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間葉系幹細胞を用いた移植治療における品質及び
安全性判定基準の確立に関する研究

平成 19 年度 総括研究報告書

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間葉系幹細胞を用いた移植治療における品質及び安全性判定基準の確立に関する研究

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研究要旨

間葉系幹細胞はさまざまな組織に分化可能な多能性幹細胞である。このため疾患、外傷などで傷害を受けた組織の修復・再生に期待され、既に臨床応用も開始されている。しかしながら間葉系幹細胞の定義が曖昧であることや施設、技術者によって抽出の方法が異なるなどの理由からその品質を保証する品質管理基準は作成されていない。また*In Vitro*の体外培養において自然発癌例が報告されるなど安全性の監視機構の構築の必要性も高まってきている。

本研究においては品質管理及び安全性監視機構を構築し、臨床試験「難治性骨壊死に対するMSC細胞移植治療」においてその妥当性を検討する。

3年間の間に研究結果を明らかにでき、患者、社会、医療従事者、行政にその情報を還元できる事の特徴としている。

A.研究目的

本研究の目的は間葉系幹細胞(Mesenchymal stem cell, MSC)を用いた細胞医療を行う際の培養(行程あるいは課程)における品質評価及び安全性監視機構を構築することである。

B.研究方法

本研究は平成19年度に京都大学整形外科で開始された臨床試験「難治性骨壊死に対するMSC細胞移植治療」と連携して実施する。品質評価項目は①MSCの多分化能評価②増殖能評価であり、安全性監視項目は③感染の監視④癌化の監視である。本研究においては*In Vitro*でこれらの4項目を定量的評価し、基準値を作成し、そのプロトコルを臨床試験にフィードバックする。最終的には臨床試験で得られた問題点を検討し、安全で品質の高い細胞移植治療プロトコルを作成する。

(倫理面への配慮)解析方法の検討に用いるドナー由来のMSCは、京都大学医学部医の倫理委員会の審査を経て実施した。臨床試験は「ヒト幹細胞を用いる臨床研究に関する指針」を遵守しインフォームドコンセントを得られた症例において個人情報保護・秘匿を遵守して行う予定である。

C.研究結果

分化能の確認は*In Vitro*で骨、軟骨、脂肪へ分化誘導を行い、特異的基質産生を定量評価するシステムを構築した。増殖能の確認は*In Vitro*におけるMSCの増殖には細胞周期調節因子p16が関与していることが明らかにした(Shibata KR,2007)。このことからp16の発現値を定量解析し、培養継続可能回数予測式を用いることで、増殖能予測の有効性を確認している。感染監視はcGMPグレードに準拠したプロトコル、SOP、定期検査方法を確立し、有効性を検証している。癌化監視は早期悪性転化予測マーカーとしてp16遺伝子転写調節領域のメチル化解析の有効性を明らかにした(Shibata KR,2007)。この方法と染色体解析、免疫不全マウスへの皮下接種を行い、それらの有効性を比較検討している。

E.結論

平成18、19年度に作成した品質評価、安全性監視のためのプロトコルを用いて平成19年度より臨床試験「難治性骨壊死に対するMSC細胞移植治療」を開始した。今後プロトコルの問題点の検討、妥当性を検証していく予定である。

F.健康危険情報

該当なし

G.研究発表

1.論文発表

- 1) Shibata KR, Aoyama T, et al. Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. *Stem Cells* 2007;25(9):2371-82.
- 2) Aoyama T, et al. Cell-specific epigenetic regulation of ChM-I gene expression: crosstalk between DNA methylation and histone acetylation. *Biochem Biophys Res Commun*. 2008; 365(1):124-30.
- 3) Fukiage K, Aoyama T, et al. Expression of vascular cell adhesion molecule-1 indicates the differentiation potential of human bone marrow stromal cells. *Biochem Biophys Res Commun*. 2008; 365(3):406-12.

2.学会発表

- 1) 青山朋樹、戸口田淳也間葉系幹細胞をバイオマテリアルとして用いるための品質管理法の確立第29回日本バイオマテリアル学会2007.11.26大阪市

H.知的財産権の出願・登録状況

- 「間葉系幹細胞の軟骨細胞分化マーカー」特許出願
「間葉系幹細胞の分化能マーカーとしてのCD106の使用」特許出願
「骨再生組成物製造器具、骨再生組成物の製造方法、骨再生組成物、および骨再生方法」特許出願

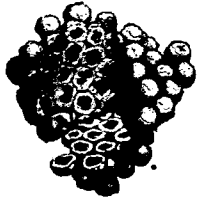
研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版地	出版年	ページ
青山朋樹、 戸口田淳也	培養細胞のエピジェネティク解析再生医療に用いられる細胞・再生組織の評価と安全性	大串 始	シーエムシー出版	東京	2007	19-31

雑誌

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shibata KR, Aoyama T et al.	Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion.	Stem Cells	25(9)	2371-82	2007
Aoyama T, Toguchida J, et al.	Cell-specific epigenetic regulation of ChM-I gene expression: crosstalk between DNA methylation and histone acetylation.	Biochem Biophys Res Commun.	365(1)	124-30	2008
Fukiage K, Aoyama T, et al	Expression of vascular cell adhesion molecule-1 indicates the differentiation potential of human bone marrow stromal cells.	Biochem Biophys Res Commun.	365(3)	406-12	2008
青山朋樹、戸口田淳也	骨再生治療の開発の現状と問題点	BIO Clinica	22	49-53	2007



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Expression of the p16INK4A Gene Is Associated Closely with Senescence of Human Mesenchymal Stem Cells and Is Potentially Silenced by DNA Methylation During In Vitro Expansion

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Expression of the p16INK4A Gene Is Associated Closely with Senescence of Human Mesenchymal Stem Cells and Is Potentially Silenced by DNA Methylation During In Vitro Expansion

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Key Words. Mesenchymal stem cells • Telomere • Methylation • p16INK4A • Senescence • BMI1

ABSTRACT

The precise biological characteristics of human mesenchymal stem cells (hMSCs), including growth regulatory mechanisms, have not yet been defined. Using 29 strains of hMSCs isolated from bone marrow, we have performed extensive analyses of the growth profiles of hMSCs in vitro. All 29 strains stopped proliferating with a mean population doubling (PD) of 28, although there was a considerable difference among strains. The mean telomere restriction fragment length of the cells passaged twice correlated well with the final number of PDs in each strain, suggesting the value of this measurement to be predictive of the growth potential of hMSCs. The expression level of the p16INK4A gene was associated closely with the PD number of each strain ($p = .00000001$). Most of the p16INK4A-positive cells were Ki67-negative and senescence associated β -galactosi-

dase-positive, and the suppression of p16INK4A gene expression by small interfering RNA in senescent hMSCs reduced the number of senescent cells and endowed them with the ability to proliferate. Twenty-five of the 29 strains showed a steady gradual increase in the expression of p16INK4A. The remaining four strains (13.8%) showed different profiles, in which DNA methylation in the promoter region occurred in vitro. One of the four strains continued to proliferate for much longer than the others and showed chromosomal aberrations in the later stages. These results indicated p16INK4A to be a key factor in the regulation of hMSC growth, and, most importantly, careful monitoring of DNA methylation should be considered during the culture of hMSCs, particularly when a prolonged and extended propagation is required. *STEM CELLS* 2007;25:2371–2382

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells are defined as plastic-adherent fibroblastic cells with the potential to differentiate into multiple mesenchymal tissues. Human mesenchymal stem cells (hMSCs) isolated from bone marrow are able to proliferate in vitro and differentiate along multiple lineages including bone, cartilage, adipose, and muscle cells [1]. Thus, hMSCs are being tested in clinical trials for tissue regeneration and engineering [2, 3]. Although infinite growth ability is one definition of stem cells, most hMSCs cease proliferating after 20–40 population doublings (PDs) [4], although precisely why has not yet been elucidated. The proliferative capacity of primary mammalian cells in vitro is finite, a concept known as the Hayflick limit [5]. In vitro cultured cells undergo a limited number of PDs before entering a state of premature growth arrest, referred to as senescence, in which cells remain alive and metabolically active but are completely refractory to mitogenic stimuli [5]. As telomere shortening, oxidative stress, DNA damage, and aberrant mitogenic signaling can all induce senescence in human primary cells, senescence is now considered to be a form of biological protection from neoplastic transformation [6].

Telomeres, tandem TTAGGG repeats that cap the ends of eukaryotic chromosomes, are forced to shorten at each DNA

replication. The shortening of telomeres plays an important role in cell aging and is regulated by a protein complex associated with telomere end [7, 8]. The maintenance of telomere ends is one of the mechanisms for self-renewal of stem cells. In undifferentiated hESCs, many proteins of the telomerase complex, including TERT, are expressed, and telomere length (8–11 kilobase [kb]) is maintained [9, 10]. In most characterized tissue stem cell subtypes such as hematopoietic stem cells (HSCs) and epidermal stem cells, telomeric shortening occurs with age despite the presence of telomerase activity [11].

Proteins regulating cell cycles are also involved in the cellular senescence process, among which p16^{INK4A} has been studied extensively. p16^{INK4A} was identified as a causative factor for familial melanomas and, simultaneously, as an inhibitor for the cell cycle kinases CDK4 and CDK6, indicating its properties as a tumor suppressor negatively regulating the cell cycle [12]. Subsequent investigations, however, have proved that the essential role of p16^{INK4A} is actually in the regulation of cellular senescence. p16^{INK4A} was found to be accumulated in many aged cells and tissues [13], and the age-induced increase in p16^{INK4A} contributed to a reduction in the size of reservoirs of self-renewing tissue stem cells [14, 15]. The precise profile of p16^{INK4A} gene expression during the in vitro culture of hMSCs has yet to be reported, and it is also unknown whether the

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shortening of telomere or the upregulation of p16^{INK4A} expression is critical for senescence in hMSCs.

The activity of p16^{INK4A} is regulated under both physiological and nonphysiological conditions. Under physiological conditions requiring the repression of p16^{INK4A} such as in tissue stem cells, the transcription of the p16^{INK4A} gene is downregulated by BMI1, a polycomb group (PcG) protein that affects stem cell fate, including self-renewal, senescence, and aging [16]. BMI1 is necessary for efficient self-renewing cell division in adult murine HSCs and neural stem cells (NSCs) [17, 18]. BMI1 represses the transcription of p16^{INK4A} as well as p14^{ARF}, which is another inhibitor of cell proliferation sharing part of the coding sequence at the *INK4A-ARF* locus [19].

Under nonphysiological conditions such as in cancer cells, the most frequent event inactivating p16^{INK4A} function is transcriptional downregulation by DNA methylation in the core promoter region [20]. In higher eukaryotes, DNA is methylated at cytosines located 5' to guanine in the CpG dinucleotide [21]. This modification has important regulatory effects on gene expression and also triggers de novo methylation in unmethylated lesions [22]. Therefore, the mapping of methylation patterns in CpG islands is an important tool for understanding both normal and pathologic gene expression events [23]. De novo methylation of the p16^{INK4A} promoter regions in vitro was reported in spontaneously immortalized rodent cells, suggesting the important role of p16^{INK4A} in bypassing the senescence. In this sense, it is interesting that the hMSCs transformed spontaneously in vitro lacked expression of the p16^{INK4A} gene [24].

Here, we investigated the role of two critical factors, telomere length and the expression of p16^{INK4A}, during life span of hMSCs in vitro and found that both are involved in different phases. Also, we found that the prolonged propagation of hMSCs may induce inactivation of the p16^{INK4A} gene by DNA methylation with chromosomal aberrations.

MATERIALS AND METHODS

Study Population

Primary hMSCs were isolated from the bone marrow taken from iliac crests of 29 donors, who received orthopedic operative procedures requiring autologous bone grafts from iliac crests (supplemental online Table 1). The donors had no history of concurrent illness or of medication that could affect bone metabolism. There were 13 males and 16 females. The median donor age was 49.1 years (range, 10–84 years). The Ethics Committee of the Faculty of Medicine, Kyoto University, approved the procedure and informed consent was obtained from each donor.

Cell Culture

Primary hMSC cultures were established by means of a previously described method [25]. Mononuclear cells were suspended in α -minimal essential medium with GlutaMAX (Invitrogen Co., Carlsbad, CA, <http://www.invitrogen.com>) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, <http://www.hyclone.com>), 100 U/ml penicillin, and 100 mg/ml streptomycin and seeded at a density of 2.5×10^5 cells per cm² in 5% CO₂ at 37°C. At 80% confluence, cells were counted and reseeded at a dilution of 1:3, as cells in the first passage. From this point, the number of PDs was calculated based on the total cell number at each passage. This procedure was repeated until the cells stopped proliferating. At this point, cell numbers were counted to calculate the final PD.

Induction of Differentiation

The potential for differentiation in the osteogenic, adipogenic, and chondrogenic directions was examined using the differentiation-induction protocol provided by Cambrex (East Rutherford, NJ,

<http://www.cambrex.com>) in each hMSC strain at the second passage. After 14 days of osteogenic differentiation, the cells were stained with 40 mM PH4.2 Alizarin Red S (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>). After 21 days of adipogenic differentiation, cells were stained with 0.3% oil red O (Nacalai Tesque, Kyoto, Japan, <http://www.nacalai.co.jp/en/index.html>). After 21 days of chondrogenic differentiation, the pellets were stained with Alcian Blue (Muto Pure Chemicals Co., LTD, Tokyo, <http://www.mutokagaku.com>).

Telomere Length Assay

High molecular weight DNA was extracted from each hMSC at the second and final passage. Telomere restriction fragment length (TRFL) was determined using Southern blot analysis. Genomic DNA (2 μ g) was digested by mixing HinfI and RsaI. The digested DNA was separated on 0.7% agarose gels. Gels were denatured and blotted onto a positively charged nylon membrane (Roche Diagnostics, Basel, Switzerland, <http://www.roche-applied-science.com>). Using the DIG Assay Kit (Roche Diagnostics), hybridization with a digoxigenin (DIG)-labeled telomere-specific probe (CCCTAA)₃ was conducted. After hybridization, membranes were washed and incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase (AP). Results were visualized using AP metabolizing chemiluminescent substrate (CSPD; Roche Diagnostics). Each assay was performed twice. Membranes were then scanned and the mean TRFL (mTRFL) was calculated.

Western Blot Analysis

Western blotting was performed as mentioned [26]. The primary antibodies used were as follows: DO-1 for p53; G175-405 for p16^{INK4A} (BD Pharmingen, San Diego, <http://wwwbdbiosciences.com>), F-5 for p21^{CIP1}, C-19 for p27 (Santa Cruz Biotechnology, Santa Cruz, CA, <http://www.scbt.com>), and clone F6 for BMI1 (Upstate, Lake Placid, NY, <http://www.upstate.com>). Blots were probed with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology) and visualized using a chemiluminescence reagent ECL Plus Detection Kit (GE Healthcare UK Ltd, Little Chalfont, U.K., <http://www.gehealthcare.com>).

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) and 1 μ g was reverse transcribed for single-stranded cDNAs using oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen).

Quantitative Reverse Transcription-Polymerase Chain Reaction

The reaction mixtures for amplification contained 100 ng of cDNA and Universal TaqMan 2X Master Mix (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>). The concentrations of primers and TaqMan probes (Biosearch Technologies Japan, Tokyo, <http://www.btjkk.co.jp>) were 900 and 250 nM. All reactions were performed in triplicate using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) under the following conditions: 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression of 18S was used as an internal control. Saos2, a human osteosarcoma cell line, expresses the p16^{INK4A} gene at a high level due to a homozygous deletion of the retinoblastoma gene [27], and by setting the expression level of p16^{INK4A} in Saos2 cells as a standard, the relative expression level of p16^{INK4A} in hMSCs was calculated. A statistical analysis was done with the *t* test after logarithmic transformation. Primers and probes for p16^{INK4A} are as described: sense, 5'-CCCAACGCACCGAATAGTTAC-3'; antisense, 5'-CACGGGTCTGGGTGAGAGT-3'; probe, FAM-AGGCCGATCCAGGTTCATGATGATGG-BHQ-1.

Methylation-Specific Polymerase Chain Reaction

The bisulfite modification of DNA samples was performed using the EpiTect Bisulfite Kit (Qiagen, Tokyo, <http://www1.qiagen.com>). Methylation-specific polymerase chain reaction (PCR) (MSP)

for p16^{INK4a} was performed as previously described [20, 28]. The methylation-specific primer sequences are as follows: sense, 5'-TTATTAGAGGGTGGGGCGGATCGC-3'; antisense, 5'-CAAC-CCCAAACCACAAC CATAA-3'. For the unmethylated region, the primers are: sense, 5'-TTATTAGAGGGTGGGGTGGATTGT-3'; antisense, 5'-CCACCTAAATCGACCTCCGACCG-3'.

Small Interfering RNA Assay

The optimum sequence for the small interfering (si)RNA targeting the p16^{INK4A} gene was obtained as ready-annealed, purified duplexes from Dharmacon Research (Lafayette, CO, <http://www.dharmacon.com>). The control "scrambled" sequence was AAGCGC-GCUUUGUAGGAUUCG; the p16^{INK4A}-targeted sequence was AAACCAGAGGCAGUAACCAUG. Transfection was carried out using the Amaxa electroporation system (Amaxa Biosystems, Cologne, MA, <http://www.amaxa.com>).

5-Bromo-2'-deoxyuridine Assay

Incorporated 5-bromo-2'-deoxyuridine (BrdU) was detected with a BrdU Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Experiments were done in triplicate.

Senescence Associated- β -Galactosidase Assay

Cells were cultured on eight-well chamber slides. The senescence associated- β -galactosidase (SA- β -Gal) assay was performed as described previously [29].

Immunocytochemistry

After SA- β -Gal staining, cells were washed with phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100, 1% bovine serum albumin in PBS. Slides were blocked with 10% nonfat milk in PBS, incubated with primary antibody for 16 hours and with secondary antibody for 1 hour in blocking solution, counterstained with 4,6-diamidino-2-phenylindole, and viewed by epifluorescence microscopy. The antibodies used were DCS-50.2, a mouse monoclonal antibody for p16^{INK4a} (Progen, Heidelberg, Germany, <http://www.progen.de>), and ab27619, a fluorescein isothiocyanate-conjugated rabbit monoclonal antibody for Ki-67 (Abcam, Cambridge, U.K., <http://www.abcam.com>).

Statistical Analyses

Statistical analyses were performed using Statcel software. Data were assessed using the Pearson product-moment correlation coefficient, Student's *t* test, Spearman's rank-correlation coefficient analysis, a one-factor analysis of variance, a multiple regression analysis, and the Mantel-Haenszel chi-squared test.

RESULTS

Long-Term Culture of Human MSCs

The primary culture of donor cells was successful in all 29 cases, and the adipogenic, chondrogenic, and osteogenic differentiation of early passage cells was confirmed in all strains (supplemental online Fig. 1). Growth profiles of hMSCs showed characteristics typical of the Hayflick model of cellular aging (Fig. 1A), and all strains stopped proliferating after a certain period in vitro, although the culture period (mean, 151.2 \pm 47.7 days; range, 90–334 days) and the final PD number (mean, 28.1 \pm 7.2; range, 13–54) differed considerably among strains (supplemental online Table 1). There was a significant correlation between the final PD number and the age of donors ($p = .0042$) with the Pearson product-moment correlation coefficient analysis. When each donor was classified by age into either a younger group (under 50 years, $n = 13$, mean, 28.5 \pm 12.2 years) (Fig. 1B) or an older group (over 50 years, $n = 16$, mean, 66.6 \pm 8.8 years) (Fig. 1C), the mean final PD number of the older group (24.8 \pm 4.7) was found to be significantly less than

that of the younger group (32.5 \pm 7.5) ($p = .0086$) (Fig. 1D). This result was confirmed by an analysis with Spearman's rank-correlation coefficient ($r = -.608$, $p = .00128$) (Fig. 1E).

Telomere Length

Most of the cells at the final passage were flattened and enlarged (Fig. 2Ab), compatible with cells entering senescence. The flattened cells were positive for SA- β -gal staining (Fig. 2Ac) but negative for the terminal deoxynucleotidyl transferase dUTP nick-end labeling staining (data not shown), suggesting that the senescence was why the proliferation ceased. It is well known that the length of telomeres contributes to cellular senescence [30], and therefore the mTRFL was determined in each hMSC strain at the second and final passage, designated as SP and FP, respectively (Fig. 2B) (supplemental online Table 1). The mTRFL of SP and FP cells was 9.4 \pm 1.0 kb and 7.5 \pm 0.6 kb, respectively, and the mean telomere-shortening rate was 109 \pm 43 base pairs (bp) (Fig. 2B). A Pearson product-moment correlation coefficient analysis showed a positive correlation ($p = .00048$) between the mTRFL of SP and final PD. This result was confirmed with Spearman's rank-correlation coefficient ($r = -.557$, $p = .00321$) (Fig. 2C). These findings suggested that calculating the mTRFL of SP roughly predicts the expandability of the hMSC strain, which will be valuable information in expanding hMSC populations for clinical purposes. We then analyzed the correlation between the mTRFL of SP and donor age, but results of a Student's *t* test ($p = .13$) (Fig. 2D) and Spearman's rank-correlation coefficient analysis ($r = -.28$, $p = .13$) (Fig. 2E) indicated that there was no correlation.

PD Number-Dependent Increase of p16^{INK4A} Gene Expression

The mTRFL of FP cells was 7.5 \pm 0.6 kb, which is much longer than the critical size of the telomere (approximately 4.5 kb) [31], suggesting the involvement of other factors in the senescence of hMSCs. p16^{INK4A}, a cell cycle regulator, is known to contribute to cellular senescence and also stem cell aging [13]. The mRNA expression of the p16^{INK4A} gene was analyzed in all hMSC strains at several time points. In most cases, the expression level of the p16^{INK4A} gene showed a gradual increase during propagation in vitro (Fig. 3A). Using the expression level of Saos2 as a reference, the expression level of the p16 gene at each point was digitalized by quantitative reverse transcription (RT)-PCR and plotted against PD number (Fig. 3B). The PD-dependent increase in p16^{INK4A} gene expression was confirmed statistically by a multiple regression analysis ($r = .78$, $p = .00000056$). A tight association was observed in both younger (Fig. 3B, circle) and older (Fig. 3B, triangle) donors.

The result of RT-PCR was confirmed by Western blotting. Seven cases were randomly selected, and proteins were extracted from cells at four time points based on the final PD number of each strain: the second passage (early); the PD number that is one third of the final PD number (early middle); the PD number that is two thirds of the final PD number (late middle); and the last passage (final). The expression of p16^{INK4A} protein at each point was analyzed as well as that of p21^{cip1}, p27, and p53 and quantified. As in the case of mRNA expression, the expression level of p16^{INK4A} protein showed a PD-dependent increase ($p = .000000001$). As for the other cell cycle regulating genes, the p21^{cip1} gene showed a nonsignificant ($p = .21$) but slight increase in expression toward the end of the cells' life span. The expression level of p27 and p53 showed no significant change in vitro (Fig. 3C, 3D).

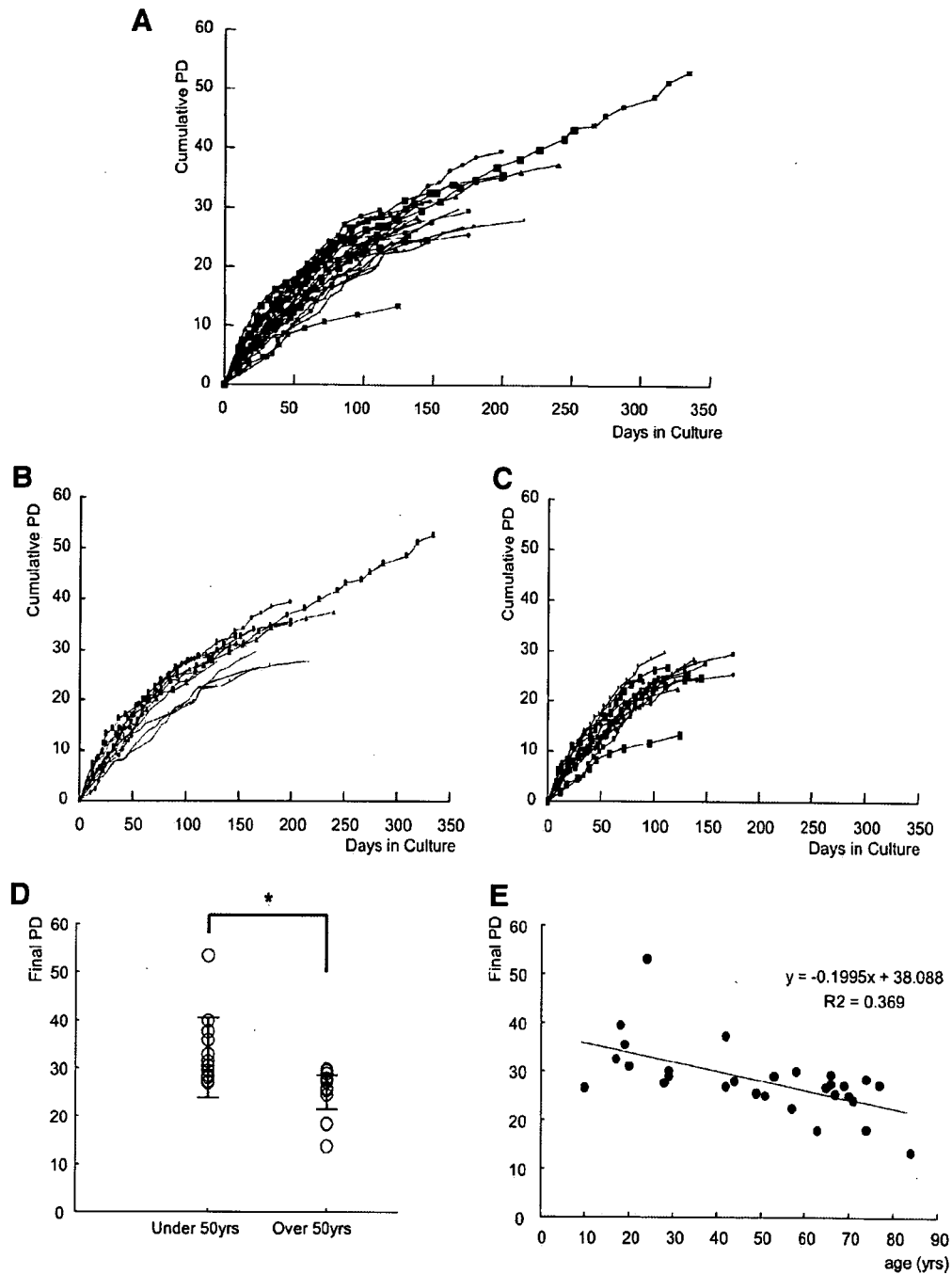


Figure 1. Growth profiles of human (h)MSCs in vitro. (A): Growth profiles of 29 hMSC strains in vitro. PD number is plotted against culture period. (B): Growth profiles of hMSCs from younger donors (<50 years, $n = 13$). (C): Growth profiles of hMSCs from older donors (≥ 50 , $n = 16$). (D): Comparison of final PD between younger and older donors with Student's t test. There was a significant difference $* p = .0086$. (E): A Spearman's rank-correlation coefficient when age was plotted as a metric variable and final PD as an objective variable ($r = -.608$, $p = .00128$). Abbreviations: PD, population doubling; yrs, years.

The Expression of p16^{INK4A} Was Positively Correlated with SA- β -Gal Staining but Not Ki67

Expression of the p16^{INK4A} gene was analyzed at the cellular level using immunocytochemical methods in three randomly selected cases (hMSC08, hMSC40, and hMSC45). The expression of SA- β -gal and Ki67 was also analyzed in these samples as a marker for senescence and proliferation, respectively. Cells positive for p16^{INK4A} were negative for Ki-67 in hMSC08 at

PD12, which was 50% of their life span in vitro (final PD number was 25) (Fig. 4Ac). Similar results were obtained in the case of hMSC45 at PD18 (final PD number was 29) (Fig. 4Af) and hMSC40 (data not shown). These data suggested that cells positive for p16^{INK4A} lost their proliferating ability.

Next, SA- β -Gal staining was performed before double staining of p16^{INK4A} and Ki-67, and cells positive for each staining were counted. Among hMSC40 cells at PD6, only 1 of 94 cells (1%) was positive for SA- β -Gal, and it was also stained by

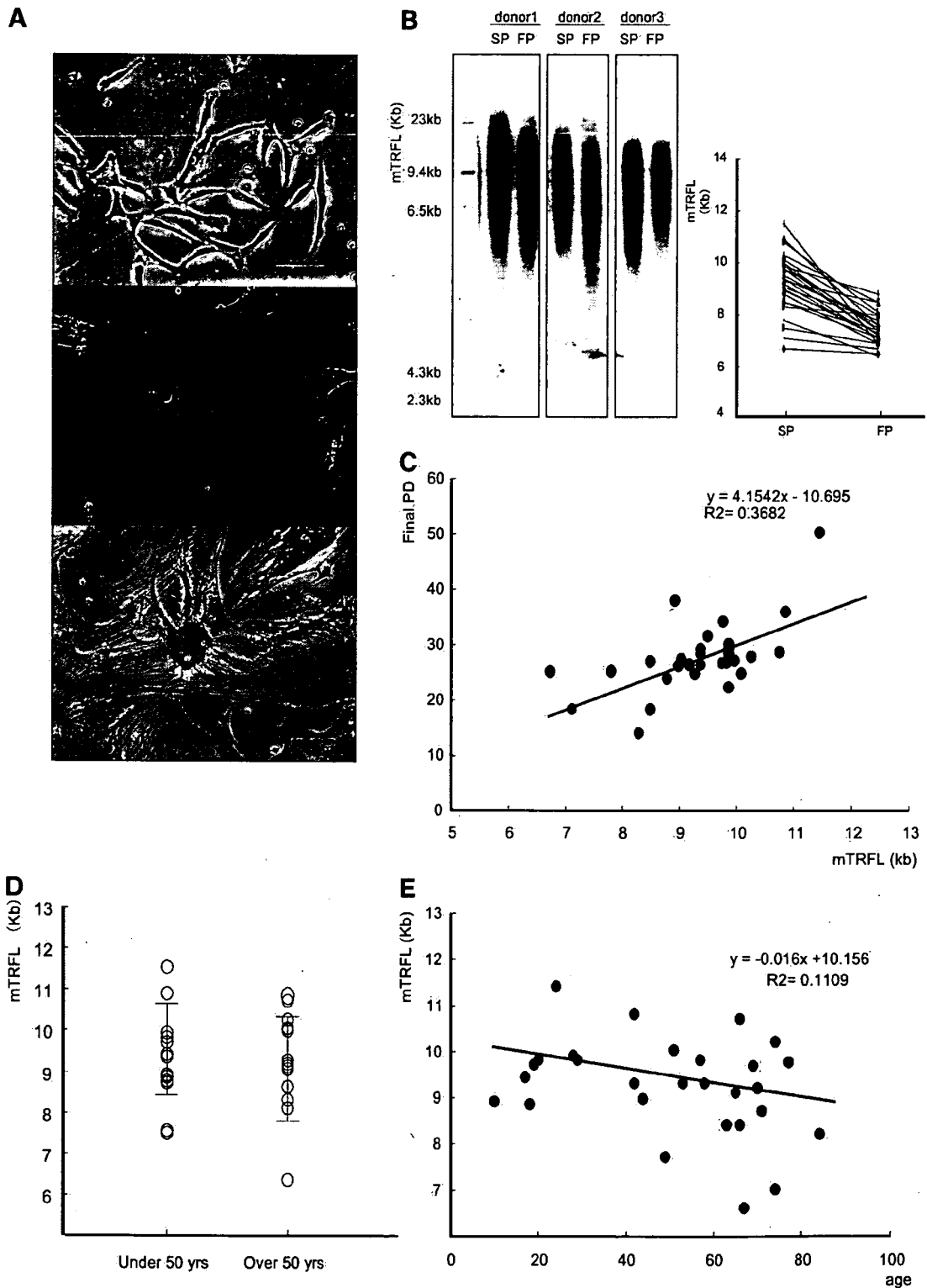


Figure 2. Telomere shortening during the in vitro life span of human (h)MSCs. (A): Phase-contrast photos of hMSC45 at (Aa) PD2 and (Ab) PD28. Most cells at PD28 were positive for SA- β -gal staining (Ac); $\times 400$ magnification. Scale bar = 100 μ m. (B): Detection of telomere restriction fragment by Southern blotting. The mTRFL in the second passage differed among donors (9.4 ± 1.0 kb) but that in late passages converged to almost the same value (7.5 ± 0.6 kb). (C): mTRFL at SP as a predictor of the final PD. A statistically significant association ($r = -.557, p = .00321$) was obtained with Spearman's rank-correlation coefficient when mTRFL at SP was plotted as a metric variable and final PD as an objective variable. (D): Donor age is not a denominator of the mTRFL at SP. No significant difference of mTRFL at SP was observed between younger and older donors ($p = .13$). (E): Spearman's rank-correlation coefficient when age was plotted as a metric variable and early mTRFL as an objective variable ($r = -.28, p = .13$). Abbreviations: FP, first passage; kb, kilobase; mTRFL, mean telomere restriction fragment length; PD, population doubling; SP, second passage; yrs, years.

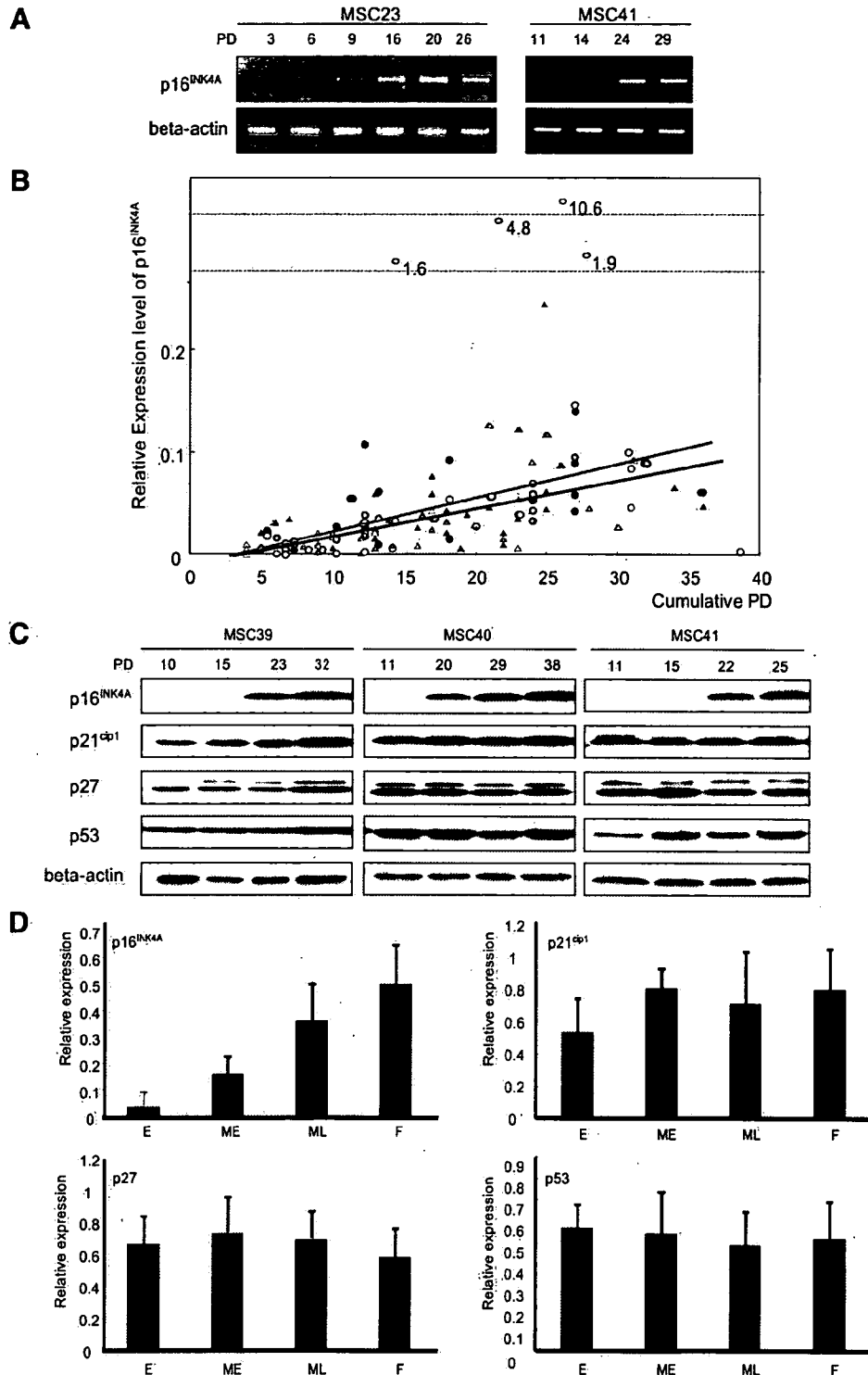


Figure 3. PD-dependent increase in the expression of p16^{INK4A}. (A): mRNA expression of p16^{INK4A} in vitro analyzed by semiquantitative reverse transcription-polymerase chain reaction. (B): Correlation of p16^{INK4A} expression with PD number. Samples derived from younger and older donors were demonstrated with circles and triangles, respectively, and samples from each donor taken at different time points are indicated in the same color. PD-dependent expression of p16^{INK4A} was confirmed by multiple regression analysis in human (h)MSC derived from both younger (red line, $r = .59$, $p = .0000015$) and older (blue line, $r = .64$, $p = .000001$) donors. (C): Expression of cell cycle regulators during the in vitro life span of hMSCs. (D): Quantitative analyses of the expression of cell cycle regulators in vitro. The relative expression of the cell cycle regulators was quantified as described in Materials and Methods as well as the abbreviation of each time point (E, ME, ML, and F). A significant increase in the expression level of p16^{INK4A} was observed toward the later stage of life (analyzed by one-factor analysis of variance, $p = .00000001$). There was no significant change in vitro in the expression of p21^{cip1} ($p = .21$), p27 ($p = .36$), and p53 ($p = .87$). Abbreviations: E, early; F, final; ME, middle early; ML, middle late; PD, population doubling.

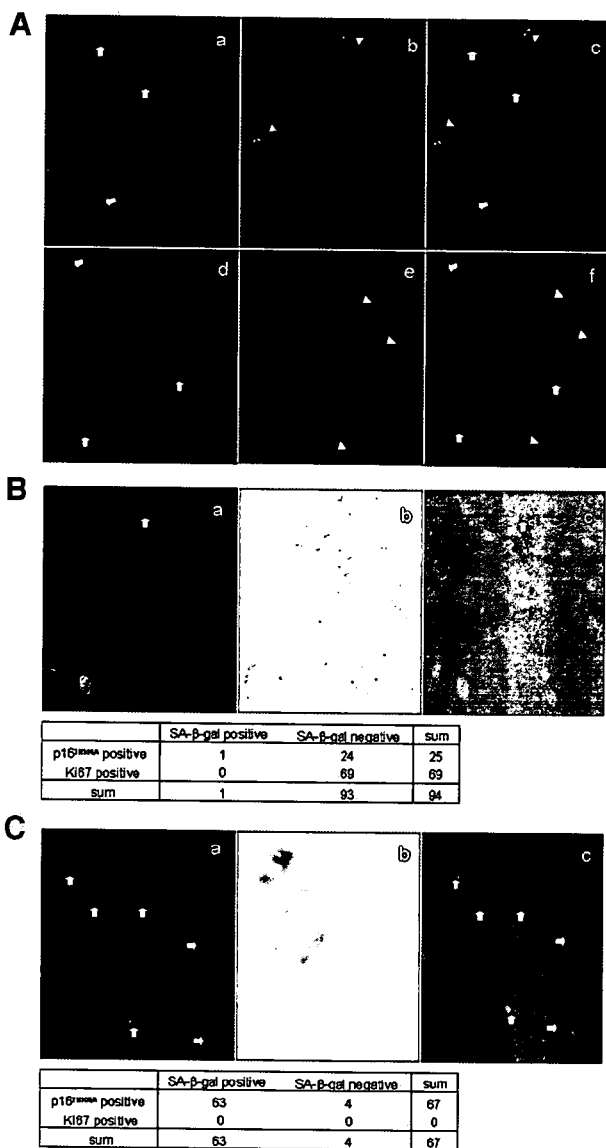


Figure 4. Association of p16^{INK4A} with a marker of senescence but not that of proliferation. (A): Immunocytochemical staining of p16^{INK4A} and Ki-67. (Aa–Ac): Human (h)MSC08 at population doubling (PD)23 (final PD was 25); (Ad–Af): hMSC45 at PD18 (final PD was 26). (Aa, Ad): p16^{INK4A} staining. Signals are demonstrated as red dots in nuclei, and arrows indicate positive cells. (Ab, Ae): Ki-67 staining. Signals are demonstrated as blue dots in nuclei, and positive cells are indicated by arrowheads. (Ac, Af): Merged images. Note that most p16^{INK4A}-positive cells were Ki-67-negative and vice versa. (B): Triple staining of p16^{INK4A}, Ki-67, and SA-β-Gal in hMSC40 at PD6. (Ba), double staining of p16^{INK4A} (red) and Ki-67 (green); (Bb), light field image of SA-β-Gal staining; (Bc), merged image of (Ba) and (Bb). Cells were classified by staining pattern, and the number of cells in each group is demonstrated in the table below the figures. (C): Identical experiments were performed using hMSC40 at a later passage (PD28). Abbreviation: SA-beta-gal, senescence associated-β-galactosidase.

p16^{INK4A} (Fig. 4B). Among SA-β-Gal-negative cells ($n = 93$), 24 (25.8%) were stained with p16^{INK4A} and 69 (73.4%) were stained with Ki67 (Fig. 4B). There were no cells that were double positive for p16^{INK4A} and Ki-67. Among the late passage cells (PD26), 63 (94%) were positive for SA-β-Gal. Among SA-β-Gal-negative cells ($n = 4$), all were stained for p16^{INK4A} and none were stained with Ki67 (Fig. 4C). Identical results

were obtained for hMSC08 and hMSC45 (data not shown), and the Mantel-Haenszel chi-squared test with continuity correction in three strains showed a positive correlation between the expression of p16^{INK4A} and SA-β-Gal ($\chi^2 = 2.75, p = .046$). These results suggested that p16^{INK4A} expression induced senescence and loss of proliferating ability.

Knockdown of p16^{INK4A} Rescued Cells from Senescence and Induced Cell Proliferation

To further investigate the role of p16^{INK4A}, its expression was inhibited with siRNA. Twenty-four hours after a single transfection with p16-siRNA in p16^{INK4A} positive hMSC (hMSC40 at PD24), effective knockdown of the gene expression was confirmed by RT-PCR, and the expression of the p14^{ARF} gene, which shares exons 2 and 3 with the p16^{INK4A} gene, was not affected by p16-siRNA (Fig. 5A). The knockdown effect at the protein level was confirmed by Western blot analysis (Fig. 5B); no change in the expression level of p21^{cip1} or p53 (Fig. 5B) was observed, indicating a specific effect on the p16^{INK4A} gene.

To investigate the effect of the downregulation of p16^{INK4A} expression on the proliferating ability of hMSC strains that have an upregulated p16^{INK4A}, p16-siRNA was introduced into hMSC39 at PD32 (final PD number was 35), and the incorporation of BrdU was assayed 24 hours later. Suppression of p16^{INK4A} gene expression by p16-siRNA was confirmed by RT-PCR (Fig. 5C, lower panel). p16-siRNA treatment led to a 4.2- and 10.5-fold increase in BrdU incorporation compared with no treatment or treatment with control-siRNA, respectively ($p < .01$) (Fig. 5C, upper panel). The knockdown of p16^{INK4A} also rescued cellular senescence. The ratio of SA-β-gal-positive cells treated with p16-siRNA ($3.1\% \pm 0.6\%$) was significantly lower than that of cells not treated ($31.8\% \pm 5.3\%$) or treated with control-siRNA ($14.2\% \pm 3.0\%$) (Fig. 5D). The reason for the decrease in the SA-β-Gal-positive ratio in control-siRNA treated cells is probably due to the decrease in total cell number (nontreatment group: 164 ± 25.7 ; control-siRNA group: 112.7 ± 19.4 , p16-siRNA group: 172.3 ± 38.6). Senescent cells expressing SA-β-Gal seemed to be vulnerable to the trypsinization process, which was required for the transfection experiments. Even in such a situation, the total cell number was increased in the p16-siRNA group. The growth stimulatory effect of p16-siRNA was confirmed by serial cell counting (Fig. 5E). The fold increase in cell number at 144 and 216 hours after transfection was higher in the p16-siRNA-treated cells than in cells without treatment or control-siRNA-treated cells (Fig. 5E).

The Expression Profile of p16^{INK4A} Is Classified into Three Types

In almost all strains, the expression of p16^{INK4A} gradually increased with the propagation in vitro (Fig. 3) of hMSC10, shown in Figure 6C. There were, however, some strains showing a different profile of expression. Based on the p16^{INK4A} expression profile in vitro, 29 cases were divided into three groups and analyzed in detail (supplemental online Table 1).

Gradual Increase Group. Twenty-five of 29 (86%) strains demonstrated this pattern of expression (Fig. 6A; hMSC23 shown in Fig. 6A). The expression level at the final passage was at least two times higher than at the first passage. The mean final PD of the gradual increase (GI) group was 28.1 ± 7.2 (18–39), and the mean culture period was 151.2 ± 47.7 days (90–240 days). The mTRFL of SP was 9.4 ± 1.0 kb (6.7–10.1 kb) and that of FP was 7.5 ± 0.6 kb (6.4–8.5 kb). The mean highest expression of p16^{INK4A} was 0.06 ± 0.04 (0.019–0.24). The expression of p16^{INK4A} was not increased more than 0.3 relative

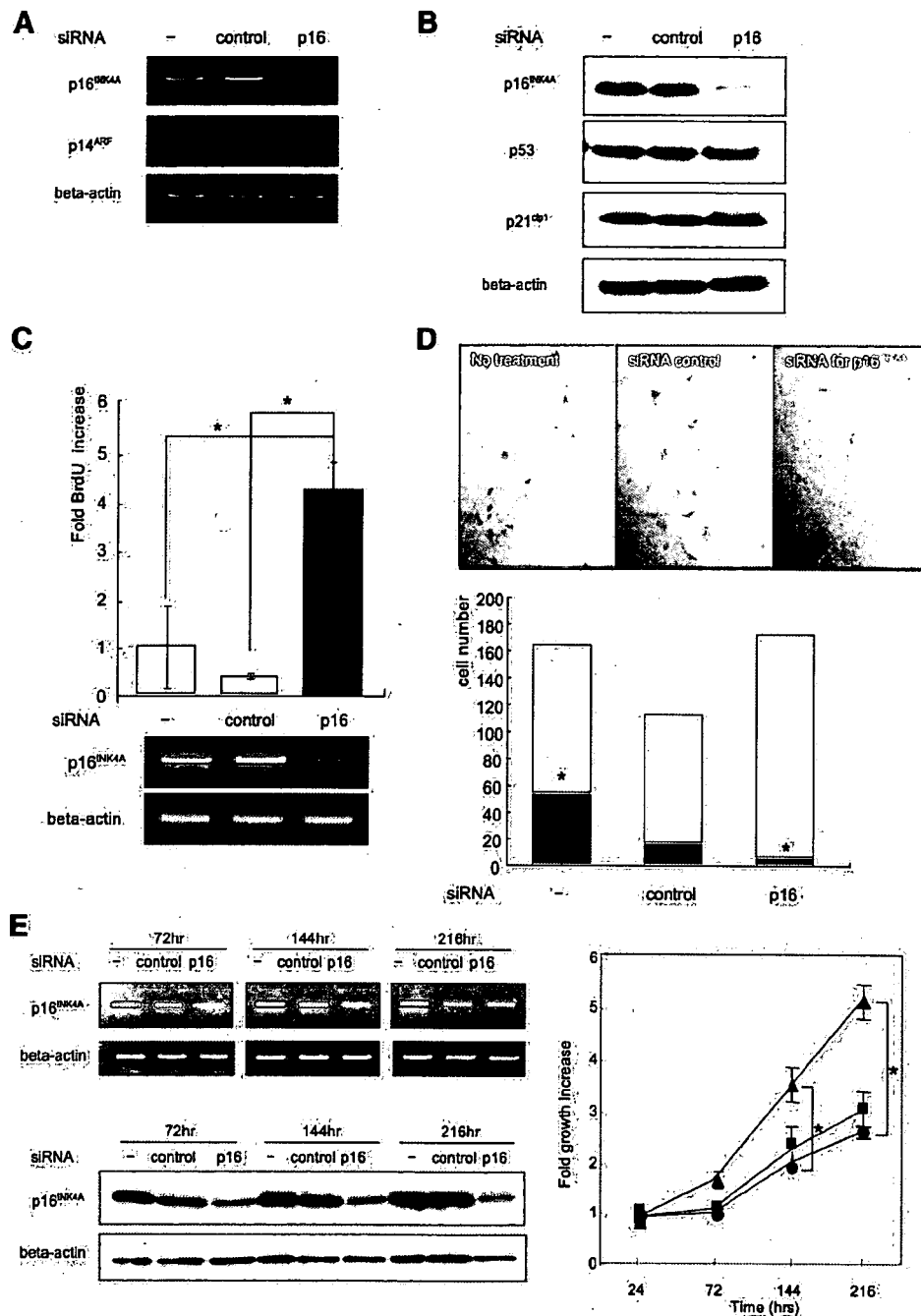


Figure 5. Knockdown of p16^{INK4A} prevented senescence and restored proliferation. (A): Inhibition of p16^{INK4A} gene expression by p16-siRNA. siRNA for a scramble sequence (control-siRNA) was used as a control. (B): Specific inhibition of p16 protein expression by p16-siRNA. (C): Incorporation of BrdU after siRNA treatment. Effective inhibition of p16^{INK4A} expression by p16-siRNA was confirmed by reverse transcription-polymerase chain reaction shown in the lower part of the figure; *, $p < .01$. (D): Senescence associated- β -galactosidase (SA- β -Gal) staining after siRNA treatment. Representative data were shown in the upper part of the figure, and SA- β -Gal-positive (black square) and negative (white square) cells were counted as described in the Materials and Methods section and are shown in the lower part; *, $p < .01$. Note that total cell number was decreased in the control-siRNA-treated cells, whereas the number in the p16-siRNA-treated cells was equal to that in untreated cells. (E): Growth profile of human MSCs after siRNA treatment. Living cells of each group (▲, treated with p16-siRNA; ●, treated with control-siRNA; ■, no treatment) were enumerated at each time point, and the fold increase compared with the number at 24 hours after siRNA treatment was demonstrated; *, $p < .01$. Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; hr(s), hours; siRNA, small interfering RNA.

to the expression of Saos2. The expression level of the BMI1 gene showed no significant change in vitro (Fig. 6Ab).

Increase Followed by Reduction Group. Two out of 29 (7%) strains showed a rapid reduction in p16^{INK4A} gene expression after an extremely high level was reached (Fig. 6B; hMSC17

shown in Fig. 6B; hMSC14, data not shown). The donor age was relatively young (hMSC14, 17 years; hMSC17, 24 years). The final PD (hMSC14, 33; hMSC17, 54) and culture period (hMSC14, 147 days; hMSC17, 334 days) were lengthened. The mTRFL of SP was almost equal to the average of the GI group

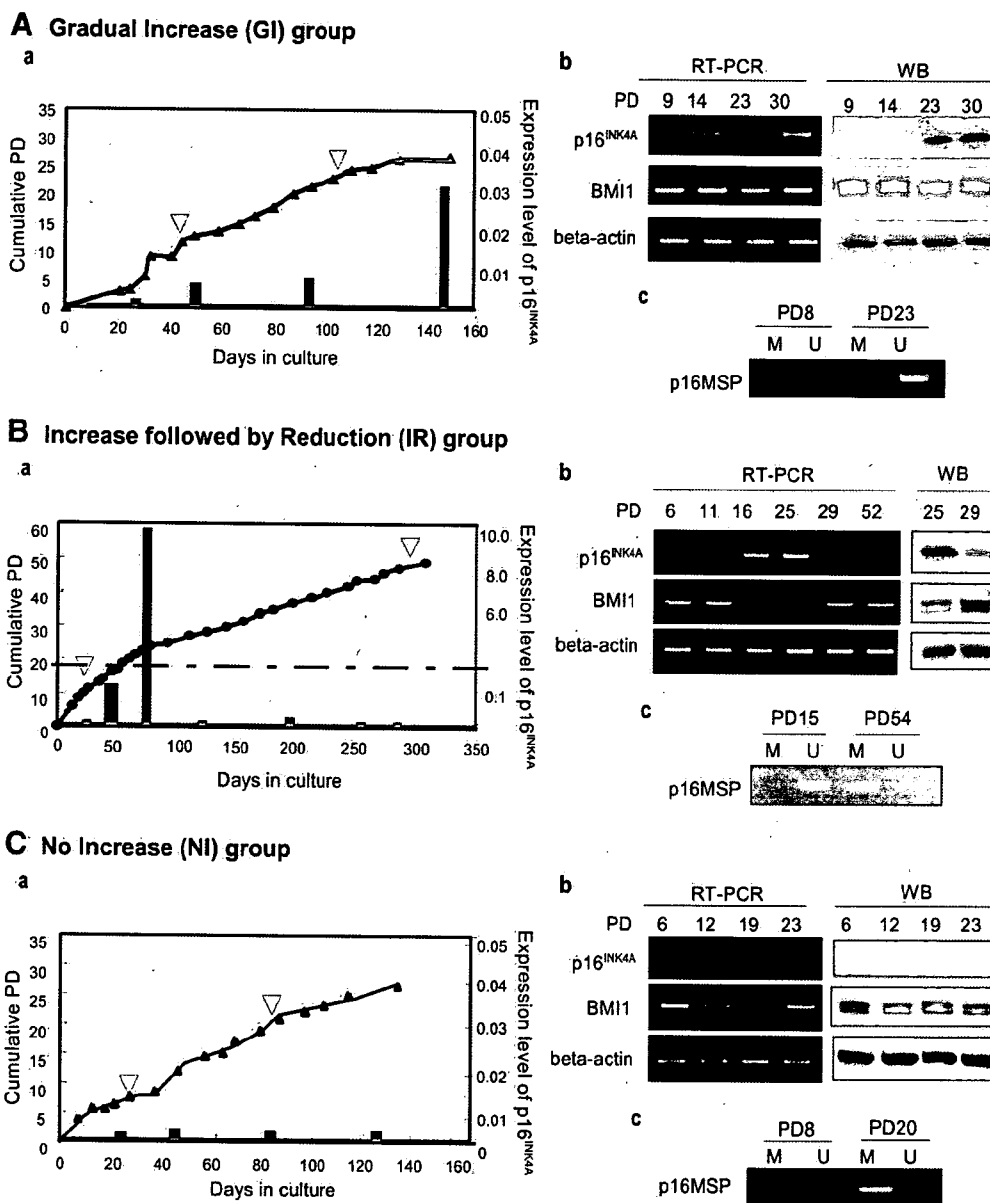


Figure 6. Classification of human (h)MSC strains by the expression of p16^{INK4A} during cell expansion. (A): GI group. (Aa): Cumulative PD (line graph) and the relative expression of the p16^{INK4A} gene (bar graph) in hMSC23. The arrowhead indicates the time point at which the methylation of the p16^{INK4A} gene was analyzed. (Ab): Expression of the p16^{INK4A} and BMI1 genes at the mRNA (left panel) and protein (right panel) levels. (Ac): MSP of the p16^{INK4A} gene. (B): IR group. Samples were derived from hMSC17. (C): NI group. Samples were derived from hMSC10. Abbreviations: GI, gradual increase; IR, increase followed by reduction; M, fragments amplified by methylated sequence-specific primers; MSP, methylation-specific polymerase chain reaction; NI, no increase; PD, population doubling; RT-PCR, reverse transcription-polymerase chain reaction; U, fragments amplified by unmethylated sequence-specific primers; WB, western blotting.

in hMSC14 (9.5 kb) but longer in hMSC17 (11.5 kb). The mTRFL of FP was equal to the average of the GI group in both strains (hMSC14, 7.6 kb; hMSC17, 6.9 kb). The highest value of p16^{INK4A} expression was much higher than the mean value of the GI group in both strains (hMSC14, 4.8; hMSC17, 10.6). Interestingly, the expression of the BMI1 gene was repressed during the period in which the expression level of the p16^{INK4A} gene was extremely high and returned to the initial level after the p16^{INK4A} gene was downregulated (Fig. 6Bb).

No Increase Group. In two out of 29 (7%) strains, the level of p16^{INK4A} remained low throughout the culture (hMSC10 shown in Fig. 6C; hMSC24, data not shown). The final PD (hMSC10, 28; hMSC24, 27) and culture period (hMSC10, 138

days; hMSC24, 170 days) were almost equal to the average for the GI group. The mTRFL of SP was almost equal to the average of the GI group in both strains (hMSC10: 10.3 kb, hMSC24: 9.0 kb). The mTRFL of FP was longer in hMSC10 (8.5 kb) but equal in hMSC24 (7.1 kb). The highest expression of p16^{INK4A} did not exceed 0.01 (hMSC10, 0.0063; hMSC25, 0.0028). The expression level of the BMI1 gene showed no significant change during the culture period.

Methylation of the Promoter of the p16^{INK4A} Gene

Several mechanisms were found as the cause of downregulation of the p16^{INK4A} expression in cancer cells, the DNA methylation in the core promoter region being the most frequent event

[28]. Several experiments were performed to investigate whether DNA methylation was involved in the downregulation of p16^{INK4A} expression observed in certain hMSC strains. First, hMSC17 cells, one of two strains in the increase-followed-by-reduction (IR) group, were treated with the demethylation drug 5-aza-dC for 96 hours, and p16^{INK4A} expression was checked by RT-PCR (supplemental Fig. 2). As seen in control U2OS cells, an osteosarcoma cell line in which p16^{INK4A} expression is downregulated by DNA methylation [32], p16^{INK4A} expression was induced in PD54 but not in PD15 (supplemental online Fig. 2A). 5-aza-dC treatment also induced a senescence phenotype in late passage hMSC17 (PD54) as SA- β -Gal-positive cells increased by twofold after 96 hours of treatment (supplemental Fig. 2B).

Next, the methylation status of the promoter region of p16^{INK4A} was analyzed by the MSP assay. MSP was performed using the genomic DNA from early and late passage cells from 10 strains of the GI group, in two strains of the IR group, and in two strains of the no increase (NI) group (supplemental online Table 1). The methylated sequence-specific fragment of the p16^{INK4A} gene was not amplified in 12 out of 12 group GI strains either in early or late passage cells, and unmethylated sequence-specific fragments were amplified (Fig. 6Ac). In the case of hMSC17 from the IR group, only unmethylated sequence-specific fragments were amplified using DNA extracted from cells at PD15, at which the level of p16^{INK4A} expression remained low (Fig. 6Bc). When DNA extracted from cells at PD54 was used for MSP, however, methylated sequence-specific fragments were amplified, and the amount of unmethylated sequence-specific fragments decreased (Fig. 6Bc). In the NI group, the methylated sequence-specific fragment was observed even in the early passage cells in addition to the unmethylated sequence-specific fragment (Fig. 6Cc). The expression of the methylated sequence-specific fragment was increased in late passage, and unmethylated sequence-specific fragment was decreased. Methylation of the target region of MSP in these samples was confirmed by bisulfite sequencing (data not shown). These results indicated that DNA methylation in the core promoter region plays a role in downregulating the expression of the p16^{INK4A} gene in vitro in hMSCs as observed in human cancers.

Chromosomal Rearrangement

Because of the extraordinarily long culture period, the karyotype of hMSC17 was analyzed using the standard G-band method, and several samples from the GI and NI groups were also analyzed. No chromosomal aberrations were detected in samples from the GI and NI groups (data not shown). The chromosomal structure was also normal in hMSC17 at early passage (PD10) (supplemental online Fig. 3A). Cells of the same strain at the later passage (PD54), however, showed a number of numerical and structural aberrations including two clonal aberrations with the same reciprocal translocation (supplemental online Fig. 3B). Therefore, both epigenetic and genetic abnormalities existed in the late passage cells of hMSC17, which might be responsible for the prolonged culture of this strain.

DISCUSSION

Unlimited self-renewal ability is a hallmark of embryonic stem cells, providing the potential to differentiate into cells of any type [33]. In the case of tissue stem cells including MSCs, however, the potential to proliferate is restricted in the Hayflick

model of cellular aging. The molecular mechanism for inducing senescence in vitro has been extensively investigated, and recent reports suggested a cell specificity-dependent mechanism [33]. In this study, we have demonstrated that telomere length and p16^{INK4A} expression are involved in different phases of cellular senescence in hMSC.

In human MSCs, telomerase activity was not detected [26, 34]. The mean telomere restriction fragment length in primary hMSCs was 9.4 ± 1.0 kb for early passage and 7.5 ± 0.6 kb for late passage cells, and the mean telomere shortening rate was 109 ± 43 bp (Fig. 2), which is close to the theoretical length of shortening in each DNA replication, suggesting that there is no mechanism to maintain telomere length in hMSCs. Therefore, it is rational that the initial mTRFL correlated well with the replicative capacity of hMSCs ($r = .677$, Fig. 2C). hMSCs transduced with the human telomerase gene hTERT exhibit a prolonged replicative capacity, evidence that the integrity of the telomere plays a vital role in regulating replicative capacity in vitro [26, 35]. However, the shortening of telomeres is not the sole factor inducing cellular senescence in hMSCs, and our current results indicated that the upregulation of the p16^{INK4A} gene expression plays an essential role in this process. This may be relevant to our previous data that the introduction of hTERT was not sufficient to immortalize hMSC, and that the inactivation of the function of p16 either by the HPVE6/E7 or BM11 genes was required [26, 36].

The relationship between growth capacity and donor age is not clear. Clearly, the hMSC strains isolated from younger donors on the average exhibited a longer life span in vitro (Fig. 1D, 1E), and the initial mTRFL correlated with the life span. There was, however, no clear relationship between the initial mTRFL and donor age (Fig. 2). Also, the expression profile of p16^{INK4A} was not correlated with donor age. These issues should be further analyzed in detail.

p16^{INK4A} is known as a biomarker of aging. This cell cycle regulator is not expressed in undifferentiated hESCs [37]. In mouse HSCs and NSCs, the expression of p16^{INK4A} was repressed by the PcG protein BMI1 [15]. But in later life, this PcG protein failed to repress the expression of p16^{INK4A} and the function of HSCs and NSCs declined [14, 15]. In the current study, BMI1 was expressed at the mRNA and protein levels in all the hMSC strains (Fig. 6). In some strains, the expression of BMI1 was negatively correlated with the expression of p16^{INK4A} (Fig. 6B, 6C). The relationship between these two molecules in hMSCs remained to be investigated. The expression of p16^{INK4A} was increased toward senescence (Fig. 3), and senescent cells expressed p16^{INK4A}. The downregulation of p16^{INK4A} expression resulted in escape from senescence and a restoration of the proliferating activity (Fig. 5). These results suggest that most hMSCs do not have a mechanism to repress the expression of p16^{INK4A} in vitro.

We found three types of passage-dependent change of the p16 gene expression during in vitro life of hMSCs of which two were associated with the methylation-induced suppression. No clear differences were found among three groups in clinical characters of donors, and the procedures for the isolation and expansion of hMSCs were the same in all cases. Subtle changes in the culture condition such as the initial density, the concentration of CO₂, and the timing of passage may influence the development of the methylation, which will be an important issue to be further investigated.

As far as we know, this is the first report to demonstrate the methylation of p16^{INK4A} in hMSCs. This phenomenon was observed in 4 of 29 strains (13.8%), which is above the negligible frequency (Fig. 6, supplemental online Table 1). Methylation of the p16^{INK4A} gene in normal cells in vitro is reported in

the case of human mammary epithelial cells showing a prolonged life span in vitro [20]. In many cases, however, this phenomenon is seen in cancer cells [38].

A line of evidence has indicated a tight association between the inactivation of p16^{INK4A} and transformation of hMSCs. The hMSCs immortalized with hTERT showed spontaneous transformation in vitro in association with the deletion of the p16^{INK4A} gene and activation of the K-ras gene [39]. The hMSC strain transformed spontaneously in vitro lacked expression of the p16^{INK4A} gene [24]. The Bmi1-transduced immortalized hMSCs, which showed no detectable p16^{INK4A} gene expression, became fully transformed after the introduction of an activated H-ras gene [36]. In this study, one case with an inactivated p16^{INK4A} gene by DNA methylation (hMSC17) showed a prolonged life span (334 days in culture with a final PD of 54) in vitro far longer than the average (151 days and 28 PD) (Fig. 6Bb). Although no apparent phenotype of transformed cells was observed in this strain, and the growth ceased with telomere shortening, the association of chromosomal aberrations (supplemental online Fig. 3) may be considered a sign of pretransformation as reported in human and murine MSC cases [24, 40, 41]. This point should be seriously considered in the clinical use of hMSCs, particularly in cases requiring prolonged and

extensive in vitro expansion. Monitoring of the DNA methylation of the p16^{INK4A} gene will serve as surveillance for the transformation of hMSCs.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

- Caplan AI, Bruder SP. Mesenchymal stem cells: Building blocks for molecular medicine in the 21st century. *Trends Mol Med* 2001;7: 259–264.
- Mazzini L, Mareschi K, Ferrero I et al. Autologous mesenchymal stem cells: Clinical applications in amyotrophic lateral sclerosis. *Neuro Res* 2006;28:523–526.
- Ohgushi H, Kotobuki N, Funaoka H et al. Tissue engineered ceramic artificial joint—ex vivo osteogenic differentiation of patient mesenchymal cells on total ankle joints for treatment of osteoarthritis. *Biomaterials* 2005;26:4654–4661.
- Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. *Exp Hematol* 2000;28:875–884.
- Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961;25:585–621.
- Itahana K, Campisi J, Dimri GP. Mechanisms of cellular senescence in human and mouse cells. *Biogerontology* 2004;5:1–10.
- Blackburn EH. Telomere states and cell fates. *Nature* 2000;408:53–56.
- Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;345:458–460.
- Carpenter MK, Rosler ES, Fisk GJ et al. Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. *Dev Dyn* 2004;229:243–258.
- Miura T, Luo Y, Khrebukova I et al. Monitoring early differentiation events in human embryonic stem cells by massively parallel signature sequencing and expressed sequence tag scan. *Stem Cells Dev* 2004;13: 694–715.
- Allsopp RC, Cheshier S, Weissman IL. Telomere shortening accompanies increased cell cycle activity during serial transplantation of hematopoietic stem cells. *J Exp Med* 2001;193:917–924.
- Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993;366: 704–707.
- Krishnamurthy J, Torrice C, Ramsey MR et al. INK4A/Arf expression is a biomarker of aging. *J Clin Invest* 2004;114:1299–1307.
- Janzen V, Forkert R, Fleming HE et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16^{INK4A}. *Nature* 2006;443: 421–426.
- Molofsky AV, Slutsky SG, Joseph NM et al. Increasing p16^{INK4A} expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* 2006;443:448–452.
- Park IK, Morrison SJ, Clarke MF. Bmi1, stem cells, and senescence regulation. *J Clin Invest* 2004;113:175–179.
- Park IK, Qian D, Kiel M et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 2003;423:302–305.
- Molofsky AV, Pardal R, Iwashita T et al. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* 2003;425:962–967.
- Jacobs JJ, Kieboom K, Marino S et al. The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the INK4A locus. *Nature* 1999;397:164–168.
- Wong DJ, Foster SA, Galloway DA et al. Progressive region-specific de novo methylation of the p16 CpG island in primary human mammary epithelial cell strains during escape from M0 growth arrest. *Mol Cell Biol* 1999;19:5642–5651.
- Holliday R, Grigg GW. DNA methylation and mutation. *Mutat Res* 1993;285:61–67.
- Ushijima T, Okochi-Takada E. Aberrant methylations in cancer cells: Where do they come from? *Cancer Sci* 2005;96:206–211.
- Ushijima T, Nakajima T, Maekita T. DNA methylation as a marker for the past and future. *J Gastroenterol* 2006;41:401–407.
- Rubio D, Garcia-Castro J, Martin MC et al. Spontaneous human adult stem cell transformation. *Cancer Res* 2005;65:3035–3039.
- Caterson EJ, Nesti LJ, Danielson KG et al. Human marrow-derived mesenchymal progenitor cells: Isolation, culture expansion, and analysis of differentiation. *Mol Biotechnol* 2002;20:245–256.
- Okamoto T, Aoyama T, Nakayama T et al. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun* 2002;295:354–361.
- Huang HJ, Yee JK, Shew JY et al. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science* 1988;242:1563–1566.
- Herman JG, Graff JR, Myohanen S et al. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996;93:9821–9826.
- Dimri GP, Lee X, Basile G et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* 1995;92:9363–9367.
- Flores I, Cayuela ML, Blasco MA. Effects of telomerase and telomere length on epidermal stem cell behavior. *Science* 2005;309: 1253–1256.
- Bodnar AG, Ouellette M, Frolkis M et al. Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998;279: 349–352.
- Park YB, Park MJ, Kimura K et al. Alterations in the INK4a/ARF locus and their effects on the growth of human osteosarcoma cell lines. *Cancer Genet Cytogenet* 2002;133:105–111.
- Rosler ES, Fisk GJ, Ares X et al. Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn* 2004;229:259–274.
- Sethi S, Scutt A, Stolzing A. Aging of mesenchymal stem cells. *Ageing Res Rev* 2006;5:91–116.
- Simonsen JL, Rosada C, Serakinci N et al. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. *Nat Biotechnol* 2002; 20:592–596.

- 36 Shima Y, Okamoto T, Aoyama T et al. In vitro transformation of mesenchymal stem cells by oncogenic H-rasV12. *Biochem Biophys Res Commun* 2007;353:60–66.
- 37 Zeng X, Rao MS. Human embryonic stem cells: Long term stability, absence of senescence and a potential cell source for neural replacement. *Neuroscience* 2007;145:1348–1358.
- 38 Rocco JW, Sidransky D. p16(MTS-1/CDKN2^{INK4A}) in cancer progression. *Exp Cell Res* 2001;264:42–55.
- 39 Serakinci N, Guldborg P, Burns JS et al. Adult human mesenchymal stem cell as a target for neoplastic transformation. *Oncogene* 2004;23:5095–5098.
- 40 Zhou YF, Bosch-Marce M, Okuyama H et al. Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. *Cancer Res* 2006;66:10849–10854.
- 41 Tolar J, Nauta AJ, Osborn MJ et al. Sarcoma derived from cultured mesenchymal stem cells. *STEM CELLS* 2007;25:371–379.



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Expression of the p16INK4A Gene Is Associated Closely with Senescence of Human Mesenchymal Stem Cells and Is Potentially Silenced by DNA Methylation During In Vitro Expansion

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Cell-specific epigenetic regulation of ChM-I gene expression: Crosstalk between DNA methylation and histone acetylation

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Abstract

The expression of the chondromodulin-I (ChM-I) gene, a cartilage-specific gene, is regulated by the binding of Sp3 to the core promoter region, which is inhibited by the methylation of CpG in the target genome in the osteogenic lineage, osteosarcoma (OS) cells. The histone tails associated with the hypermethylated promoter region of the ChM-I gene were deacetylated by histone deacetylase 2 (HDAC2) in three ChM-I-negative OS cell lines. Treatment with an HDAC inhibitor induced the binding of Sp3 in one cell line, which became ChM-I-positive. This process was associated with acetylation instead of the dimethylation of histone H3 at lysine 9 (H3-K9) and, surprisingly, the demethylation of the core promoter region. The demethylation was transient, and gradually replaced by methylation after a rapid recovery of histone deacetylation. These results represent an example of the plasticity of differentiation being regulated by the cell-specific plasticity of epigenetic regulation.

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The expression of cell type-specific genes is tightly regulated by a hierarchical mechanism composed of genetic and epigenetic factors. In some cases, a single transcription factor may determine cell fate. In other instances, however, the expression of such a master gene is found in cells in other tissue-lineages. For example, the expression of the SOX9 gene, which is the master gene for chondrogenic cell lineage, is also found of in cells of the osteogenic lineage [1]. Transcription factors regulating the expression positively or negatively must bind to specific DNA elements to exert their function, and the factors regulating such binding sig-

nificantly affect their function and therefore the expression of each gene.

Chondromodulin-I (ChM-I) is a potent vascular endothelial cell growth inhibitor purified from bovine epiphyseal cartilage [2], the expression of which in matured limbs is limited to cells in the resting, proliferating, and early hypertrophic zone of the growth plate [2], and not in other mesenchymal tissues including bone. Therefore, it is a suitable material for analyzing the molecular mechanism regulating cell-specific genes, and we have shown that the Sp3 transcription factor is a major driver of binding to a specific portion in the core promoter region [1]. Because Sp3 is a universal transcription factor ubiquitously expressed in many tissues, the cell-specific expression of the ChM-I gene requires additional factors. Using osteosarcoma (OS) cells, which are malignant mesenchymal

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tumor cells with features of osteogenic lineage cells, we have shown that DNA methylation of the Sp3-binding sequence is one such mechanism to shut down the expression in cells of the osteogenic lineage [1]. Treatment with a demethylating reagent, however, failed to restore the expression of the ChM-I gene in some OS cells, suggesting the presence of other regulatory mechanisms [1].

Modification of the histone tail is another epigenetic regulatory mechanism [3]. Histone proteins assemble into nucleosomes, which function as DNA packaging. The amino-terminal tails of histone protrude from the nucleosome and are subject to chemical modifications including phosphorylation, ubiquitination, acetylation, and methylation [4]. The acetylation of lysine residues on histone H3 and H4 leads to the formation of an open chromatin structure and allows regulatory factors to easily access the chromatin [4], which is regulated by the stochastic balance of histone acetyltransferases (HAT) and histone deacetylases (HDAC). Notably, dimethylation of histone H3 at lysine 9 (H3-K9) tightly inhibits the gene expression, correlated with the methylation of CpG [5,6]. These modifications of the histone tail and CpG methylation status determine the differentiation [7].

Here, we investigated the epigenetic regulation of the ChM-I gene in a variety of OS cell lines and found that modifications of the histone tail determine the expression. We found evidence for crosstalk between the modification of histones and methylation of CpG, in which histone acetylation can inhibit the maintenance of CpG methylation by substituting H3-K9 dimethylation with H3-K9 acetylation.

Materials and methods

Cell lines and culture conditions. The human cell lines Saos2, HuO, HOS, MG63, and U2OS were obtained from ATCC or Japanese Cancer Research Resources Bank. The human osteosarcoma cell lines TAKAO and ANOS were established in our laboratory. All the cell lines used in this study were maintained in DMEM (Sigma) with 10% fetal bovine serum (Hyclone, South Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin, in 5% CO₂ at 37 °C.

SDS-PAGE. Western blotting was performed as previously mentioned [1]. The primary antibodies used were as follows: acetylated H3, acetylated H4, dimethylated H3-K9, acetylated H3-K9, and H3 from Upstate Biotechnology (Lake Placid, NY), HDAC2, HDAC3, and HDAC6 from Zymed Laboratory (San Francisco, CA), and Sp1 and Sp3 from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse transcription (RT)-PCR and quantitative RT-PCR. RNA was isolated using Trizol Reagent (Life Technologies, Rockville, MD) following the manufacturer's directions from frozen tumor tissues and the cultured cell lines. All RT reactions were performed using 1 µg of total RNA with a Super Script First Strand Synthesis System for RT-PCR kit (Life Technologies) according to the instructions supplied. RT-PCR amplification was performed as previously mentioned [1].

Demethylation drug treatment. The cells (1×10^5) were seeded on 60-mm dishes in DMEM with 10% FBS. After cells attaching to the dishes, the cells were treated with 1 µM of 5-Aza-2'-deoxycytidine (5-Aza-dC) (Sigma-Aldrich) for 96 h.

Histone deacetylase inhibitor (HDACi) treatment. The cells (1×10^5) were seeded on 60-mm dishes in DMEM with 10% FBS. After attaching to the dishes, they cells were treated with histone deacetylase inhibitors; MS-

275 provided by Nihon Scherring (Chiba, Japan). The cells were harvested after 24 h.

Bisulfite genomic sequencing. The bisulfite modification of DNA samples was performed using the EpiTect bisulfite kit (Qiagen, Tokyo, Japan). Bisulfite-modified DNA spanning residues -297 to -104 relative to the transcription start point [1] was amplified, cloned into the TA-vector (Invitrogen), and sequenced using an ABI 377 semiautomatic sequencer (PE Applied Biosystems).

Chromatin immunoprecipitation (ChIP). The suitability of each antibody for the ChIP assay was confirmed by immunoprecipitation-Western blotting (data not shown). Tissue samples were treated using an EpiQuik tissue ChIP kit according to the manufacturer's directions (Epigentek Group Inc. Brooklyn, NY). Cells were harvested and mixed with formaldehyde at a final concentration of 1.0% for 10 min at 37 °C to cross-link protein to DNA. Cells then were suspended in 0.2 ml of SDS lysis buffer and settled on ice for 10 min. DNA cross-linked with protein was sonicated into fragments of 200–1000 bp. One-tenth of the sample was set aside as an input control, and the rest was precleared with salmon sperm DNA protein A-Sepharose beads (Upstate Biotechnology) for 30 min with agitation. The soluble chromatin fraction was collected with each antibody at 4 °C overnight with rotation. Immune complexes were collected with salmon sperm DNA protein A-Sepharose beads and washed with the manufacturer's low salt, high salt, and LiCl buffers and then washed twice with TE buffer (10 mM Tris-HCl and 1 mM EDTA). The chromatin-antibody complexes were eluted with elution buffer (1% SDS and 0.1 M NaHCO₃). Protein-DNA cross-links were reversed with 5 M NaCl at 65 °C for 4 h, proteinase K treatment and phenol-chloroform extraction were carried out, and then the DNA was precipitated in ethanol. PCR amplification was performed using primers specific for the ChM-I promoter (sense, 5-GAATGCAGGCCAGTGAGAAGGT-3; antisense, 5-GCACCTGGGATCTGTCCCGCT-3). The reaction was performed with an initial denaturation of 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 63 °C, and 1 min at 72 °C with a final extension at 72 °C for 7 min.

Results

The promoter lesion of ChM-I was deacetylated by binding of HDAC2 in ChM-I-negative OS cell lines

Three of seven OS cell lines (TAKAO, Saos2, and MG63) showed no expression of the ChM-I gene even in the RT-PCR analyses, among which two (TAKAO and Saos2) turned out to be positive on treatment with 5-Aza-dC (Fig. 1A). The state of the histone tails of these seven OS cell lines was analyzed by Western blotting (Fig. 1B) and ChIP assay (Fig. 1C). The acetylation of global lesions of the histone H3 and H4 tails did not differ between the ChM-I-positive and negative cell lines (Fig. 1B). But the histone H3 and H4 tails associated with the lesion in the promoter of ChM-I was locally acetylated in ChM-I-positive cells, and deacetylated in the negative cells (Fig. 1C), indicating that the status of histone acetylation correlated with the expression of the ChM-I gene. To find the HDAC responsible for the deacetylation, the expression of HDAC2, HDAC3, and HDAC6 was analyzed by Western blotting, which showed no clear correlation with the expression of ChM-I gene (Fig. 1D). The ChIP assay, however, showed that HDAC2, but not HDA3 or HDAC6, bound to the promoter region in the ChM-I-negative cell lines (Fig. 1E). These results suggested that HDAC2 was