have a specific cell surface marker such as the CD34 in hematopoietic stem cells. A number of CD antigens and other cell surface molecules have been proposed as candidates and attempts have been made to concentrate MSC populations using such markers by cell sorting techniques. STRO-1 is one classical candidate for an MSC-related cell surface marker, and the fraction of BMSCs sorted with anti-STRO-1 antibody was shown to be rich in MSC-like cells [4]. CD105/endoglin, formerly known as SH2, is also used as an MSC-associated marker to evaluate the efficacy of isolation procedures [5]. Recently, low affinity nerve growth factor receptor (LNGFR) has been proposed as an MSC-associated marker. LNGFR$^+$ mononuclear cells from bone marrow were rich in clonogenic precursors and differentiated into multiple lineages [6]. The modulation of antigen expression was analyzed until the 10th passage, and BMSCs

* Corresponding author. Fax: +81 75 751 4646.
E-mail address: togjun@frontier.kyoto-u.ac.jp (J. Toguchida).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.10.149

retained high levels of CD29, CD44, CD90, and CD105 [7]. Expression of CD106 and CD166 was lost gradually through long-term culture [7,8]. $CD106^+$ cells in bone marrow also demonstrated high clonogeneity and the potential to differentiate in potential to differentiate in multiple directions [9].

In spite of these extensive studies, no definite answer has been yet obtained. This is, at least partially, due to the heterogeneity of cell populations analyzed as MSCs. The clonal analysis of BMSCs is a rational approach to identifying cell surface markers associated with multi-directional differentiation potential. This approach, however, requires a considerable number of cell divisions in vitro, which is not feasible for BMSCs with a limited growth potential. Several groups including ours have established immortalized human BMSCs (ihBMSCs) [10–12]. When analyzed as a whole, ihBMSCs demonstrated multi-directional differentiation potential encompassing osteogenic, adipogenic, and chondrogenic lineages. Clonal analyses, however, revealed that ihBMSC clones were heterogeneous in terms of differentiation potential [10]. This heterogeneity would be useful for identifying differentiation-associated cell surface markers. The identification of such markers may contribute to the clinical application of BMSCs. Most current procedures to induce differentiation into a particular lineage require considerable time. For example, the osteogenic or adipogenic differentiation potential of isolated BMSCs is not known until 2 weeks after the induction of differentiation. This can cause serious problems if the quality turns out to be not good enough for use. Therefore, it would be useful for regeneration therapy using BMSCs to have markers with which to predict the differentiation potential of isolated BMSCs before the induction of differentiation.

Here we addressed this issue using clonal ihBMSCs established through the introduction of hTERT and Bmi1 genes [13], because the karyotype of this cell line is near diploid with less chromosomal aberrations than the ihBMSCs established with hTERT and HPVE6E7 [11,13]. Then, we clarified the relationship between CD106/VCAM-1 expression and differentiation potential of BMSC.

Materials and methods

Cells and tissue samples

ihBMSCs were established by sequential transduction of the hTERT and Bmi1 genes [13], and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, St. Louis, MI) with 10% fetal bovine serum (FBS, Hyclone, South Logan, UT). Subcloning of ihBMSCs was performed by limited dilution, and 100 clones were established and cultured under the same conditions.

Primary human BMSCs (designated as huBM) were isolated from the bone marrow taken from iliac crests of donors, who received orthopedic operative procedures, and cultured by means of a previously described method [14,15]. Briefly, mononuclear cells were isolated from the bone marrow aspirates by density centrifugation, and then washed twice, and suspended in DMEM supplemented with 10% FBS. All procedures were approved by the Ethics Committee of the Faculty of Medicine, Kyoto University, Japan, and informed consent was obtained from each donor.

Differentiation procedures and qualitative and quantitative evaluation

Adipogenic differentiation. Adipogenic differentiation induction was performed by the standard method described previously [14]. Cells were then fixed in 10% buffered formalin and stained with 0.3% Oil-Red-O (Nacalai Tesque, Kyoto, Japan). Clones were categorized as adipogenic when Oil-Red-O positive mature lipoblasts were found in the dish regardless of the number. To quantify the differentiation potential, cells were lysed in 0.1% Thesit (Fluka Chemika, Buchs, Switzerland), and then triglyceride (TG) amounts in the cells were quantified by serum triglyceride determination kit (Sigma–Aldrich). For same samples, protein amounts were quantified using BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). The assays were performed according to the manufacturer's directions.

Osteogenic differentiation. Osteogenic differentiation induction was performed by the standard method described previously [14]. To visualize calcium deposits, cultures were fixed in 100% ethanol (Nakalai Tesque), and stained with 1% Alizarin-Red (Waldeck GmbH&Co, Muenster, Germany). Clones were categorized as osteogenic when Alizarin-Red positive nodules were found in the dish regardless of the number. Alkalinephosphatase (ALP) staining was performed by a method previously described [16]. ALP activity was measured with a Phosphate Substrate kit (BioRad, Hercules, CA). Amounts of DNA were also quantified by PicoGreen dsDNA Quantitation kit (Invitrogen, Carlsbad, CA). The assays were performed according to the manufacturer's directions.

Chondrogenic differentiation. Chondrogenic differentiation induction was performed by the standard method described previously [14]. Cryosections were stained with Alcian blue (Muto Pure Chemicals, Tokyo, Japan). Clones were categorized as chodrogenic when an Alcian blue-positive matrix was found in the pellet regardless of the amount. Glycosaminoglycan (GAG) contents in the pellets were quantified by BLYSCAN Dye and Dissociation reagents (BIOCOLOR, Belfast, UK) according to manufacturer's directions. Amounts of DNA were also quantified by PicoGreen dsDNA Quantitation kit.

Reverse transcription (RT)-PCR

Total RNA was extracted from cells using an RNeasy plus mini kit (Qiagen, Hilden, Germany), and reverse-transcribed with the SuperScript III first strand system (Invitrogen). The synthesized cDNA was used as a template for each PCR, and the products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Information on the primer pairs is available upon request.

Complementary DNA for quantitative RT-PCR (QRT-PCR) was synthesized by the same method as above. For each sample and gene, 25 μl reactions were set up in triplicate, each with 12.5 μl of Syber Green supermix (Applied Biosystems, Foster City, CA), 0.5 pmol of each primer, and 0.3 μl of cDNA. The levels of the CD106 and β-actin genes were determined with an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the same primer pairs as in RT-PCR.

Flow cytometry (FCM)

PE-conjugated antibody against human VCAM-1 and PE-conjugated non-immune mouse IgG (isotype control) were purchased from BD Biosciences (BD, San Diego, CA). Before FCM, cells were trypsinized and washed in PBS with 0.5% BSA and 2 mM EDTA. The cells (1×10^5) were incubated with antibody (2 μl) or isotype control. The acquisition was performed in FACS Calibur (BD) using CellQuest software (BD).

Magnet activated cell sorting (MACS)

Trypsinized cells were washed with buffer consisting of PBS with 0.5% BSA and 2 mM EDTA. The cells (1×10^6) were incubated with 20 μl of PE-conjugated anti-human VCAM-1 antibody (BD), and then with 20 μl of anti-PE magnet beads (Miltenyi Biotec, Bergisch Gladbach, Germany). After being washed at each step, the cells were applied to the sorting

column. An MS column (Miltenyi Biotec) for positive sorting and an LD column (Miltenyi Biotec) for negative sorting were used.

Statistical analyses

Statistical analyses were performed using Statcel software. Data were assessed using the Pearson product-moment correlation coefficient and Student's *t*-test.

Results

Heterogeneous differentiation potential of ihBMSC clones

One hundred single-cell derived clones were established from the parental ihBMSC by limited dilution. No significant difference was observed among clones in terms of growth potential (data not shown). The adipogenic, osteogenic, and chondrogenic potential of each clone was determined by the standard induction method and categorized as either positive or negative based on the definition described in Materials and methods section. Five clones showed tri-directional differentiation, and 78 clones showed the potential to differentiate into either two or one lineage, and 17 clones showed no differentiation potential (Supplementary Table 1). This heterogeneity concerning differentiation potential was essentially the same as we previously described in ihBMSCs established with hTERT and HPVE6E7 [10].

Expression of CD106 correlated with the differentiation property of immortalized clones

The expression of 13 CD antigens, among which 10 were known to be positive in hMSC and three were negative [17,18], in 100 clones was analyzed by RT-PCR using gene specific primers (Supplementary Fig. 1). All three MSC-negative antigens were negative or only weakly positive and all 10 MSC-positive antigens were positive in all clones except CD10 and CD106. The expression of CD10 and CD106 was positive in 65 and 35 clones, respectively. There was no clear association between the expression of CD10 and any of the three differentiation potentials. But, the expression of CD106 showed an association with the differentiation potential of ihBMSC clones, especially with osteogenic differentiation potential (Fig. 1A). Then the expression level of CD106 in each clone was evaluated by QRT-PCR. CD106 expression negatively correlated with the osteogenic differentiation potential ($P < 0.01$, Fig. 1B). In case of adipogenic differentiation the expression of CD106 was higher in clones with differentiation

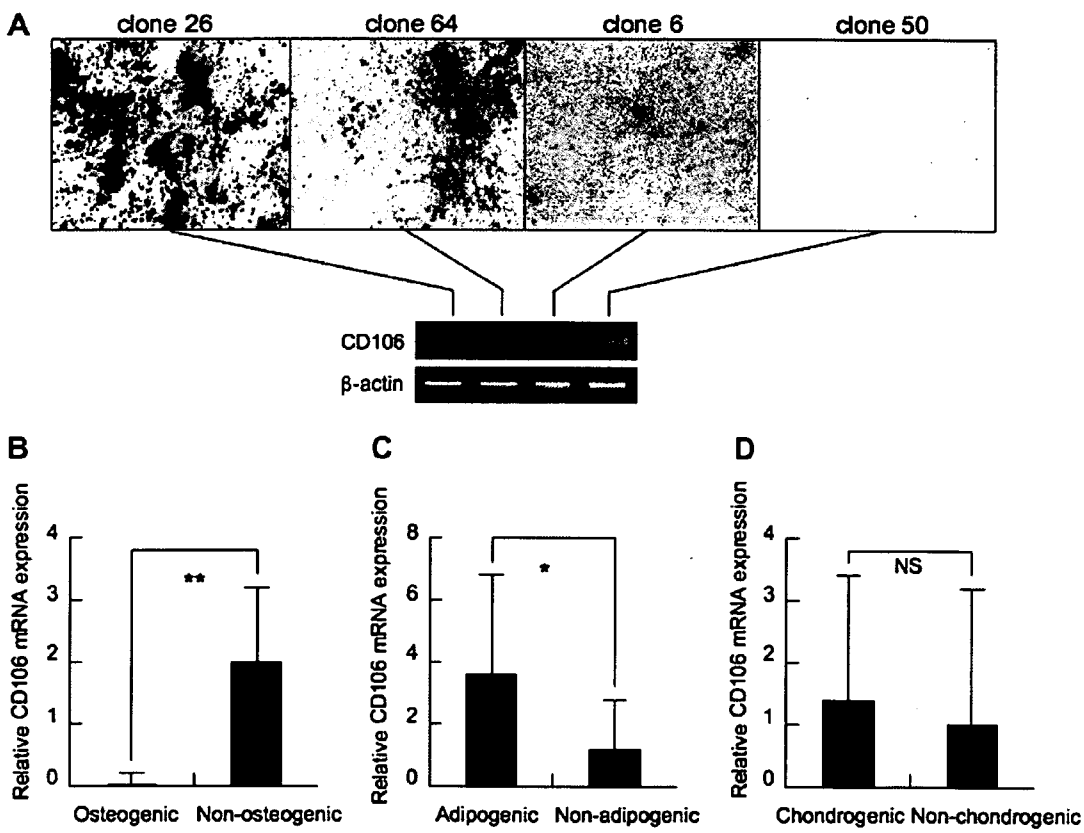

Fig. 1. The expression of CD106 correlated with the differentiation potential in ihBMSC clones. (A) Alizarin-Red staining and the mRNA expression of the CD106 gene in 4 representative clones that differ in osteogenic potential. The mRNA expression of the CD106 relative to the control gene (β-actin) was quantified by QRT-PCR in (B–D). (B) The average expression of CD106 in 29 osteogenic ihBMSC clones was significantly weaker than that in 71 non-osteogenic clones (**$P < 0.01$). (C) The average expression of CD106 in 46 adipogenic ihBMSC clones was significantly higher than that in 54 non-adipoogenic clones (*$P < 0.05$). (D) The average expression of CD106 was compared in chondrogenic and non-chondrogenic clones (NS, not significant).

potential than those without differentiation potential ($P < 0.05$, Fig. 1C), but there was no significant correlation between chondrogenic differentiation potential and the expression of CD106 in ihBMSC clones (Fig. 1D). Cell surface expression of CD106 in ihBMSC clones determined by FCM correlated with the data of QRT-PCR (data not shown). These results suggested that the ihBMSC clones positive for CD106 were less osteogenic and more adipogenic than those negative for CD106.

Expression of CD106 correlated with the differentiation property of primary BMSC

To confirm that the association of CD106 with differentiation potential was not a product of the immortalization process, the differentiation property of primary BMSC sorted by the expression of CD106 was analyzed. Both early (2nd) and late (passage 12th) cells were available for the analysis in six huBM strains. The fraction of CD106-positive cells at early passages showed a considerable difference among strains, ranging from 30% to 95% (Fig. 2A), and all strains showed a passage-dependant reduction in numbers of CD106-positive cells (Fig. 2B).

MACS was performed using antibody against CD106 in each huBM at an early passage. In the case of huBM49 at PN5, the population of CD106-positive cells was 80% before sorting, and the purity of positive-sorting and negative-sorting was 97% and 75%, respectively (Fig. 2C). When cells in each group were subjected to osteogenic induction, CD106-positive-sorted cells showed fewer number of Alizarin-Red-positive cells (data not shown), and less activity of ALP (Fig. 2D) than CD106-negative-sorted cells. On the other hand, after the adipogenic induction, CD106-positive-sorted cells included many Oil-red-O-positive cells (data not shown) and larger amounts of TG (Fig. 2E) than those of negative-sorted cells. Essentially the same results were obtained in additional three huBM strains (data not shown). These results were compatible with the results obtained in ihBMSCs, indicating that primary BMSCs were heterogeneous in terms of the expression of CD106, which correlated with the osteogenic and adipogenic differentiation potential of each BMSC.

Prediction of differentiation property based on CD106 expression in primary BMSCs

Above data suggested that the evaluation of the CD106 fraction at early passages may predict the differentiation potential of each huBM strain. As for osteogenic potential, ALP activity of each huBM strain at early passage did not correlate with the ALP activity of the corresponding huBM strain after the osteogenic induction (Fig. 3A). On the other hand, the fraction of CD106-positive cells at early passage showed a clear negative correlation with the ALP activity after osteogenic induction (Fig. 3B). The TG was almost undetectable in all huBM strains at early passage, indicating no value for the prediction of adipogenic differentiation (Fig. 3C), whereas there was a clear positive correlation between the fraction of CD106-positive cells at early passage and the amount of TG after adipogenic induction (Fig. 3D). These results indicated that the evaluation of CD106 at early passage is useful for predicting the differentiation potential of BMSCs.

Discussion

CD106/VCAM-1 is a cell surface glycoprotein, which binds to the $\alpha4\beta1$ and $\alpha4\beta7$ integrins [19]. A deficiency of CD106/VCAM-1 results in embryonic death with the absence of chorion-allantois fusion or multiple abnormalities in the heart in mice, but no significant abnormality was found in mesenchymal tissues during the embryonic stage [20]. CD106/VCAM-1 is associated with homing of HSCs, and CD106/VCAM-1-positive BMSCs, which may be MSCs, keep HSCs in their niche [21,22]. The binding to CD106/VCAM-1 activates integrin signaling, and cell migration or cytoskeletal organization is promoted [23]. But it is still unknown whether the integrin signaling influences the differentiation potential of BMSCs.

We have found that the fraction of CD106-positive cells in primary cultured-BMSC varied considerably among donors, although the same procedure was applied in all cases. The fraction of CD106 did not show any significant correlation with the sex or age of the donor (data not shown). Expression of CD106 is regulated by several cytokines [24], and some of them are produced by hematopoietic cells in bone marrow, which therefore may determine the number of CD106-positive cells at the beginning of the culture.

The negative correlation between CD106/VCAM-1 expression and the osteogenic property of ihBMSC seemed to contradict the idea of CD106/VCAM-1 as an MSC marker. We have no clear explanation for this phenomenon, but it should be considered that a considerable number of BMSCs have already committed to the osteogenic lineage before in vitro culture, among which some are ALP-positive but others may not be [25]. The expression of CD106/VCAM-1 was lost during the osteogenic differentiation (data not shown), suggesting the loss of CD106/VCAM-1 expression in osteogenic precursors. Therefore the osteogenic potential of each huBM strain evaluated in the current study may be determined by the number of osteogenic precursors negative for CD106/VCAM-1, but not of MSCs positive for CD106/VCAM-1, which would lead to the negative correlation observed in this study.

We also have found that the fraction of CD106-positive cells was positively correlated to the production of adipogenesis, which is compatible with the idea that CD106-positive cells include stem cells with adipogenic potential. As far as we know, there are no useful markers for predicting the adipogenic potential of BMSCs. Irrespective of the adipogenic potential, the amount of TG was below the detectable level (Fig. 3C), and no expression of the PPARγ

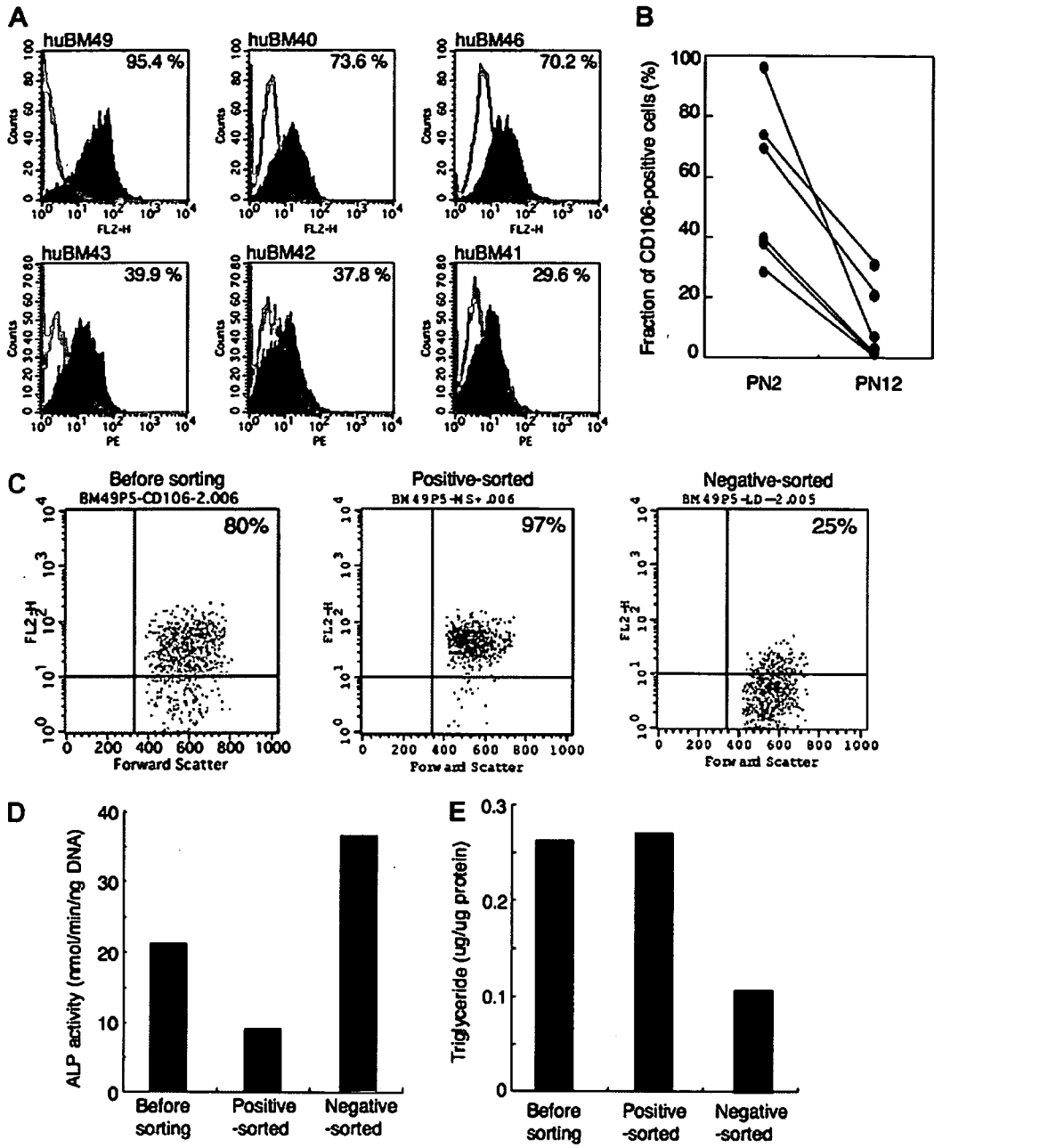

Fig. 2. The expression of CD106 correlated with the differentiation potential in primary BMSC. (A) Fraction of CD106-positive cells in six huBM strains. (B) Passage-dependent reduction of CD106-positive cells in primary BMSC. (C) Fraction of CD106 positive cells in huBM49 before and after the sorting by anti-CD106 Ab. (D) ALP activity after osteogenic induction in each cell population. (E) TG amounts after adipogenic induction in each cell population.

gene was observed (data not shown) in any BMSC strains before the adipogenic induction. Therefore, CD106 is the first cell surface marker to indicate the adipogenic potential of BMSC. Whether CD106/VCAM-1 is functionally involved in the promotion of adipogenesis is not known. That CD106/VCAM-1 knockout mice showed an apparently normal generation of mesenchymal tissue does not support a functional role for CD106/VCAM-1 in adipogenesis [20].

Whatever the mechanisms, the predictive power of CD106/VCAM-1 for osteogenic potential is quite valuable in the clinical application of BMSCs for bone regeneration. As far as we know, there are no useful markers for predicting the differentiation potential of BMSCs. The establishment of a standard method to evaluate osteogenic potential is one of the topics in bone regeneration therapy using MSCs, and a number of candidates have been proposed including ALP activity [26]. We have found,

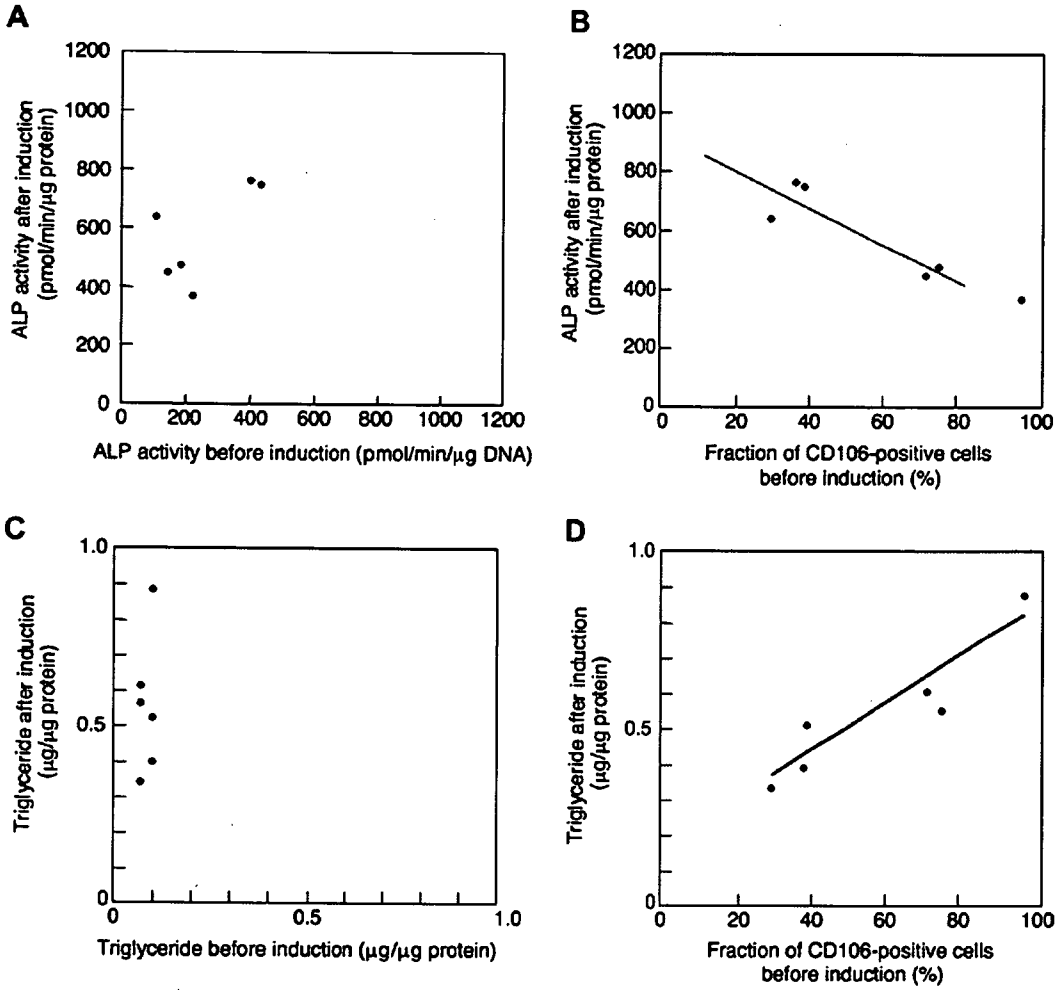

Fig. 3. Prediction of differentiation property based on CD106 expression in primary BMSCs. (A) Relationship between the ALP activity after the osteogenic induction and the fraction of CD106-positive cells before the induction in six huBM strains. No definite association was found. (B) Relationship between the ALP activity after the osteogenic induction and the fraction of CD106-positive cells before the induction. A negative association was found ($r = 0.86$, $P = 0.005$). (C) Relationship between the amount of TG before and after the adipogenic induction. No definite association was found. (D) Relationship between the amount of TG after the adipogenic induction and the fraction of CD106-positive cells before the induction. A positive association was found ($r = 0.91$, $P = 0.005$).

however, that the ALP activity of BMSCs at early passage was not a reliable indicator for osteogenic potential (Fig. 3A). CD106 may be a reliable indicator for the osteogenic differentiation potential of BMSC.

Acknowledgments

The authors thank Drs. Koichi Nishijo, and Tatsuya Ishibe for advice, and Drs. Takeshi Sakamoto and Hiromu Ito for clinical samples. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) with a project entitled Development of Evaluation Technology for Early Introduction of Regenerative Medicine, and also by Grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Education, Culture, Sports, Science, and Technology, and from the Ministry of Health, Labour, and Welfare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.149.

References

[1] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.

[2] E.M. Horwitz, P.L. Gordon, W.K.K. Koo, J.C. Marx, M.D. Neel, R.Y. McNall, L. Muul, T. Hofmann, Isolated allogenic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone, Proc. Natl. Acad. Sci. USA 13 (2002) 8932–8937.

[3] R. Kuroda, K. Ishida, T. Matsumoto, T. Akisue, H. Fujioka, K. Mizuno, H. Ohgushi, S. Wakitani, M. Kurosaka, Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete

with autologous bone-marrow stromal cells, Osteoarthr. Cartilage 15 (2007) 226–231.
[4] S. Gronthos, P.J. Simmons, The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro, Blood 4 (1995) 929–940.
[5] H. Aslan, Y. Zilberman, L. Kandel, M. Liebergall, R.J. Oskouian, D. Gazit, Z. Gazit, Osteogenic differentiation of noncultured immunoisolated bone marrow-derived $CD105^+$ cells, Stem Cells 24 (2006) 1728–1737.
[6] N. Quirici, D. Soligo, P. Bossolasco, F. Servida, C. Lumini, G.L. Deliliers, Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies, Exp. Hematol. 30 (2002) 783–791.
[7] K. Mareschi, I. Ferrero, D. Rustichelli, S. Aschero, L. Gammaitoni, M. Aglietta, E. Madon, F. Fagioli, Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow, J. Cell. Biol. 97 (2006) 744–754.
[8] M. Honczarenko, Y. Le, M. Swierkowski, I. Ghiran, A.M. Glodek, L.E. Silberstein, Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors, Stem Cells 24 (2006) 1030–1041.
[9] S. Gronthos, A.C.W. Zannettiono, S.J. Hay, S. Shi, S.E. Graves, A. Kortesidis, P.J. Simmons, Molecular and cellular characterization of highly purified stromal stem cells derived from human bone marrow, J. Cell. Sci. 116 (2003) 1827–1835.
[10] T. Okamoto, T. Aoyama, T. Nakayama, T. Nakamata, T. Hosaka, K. Nishijo, T. Nakamura, T. Kiyono, J. Toguchida, Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells, Biochem. Biophys. Res. Commun. 295 (2002) 354–361.
[11] T. Mori, T. Kiyono, H. Imabayashi, Y. Takeda, K. Tsuchiya, S. Miyoshi, H. Makino, K. Matsumoto, H. Saito, S. Ogawa, M. Sakamoto, J. Hata, A. Umezawa, Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential, Mol. Cell. Biol. 12 (2005) 5183–5195.
[12] B.M. Abdallah, M. Haack-Sorensen, J.S. Burns, B. Elsnab, F. Jakob, P. Hokland, M. Kassem, Maintenance of differentiation potential of human bone marrow mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene in despite of extensive proliferation, Biochem. Biophys. Res. Commun. 326 (2005) 527–538.
[13] Y. Shima, T. Okamoto, T. Aoyama, K. Yasura, T. Ishibe, K. Nishijo, K.R. Shibata, Y. Kohno, K. Fukiage, S. Otsuka, D. Uejima, T. Nakayama, T. Nakamura, T. Kiyono, J. Toguchida, In vitro transformation of mesenchymal stem cells by oncogenic $H\text{-}ras^{Val12}$, Biochem. Biophys. Res. Commun. 353 (2007) 60–66.
[14] K.R. Shibata, T. Aoyama, Y. Shima, K. Fukiage, S. Otsuka, M. Furu, Y. Kohno, K. Ito, S. Fujibayashi, M. Neo, T. Nakayama, T. Nakamura, J. Toguchida, Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells, and potentially silenced by DNA methylation during in vitro expansion, Stem Cells 9 (2007) 2371–2382.
[15] E.J. Caterson, L.J. Nesti, K.G. Danielson, R.S. Tuan, Human marrow-derived mesenchymal progenitor cells: isolation, culture expansion, and analysis of differentiation, Mol. Biotechnol. 20 (2002) 245–256.
[16] L.J. Foster, P.A. Zeemann, C. Li, M. Mann, O.N. Jensen, M. Kassem, Differential expression profiling of membrane proteins by quantitative proteomics in a human mesenchymal stem cell line undergoing osteoblast differentiation, Stem Cells 23 (2005) 1367–1377.
[17] R.J. Deans, A.B. Moseley, Mesenchymal stem cells: biology and potential clinical uses, Exp. Hematol. 28 (2000) 875–884.
[18] E.A. Jones, S.E. Kinsey, A. English, R.A. Jones, L. Straszynski, D.M. Meredith, A.F. Markham, A. Jack, P. Emery, D. McGonagle, Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells, Arthritis Rheum. 12 (2002) 3349–3360.
[19] J.M. Bergelson, M.E. Hemler, Integrin-ligand binding. Do integrins use a 'MIDAS touch' to grasp an Asp?, Curr. Biol. 6 (1995) 615–617.
[20] L. Kwee, S. Baldwin, H.M. Shen, C.L. Stewart, C. Buck, C.A. Buck, M.A. Labow, Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice, Development 121 (1995) 489–503.
[21] I.B. Mazo, U.H. von Andrian, Adhesion and homing of blood-borne cells in bone marrow microvessels, J. Leukoc. Biol. 66 (1999) 25–32.
[22] T. Lapidot, I. Petit, Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells, Exp. Hematol. 30 (2002) 973–981.
[23] C. Kummer, M.H. Ginsberg, New approaches to blockade of alpha4-integrins, proven therapeutic targets in chronic inflammation, Biochem. Pharmacol. 72 (2006) 1460–1468.
[24] T. Collins, M.A. Read, A.S. Neish, M.Z. Whitley, D. Thanos, T. Maniatis, Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers, FASEB J. 9 (1995) 899–909.
[25] M.W. Long, J.A. Robinson, E.A. Ashcraft, K.G. Mann, Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors, J. Clin. Invest. 95 (1995) 881–887.
[26] H. Kotobuki, M. Hirose, H. Machida, Y. Katou, K. Muraki, Y. Takakura, H. Ohgushi, Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells, Tissue Eng. 11 (2005) 663–673.