higher in the hypoxic culture. Similar results were obtained with the other three hBM cells (Fig. 1), and the final number of PD in normoxic and hypoxic cultured-cells was 17 and 25 in hBM72, 25 and 39 in hBM77, and 24 and 45 in hBM80.

Hypoxia rescued MSCs from cellular senescence

In the case of hBM77, most of the normoxic cultured-cells at PD25 were positive for SA-β-gal, whereas few were stained in the hypoxic culture at PD28 (Fig. 1B), and the difference was highly significant (Fig. 1C). Similar results were obtained with the other three hBM cells (Fig. 1C), indicating that the hypoxic culture protected MSCs from cellular senescence, which may be the cause of the increase in life span *in vitro*.

Hypoxia inhibited the up-regulation of p16 expression

We have demonstrated that the induction of cellular senescence in MSCs is tightly associated with the up-regulation of *p16* gene expression [20]. Consistent with previous findings, the level of p16 increased with the life span of normoxic cultured-hBM66, which was seven-times higher at PD20 than at PD3 (Fig. 2, upper panel). In contrast, hypoxic cultured-hBM66 retained a lower level of the p16 expression, which was only two-times higher at PD20 than at PD3 (Fig. 2, upper panel). Similar results were obtained with the other three hBM cells (Fig. 2), suggesting that hypoxia inhibited the induction of *p16* gene expression, which then protected cells from cellular senescence.

Hypoxia affected the ability to differentiate into chondro- and adipogenic, but not osteogenic, lineage

The ability to differentiate into osteo-, chondro-, and adipogenic lineages in the early phase was confirmed in all four hBM cells (data not shown). After long-term culture (about 100 days), no significant difference in osteogenic differentiation, measured based on Ca content, was observed between normoxic and hypoxic cultured-MSCs (Fig. 3A). In contrast, the ability to differentiate into the chondrogenic (Fig. 3B) and adipogenic (Fig. 3C) lineages, measured from GAG and TG content, respectively, was significantly more superior in hypoxic than normoxic conditions.

Hypoxia inhibited the activation of ERK

In the case of hBM66, the expression of molecules related to MAPK signals showed no significant difference between normoxic

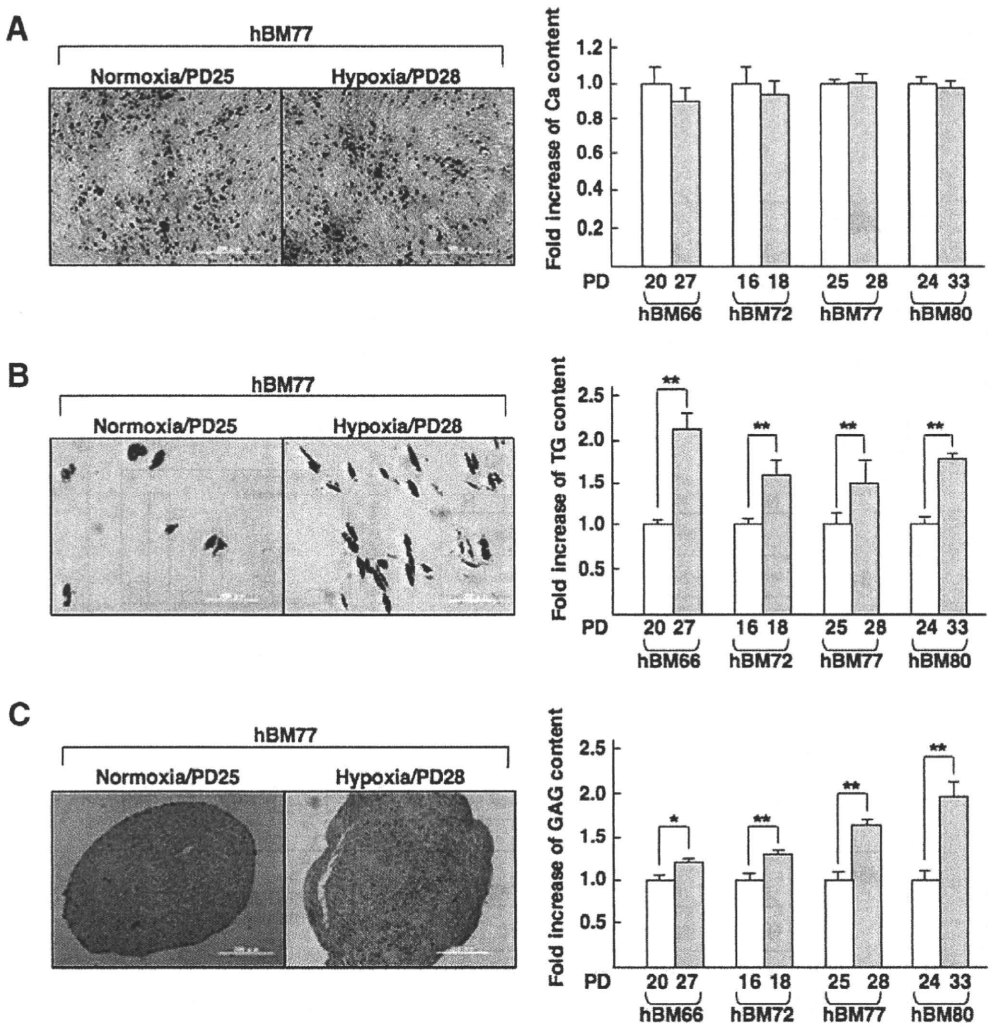

Fig. 3. Hypoxia enhanced differentiation properties of MSCs. Each preparations of MSCs was cultured for approximately 100 days under either normoxic or hypoxic conditions and then induced to undergo (A) osteogenic, (B) chondrogenic, or (C) adipogenic differentiation under normoxic conditions. The results were quantified based on the content of Ca, GAG, or TG, respectively. PD numbers of the MSCs used for these experiments are indicated. White and gray box indicate normoxic and hypoxic conditions, respectively.

and hypoxic cultured-cells except that of ERK (Fig. 4A). Levels of both phospho-ERK1 and phospho-ERK2 were much lower in hypoxic cultured-cells than in normoxic cultured-cells throughout the culture period, and similar results were obtained with the other three hBM cells (Fig. 4B). nPG is an antioxidant, which induces the production HIF-1α protein [24]. Treatment of hBM72 cells (PD3) cultured under normoxic conditions with nPG successfully induced the expression of HIF-1α, but failed to reduce the level of phospho-ERK1/2 (Fig. 4C), indicating that the reduction in phospho-ERK is not a direct consequence of HIF-1α's activation. To investigate the relationship between ERK and p16, the activity of MEK, by which ERK is phosphorylated, was inhibited by U0126. When hBM72 cells (PD3) cultured under normoxic conditions were treated with U0126 for 4 days, activation of ERK was inhibited throughout the culture period (Fig. 4D). During this period, the level of *p16* increased two-fold in control cells, but only slightly in U0126 treated-cells (Fig. 4D), suggesting that the ERK signal is one of the factors inducing the expression of the *p16* gene.

Discussion

A number of studies have been published regarding the effects of hypoxia on the growth and differentiation of MSCs [12–17], but the results differed considerably. The discrepancies may be at least in part due to differences in species, the concentration of oxygen, and/or the length of culture periods, and the discussion hereafter focuses on the data for human MSCs. As for short-term effects, some studies showed that hypoxia increased the proliferation of MSCs by promoting progression of the cell cycle [16], but others showed no or even the opposite effect [14], consistent with the results of the current study. Two studies have analyzed the long-term effects of hypoxia on human MSCs. Grayson et al. showed that human MSCs displayed enhanced proliferation under hypoxic conditions (2% pO_2) for seven passages over 6 weeks, resulting in a 30-fold increase in cell number compared with that under normoxic conditions [15]. Fehrer et al. showed that MSCs cultured under hypoxic conditions (3% pO_2) for up to 100 days had a higher number of final PD than those cultured under normoxic conditions by ten [17], which agreed with the results of the current study. Because the PD time showed no difference during the early phase of growth (Fig. 1A), our data indicate that hypoxia did not affect the growth of cells, but extended their life span, and the marked difference in the number of SA-β-gal-positive cells between hypoxic and normoxic conditions clearly indicates that the lengthening of life span by hypoxia is due to the escape from cellular senescence. We have shown that the up-regulation of *p16* gene expression is key to inducing cellular senescence in human MSCs [20]. In the current study, we showed that hypoxia inhibited the up-regulation of *p16* gene expression. ROS induces *p16* gene expression [21,22], and the p16-Rb pathway then induces the production of ROS, which leads to cellular senescence [25].

Regarding the effects of hypoxia on the differentiation of MSCs, published results vary. To evaluate the ability of MSCs to differentiate, osteo-, chondro-, or adipogenic differentiation has been analyzed in most studies [12–17], but no studies have examined all three lineages in MSCs cultured under hypoxic conditions long term. Martin-Rendon et al. analyzed the short-term effects (24 h) of hypoxia (1% pO_2) on the differentiation into three lineages and found that only chondrogenic differentiation was improved [16]. Our data indicated that chondrogenic, as well as adipogenic, but not osteogenic, differentiation was improved in MSCs cultured under hypoxic conditions for long term (about 100 days). Although

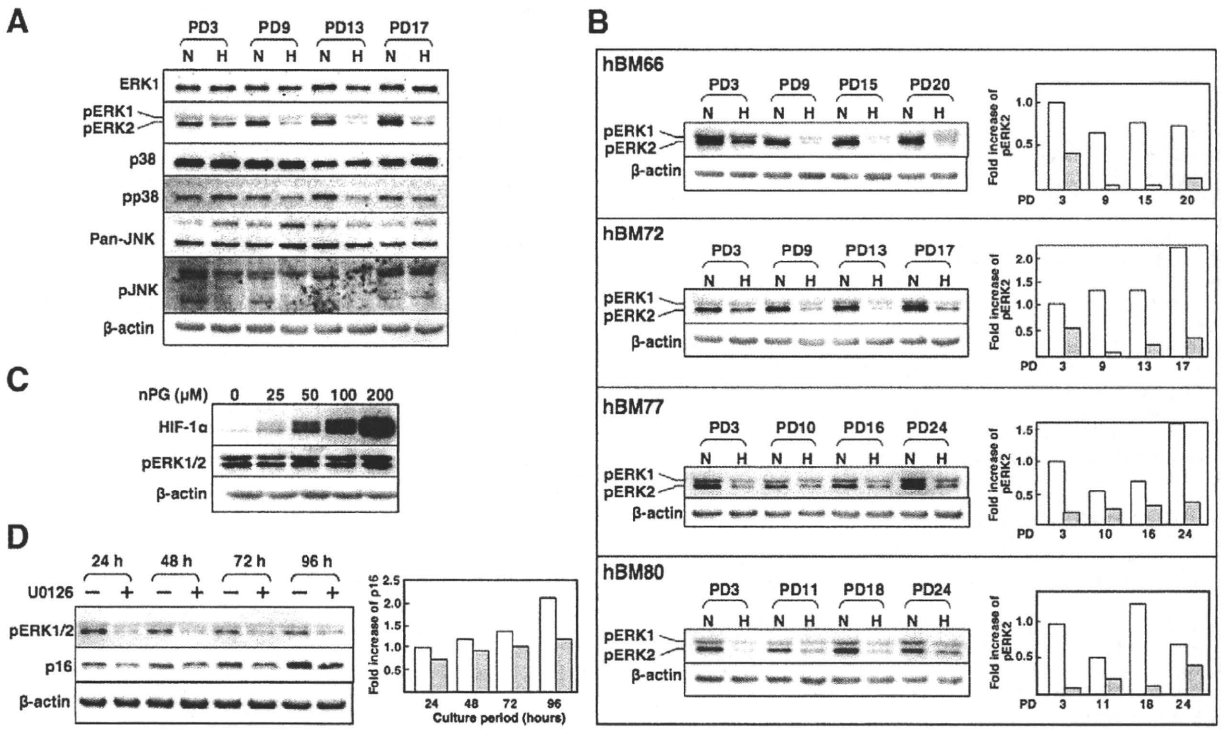

Fig. 4. Hypoxia down-regulated the activation of ERK in MSCs. (A) Expression of molecules related to MAPK signaling pathways of huBM72 at the indicated PD. (B) Expression of pERK1/2 during the life span of MSCs *in vitro*. The relative level of pERK2 was determined using the value at PD3 of normoxic cells as a standard. White and gray box indicate normoxic and hypoxic conditions, respectively. (C) Effect of nPG treatment on the expression of HIF-1α and pERK1/2. huBM72 cells cultured under normoxic conditions at PD3 were treated with nPG at the indicated concentration for 4 h, and proteins were extracted for Western blotting. (D) Effect of a MEK inhibitor on the expression of pERK1/2 and p16. huBM72 cells cultured under normoxic conditions at PD3 were treated with U0126 (10 μM) for up to 96 h, and proteins were extracted for Western blotting. The relative expression level of p16 was determined using the value of normoxic cultured-cells at 24 h as a standard. White and gray box indicate normoxic and hypoxic conditions, respectively.

the molecular mechanisms underlying this change in the behavior of MSCs are not known, the down-regulation of phospho-ERK expression caused by a reduction in oxygen is an intriguing new finding of the current study. MAPK signaling pathways have profound effects on the growth and differentiation of MSCs [26,27], and the signaling through ERK has been investigated intensively. Activation of the ERK signal triggers osteogenic differentiation [28,29]. The up-regulation of MAPK signals promoted chondrogenesis by inducing the expression of the *Sox9* gene [30]. The inhibition of ERK signals reduced the adipogenic differentiation [31]. Therefore, the ERK signal is essential to the differentiation of MSCs. In other words, inhibition of the ERK signal may restrict the "spontaneous" differentiation which maintains MSC in an undifferentiated state. The ERK signal also plays a role as a mitogenic stimulus, which promotes growth, but at the same time induces cellular senescence of MSCs. Inhibition of the ERK signal by a MEK inhibitor reduced the up-regulation of *p16* gene expression. Therefore, down-regulation of phospho-ERK expression may also help cells to escape from cellular senescence during propagation *in vitro*. Further study of mechanisms by which hypoxia down-regulates the ERK signal may provide a new method of culturing MSCs.

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Trail-Walking Exercise and Fall Risk Factors in Community-Dwelling Older Adults: Preliminary Results of a Randomized Controlled Trial

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OBJECTIVES: To evaluate the effects of a trail-walking exercise (TWE) program on the rate of falls in community-dwelling older adults.

DESIGN: Pilot randomized controlled trial (RCT).

SETTING: This trial was conducted in Japan and involved community-dwelling older adults as participants.

PARTICIPANTS: Sixty participants randomized into a TWE group (n = 30) and a walking (W) group (n = 30).

INTERVENTION: Exercise class combined with multicomponent trail walking program, versus exercise class combined with simple indoor walking program.

MEASUREMENT: Measurement was based on the difference in fall rates between the TWE and W groups.

RESULTS: Six months after the intervention, the incidence rate ratio (IRR) of falls for the TWE group compared with the W group was 0.20 (95% confidence interval (CI) = 0.04–0.91); 12 months after the intervention, the IRR of falls for the TWE group compared with the W group was 0.45 (95% CI = 0.16–1.77).

CONCLUSION: The results of this pilot RCT suggest that the TWE program was more effective in improving locomotion and cognitive performance under trail-walking task conditions than walking. In addition, participants who took part in the TWE demonstrated a decrease in the incidence rate of falls 6 months after trial completion. Further confirmation is needed, but this preliminary result may promote a new understanding of accidental falls in older adults. **J Am Geriatr Soc 58:1946–1951, 2010.**

Key words: fall prevention; trail walking task; falls; RCT; older adults

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Falls in older adults are a major health problem. Approximately 30% of community-dwelling adults aged 65 and older fall at least once a year, and 6% of these falls result in fractures.[1,2] Recent meta-analyses of fall prevention programs have convincingly shown that exercise interventions are effective at reducing the risk of falls and fall injuries.[3,4]

Although the cause of falls is typically multifactorial, performance-based measures are often used to assess how balance and gait impairments contribute to fall risk, but in community-dwelling older adults, performance-based measures are not always predictive of fall risk, particularly in a high-functioning population.[5] Concurrent cognitive or motor tasks, such as talking or carrying objects, are crucial for mobility in daily life. Because of the increasingly recognized role of cognition in postural control and gait, many researchers have used dual-task (DT) paradigms incorporating a concurrent cognitive task to improve studies investigating fall risk.[6] For example, a concurrent cognitive task during standing postural control tasks has been used to distinguish between older adults with and without a history of falls.[7] In addition, complex walking tasks such as DT walking may be more sensitive than simple walking tasks for identifying early declines in postural control in nondisabled older adults without apparent mobility limitations.[8]

The ability to modulate attention may also play an important role in the acquisition of complex-task coordination skills. Therefore, a trail-walking test (TWT), in which a person walks from numbered flags in an ascending or descending order, was developed to evaluate cognitive and motor function simultaneously.[9]

This randomized controlled trial (RCT) aimed to evaluate whether the trail-walking exercise (TWE), a new 16-week exercise program, would be effective in reducing falls in community-dwelling older adults. It was hypothesized that complex-task walking is improved to a greater extent with TWE than regular walking, suggesting that TWE is more effective in preventing falls.

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METHODS

Participants

Participants were recruited using an advertisement in the local press. The following criteria were used to screen participants in an initial interview: aged 65 and older, community-dwelling, had visited a primary care physician within the previous 3 years, a Mini-Mental State Examination (MMSE)[10] score of 24 or greater, able to walk independently (or with a cane), willingness to participate in group exercise classes for at least 6 months, access to transportation, minimal hearing and vision impairments, and no regular exercise in the previous 12 months.

The interview was also used to exclude participants based on the following exclusion criteria: severe cardiac, pulmonary, or musculoskeletal disorders; pathologies associated with greater risk of falls (e.g., Parkinson's disease or stroke); osteoporosis; and use of psychotropic drugs. Written informed consent was obtained from each participant in the trial in accordance with the guidelines approved by the Kyoto University Graduate School of Medicine and the Declaration of Human Rights, Helsinki, 1975.

Study Design and Randomization

Participants were block randomized in blocks of four. Using this sequence, opaque envelopes bearing group names were numbered and the 60 participants were then randomly assigned to the TWE (n = 30) or walking (W) group (n = 30).

Intervention

All participants received 90 minutes of group training sessions once a week for 16 weeks. Participants were randomly assigned to one of the two training groups: standardized training with TWE group and standardized training with W group.

The exercise class was individualized for each group and supervised by a physiotherapist. Each exercise class used a standardized format that included 20 minutes of moderate-intensity aerobic exercise, 20 minutes of progressive strength training, 10 minutes of flexibility and balance exercises, and 10 minutes of cool-down activities, followed by exercises known to decrease fall risk.[11] The aerobic exercise involved movement of the legs, trunk, and arms to involve all joints and major muscle groups, such as dance. Strength training involved progressive resistive exercises using an elastic band. A sequence of progressively more-difficult exercises was also performed to improve static and dynamic balance. Although exercises could be performed in a sitting position, the importance of performing them in a standing position to improve balance was stressed. Physiotherapists evaluated the participants twice during the study period to ensure adherence with exercise protocols during classes.

Trail-Walking Exercise

In the TWE training field, flags were set randomly at each of 15 positions in a 25-m^2 area (5 m × 5 m; Figure 1A). Participants in the TWE group were asked to sequentially pass from number 1 to 15 (Figure 1B). A circle with a 30-cm diameter was drawn on the ground around each flag, and the participant was required to step in the circle to pass the flag. The height of the flag was 30 cm. The tester gave the following instructions to participants, "Please move to number 15 as quickly and correctly as possible."

The 16-week program included a progressive aspect of the TWT. The participants were asked to pass sequentially from number 1 to 15 during Weeks 1 to 8 and then were asked to pass sequentially from number 15 to 1 during Weeks 9 to 16. Flag positions were changed for each day of training.

Walking Exercise

Participants in the W group were instructed to attend a supervised indoor walking session. These sessions were designed as controls for the additional physical activity in the TWE session. The participants were instructed to walk as comfortably and for as long as possible for up to 30 minutes. They were permitted to stop and take breaks during the session. The indoor walking course consisted of a 300-foot loop.

Falls

The primary outcome of this trial was the occurrence of falls, which were measured 6 and 12 months after trial completion. A fall was defined as an event that resulted in a

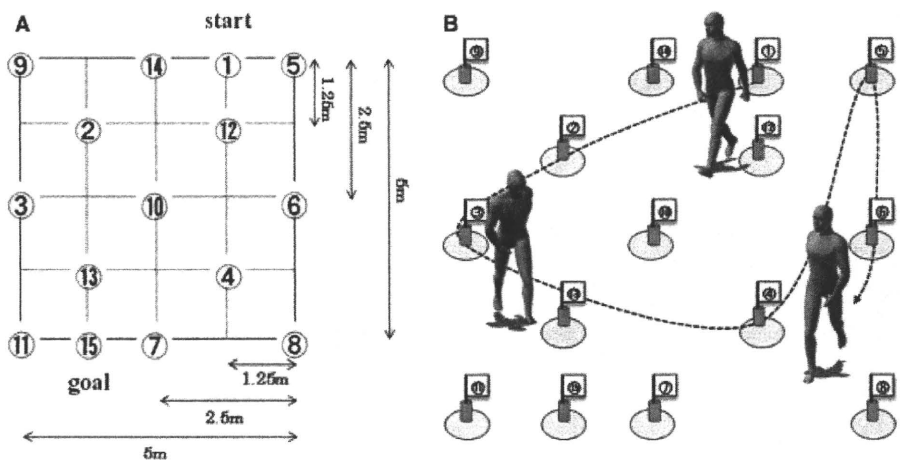

Figure 1. Schematic representation of the trail-walking test. (A) Participants are asked to pass sequentially from number 1 to 15. (B) Image scheme of a task excursion.

person unintentionally coming to rest on the ground, floor, or other lower level with or without loss of consciousness or injury.[12] Falls resulting from extraordinary environmental factors (e.g., traffic accidents or falls while riding a bicycle) were excluded. The participants were asked to record any falls in fall diaries that were mailed to the research assistants every month. If participants failed to send in the fall diaries, research assistants collected data on falls over the telephone. All participants who had fallen were interviewed during these calls using a structured questionnaire about the fall event and its consequences.

Secondary Outcome Measures

All participants underwent seven measurements: the Timed Up and Go (TUG) Test,[13] the functional reach (FR) test,[14] the one-leg stand (OLS) test, 10-m walking time under single-task conditions (ST walking),[15] 10-m walking time under DT conditions,[16] the TWT,[9] and the Trail-Making Test (TMT) Part A.[17] A physiotherapist blinded to group allocation administered these measures at baseline, upon completion of the 16-week intervention (16 weeks), and 6 and 12 months after completion of the intervention (6 months, 12 months). All baseline measures were completed before randomization. All pretest measures were completed before randomization. Before commencing the study, all staff members received training on correct protocols for administering all assessment measures included in the study from one of the authors (MY). If a walking aid was normally used at home, this aid was used during the TUG Test, ST walking, DT walking, and TWT.

In the TUG Test, participants were asked to stand up from a standard chair with a seat height of 40 cm, walk a distance of 3 m at a normal pace, turn, walk back to the chair, and sit down. The time recorded from two trials was averaged to obtain the TUG score.

In the FR test, each participant was positioned next to a wall with one arm raised at 90° and fingers extended. A meterstick was mounted on the wall at shoulder height. The distance that a participant could reach while extending forward from an initial upright posture to the maximal anterior leaning posture without moving or lifting the feet was visually measured in centimeters according to the position of the tip of the third finger against the mounted meterstick. The distances measured in two trials were averaged to obtain the FR score.

In the OLS test, participants were instructed to start from a standing position with a comfortable base as support with eyes open and arms at their sides. They were then instructed to stand unassisted on either leg. OLS was measured in seconds from the time one foot was lifted from the floor to when it touched the ground or the standing leg.

In ST walking, participants walked 15 m at a speed they felt comfortable with. A stopwatch was used to record the time required to reach the 10-m point that was marked in the middle of this walk. The time recorded in two trials was averaged as the ST walking score.

In DT walking, participants walked 15 m at a speed they felt comfortable with while counting backwards from 50 out loud as the DT. All participants were asked to walk and count simultaneously to the best of their ability without prioritizing either task. Possible counting mistakes were not corrected.[16]

The field for the TWT was the same as that used for the TWE. The test–retest reliability using the intraclass correlation coefficient was 0.945.[9] The positions in which the flags were placed are shown in Figure 1. The tester gave the following instruction to participants, "Please move to number 15 as quickly and as correctly as possible," and timed the trial using a stopwatch to the nearest 0.01 s following a standard procedure. The TWT was performed only once for each participant at each time point.

Executive function was assessed using the TMT, a well-established psychomotor test originally developed as part of the Army Individual Test Battery.[15] Part A of the TMT is a visual scanning task. The participant is required to draw lines sequentially connecting consecutively numbered circles (1–25) randomly arranged on a page as quickly as possible.

Statistical Analysis

Baseline characteristics of the TWE and W groups were compared to examine comparability of the two.

Differences in the physical function variables between the TWE and W groups were analyzed using the Student *t*-test or the chi-square test. The Kolmogorov-Smirnov test and the Mann-Whitney *U*-test were used to test the normality of distributions and differences in physical function variables between the groups, respectively.

The number of falls was calculated from the beginning of the study period to the participant's death, withdrawal from the trial, or the end of the 12-month follow-up period. Confidence intervals (CIs) for the fall rate were calculated assuming that the number of falls followed a negative binomial distribution.

Incidences of falls with their 95% CIs were calculated for participants in the TWE and W groups and compared using negative binomial regression analysis. Results were presented using incident rate ratios (IRRs) with their 95% CIs. The effect of exercise on outcome measurements was analyzed using mixed 2 × 4 (group (TWE and W groups) × time (pretraining, posttraining, after 6 months, and after 12 months)) analysis of variance. Post hoc Bonferroni tests were used to assess which group or time periods showed significant differences.

Data were entered and analyzed using the SPSS (Windows version 11.0, SPSS, Inc., Chicago, IL). $P<.05$ was considered statistically significant for all analyses.

RESULTS

Eighty-two people were screened, and 60 (73%) who met the inclusion criteria for the trial and agreed to participate were enrolled (Figure 2). Of individuals not meeting the inclusion criteria (n = 22), most were excluded because they had exercised regularly for 6 months before screening. Three people who may have been eligible for the study declined after a telephone screening. Of the 60 individuals who enrolled in the study, 58 (97%) completed the 12-month follow-up and returned for the exit interview and final tests: 29 in the TWE group (97%) and 29 in the W group (97%).

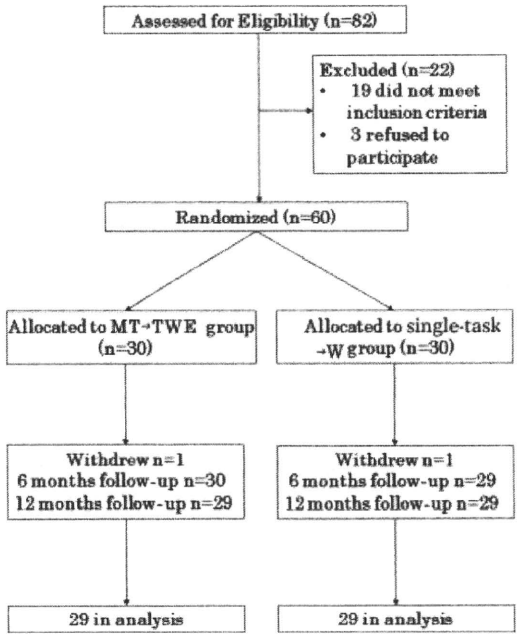

Figure 2. A flow chart showing the distribution of participants throughout the trial.

All 16 of the scheduled intervention session were completed. The median relative adherence was 100% (25th–75th percentile, 94–100%) for the TWE group and 100% (94%–100%) for the W group. No health problems, including cardiovascular or musculoskeletal complications, occurred during training sessions or testing. Minor problems observed in both groups included aching muscles after the first training sessions and fatigue. All problems were managed easily using adjustment of the intervention and improved during intervention.

Baseline Characteristics

Participants in the TWE and W groups were comparable and well matched with regard to their baseline characteristics. There were no significant differences between group means for age (TWE = 79.5 ± 6.2, W = 81.4 ± 4.9; P = .20), body weight (TWE = 52.3 ± 9.9 kg, W = 50.7 ± 7.1 kg; P = .47), height (TWE = 146.7 ± 7.3 cm, W = 146.6 ± 6.9 cm; P = .96), number of medications (TWE = 3.8 ± 2.8, W = 3.7 ± 2.9; P = .89), experiencing a fall within the past year (TWE = 37.9%, W = 34.5%; P = .50), or MMSE score (TWE = 27.8 ± 2.1 points, W = 28.0 ± 1.7 points; P = .79).

Effects of the Interventions on Fall Rate 6 and 12 Months After Trial Completion

Two participants (6.9%) in the TWE group and 10 (34.5%) in the W group had experienced a fall by 6 months after trial completion. During the 6-month follow-up period, the IRR of falls for the TWE group compared with the W group was 0.20 (95% CI = 0.04–0.91). Five participants (17.2%) in the TWE group and 11 (37.9%) in the W group had experienced a fall by 12 months after trial completion. During the 12-month follow-up period, the IRR of falls for the TWE group compared with the W group was 0.45 (95% CI = 0.16–1.77).

Effects of the Interventions on Secondary Outcome Measures 6 and 12 Months After Trial Completion

Participants in the TWE group had significantly greater improvements in secondary outcome measures including TWT, TUG, TMT, DT walking time, and DT walking step, although there were no significant group and time effects without the group × time interactions (Table 1). Post hoc Bonferroni tests showed significant differences in favor of the TWE group in several cases: TWT after the intervention and 6 months after the intervention, TMT after the intervention, and DT walking time 6 months after the intervention.

DISCUSSION

The TWE program is a multicomponent walking program that was designed to address multiple domains such as attention, short-term memory, and balance that, when impaired, have been shown to increase fall risk.[9] The multidirectional steps in the forward, backward, lateral, and oblique directions during TWE lead to better activation of synergistic and agonistic leg muscles. Therefore, it is possible that the TWE regimen improves many aspects of the functional fitness of the lower extremities.

The differences in fall rates between the TWE and W groups were significant 6 months after the intervention but not 12 months after the intervention. Balance, gait, and coordination have been practiced in many studies by performing isolated exercises. In contrast, in this trial, these physical tasks were practiced in an exercise environment that simulated complex situations. In this way, participants may also learn to recognize potentially hazardous situations and adopt strategies to minimize the risk of falling. The results of this trial show that the intervention resulted in functional changes related to locomotion skills under complex-task conditions. Several cognitive training programs have been shown to be effective at improving memory, reasoning, and speed of processing of older adults,[18,19] and previous work has shown that community-dwelling older adults living with a history of falling have significantly slower TMT times than nonfallers.[20] The training effect on TUG and DT walking, which are useful for evaluating the risk of falling,[13,16] were also better after the intervention in the TWE group. These results may indicate the importance of instructions when training under complex-task conditions and suggest that effectively improving these functions could lead to a decrease in fall rate. The maintenance periods of an improvement in physical function and that of fall prevention were the same 6 months after trial completion.

This trial has notable limitations. First, these findings should be seen as preliminary because of the small sample size. Second, the statistical analysis of each of the outcome measures, including the physical performance tests, cognitive tests, and fall rate, were performed separately, which increases the risk of false-positive findings. Third, participants in both groups were probably more motivated and showed greater interest in health issues and fall risk than the general population of older adults.

The results of this pilot RCT suggest that the TWE strategy was more effective in improving locomotion

Table 1. Fitness Items According to Group Before and After the Intervention

Item	Mean ± Standard Deviation				*F*-Value
	Before the Intervention	After the Intervention	6 Months After the Intervention	12 Months After the Intervention	1.Time Effect 2. Group × Time
Trail-walking test, s					
TWE group	66.9 ± 15.4	54.1 ± 9.9†	61.0 ± 10.1†	67.1 ± 14.5	17.93**
W group	67.8 ± 10.1	70.2 ± 11.7‡	69.4 ± 11.4‡	69.9 ± 10.9	12.06**
Trail-Making Test, n					
TWE group	81.2 ± 47.6	63.6 ± 29.6†	67.2 ± 32.0†	80.3 ± 46.8	12.67**
W group	83.6 ± 39.3	82.1 ± 3.59‡	82.6 ± 33.7	84.3 ± 39.2	8.40**
Timed Up and Go Test, seconds					
TWE group	12.3 ± 2.6	11.0 ± 2.0†	11.5 ± 2.0	11.6 ± 2.6	5.97**
W group	12.4 ± 2.7	12.1 ± 2.4	12.8 ± 2.9	13.0 ± 3.4	2.98*
Functional reach, cm					
TWE group	24.9 ± 6.4	22.2 ± 5.9	25.3 ± 6.7	24.6 ± 6.4	1.62
W group	23.7 ± 6.1	24.3 ± 6.5	24.4 ± 4.8	24.7 ± 4.1	1.77
One-leg stand, seconds					
TWE group	6.4 ± 5.5	5.4 ± 4.8	6.7 ± 6.3	5.9 ± 4.9	0.49
W group	5.4 ± 5.0	4.8 ± 2.3	4.6 ± 5.4	5.2 ± 5.7	0.49
10-m walking time, seconds					
TWE group	11.5 ± 2.6	11.4 ± 2.2	11.5 ± 2.8	11.2 ± 2.9	0.57
W group	11.4 ± 3.0	11.5 ± 2.7	12.0 ± 2.9	12.5 ± 3.6	1.53
10-m walking time, steps					
TWE group	22.3 ± 4.1	22.7 ± 32	22.5 ± 3.8	22.3 ± 3.7	0.52
W group	22.8 ± 5.1	22.1 ± 4.8	22.8 ± 4.5	23.2 ± 4.7	1.46
Dual-task 10-m walking time, seconds					
TWE group	17.4 ± 6.5	14.9 ± 3.4†	15.9 ± 5.3‡	16.1 ± 5.3	2.48
W group	16.4 ± 3.7	16.3 ± 4.6	18.6 ± 3.6	18.2 ± 4.6	2.85*
Dual-task10-m walking step, steps					
TWE group	23.2 ± 3.5	22.6 ± 3.2	22.5 ± 3.4	22.6 ± 3.5	1.05
W group	22.6 ± 4.8	23.4 ± 5.8	24.3 ± 3.6	24.6 ± 4.6	2.67*

† As calculated by comparing preintervention values.
‡ As calculated by group comparison.
$P<$**.01, *.05.
TWE = trail-walking exercise; W = walking.

and cognitive performance under complex-task conditions than walking only. In addition, participants who received individualized complex-task training combined with a traditional intervention demonstrated a lower incident rate of falls 6 months after trial completion. Further research is needed, but this preliminary result may lead to a new intervention for fall prevention.

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Author Contributions: All of the authors took significant role in every aspect of this study.

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Histone Modifiers, YY1 and p300, Regulate the Expression of Cartilage-specific Gene, Chondromodulin-I, in Mesenchymal Stem Cells*[S]

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Elucidating the regulatory mechanism for tissue-specific gene expression is key to understanding the differentiation process. The chondromodulin-I gene (*ChM-I*) is a cartilage-specific gene, the expression of which is regulated by the transcription factor, Sp3. The binding of Sp3 to the core-promoter region is regulated by the methylation status of the Sp3-binding motif as we reported previously. In this study, we have investigated the molecular mechanisms of the down-regulation of *ChM-I* expression in mesenchymal stem cells (MSCs) and normal mesenchymal tissues other than cartilage. The core-promoter region of cells in bone and peripheral nerve tissues was hypermethylated, whereas the methylation status in cells of other tissues including MSCs did not differ from that in cells of cartilage, suggesting the presence of inhibitory mechanisms other than DNA methylation. We found that a transcriptional repressor, YY1, negatively regulated the expression of *ChM-I* by recruiting histone deacetylase and thus inducing the deacetylation of associated histones. As for a positive regulator, we found that a transcriptional co-activator, p300, bound to the core-promoter region with Sp3, inducing the acetylation of histone. Inhibition of YY1 in combination with forced expression of p300 and Sp3 restored the expression of *ChM-I* in cells with a hypomethylated promoter region, but not in cells with hypermethylation. These results suggested that the expression of tissue-specific genes is regulated in two steps; reversible down-regulation by transcriptional repressor complex and tight down-regulation via DNA methylation.

The expression of cell lineage-specific genes is a key to initiating the differentiation of stem cells into a particular cell lineage, and the down-regulation of such genes in cells of other lineages is also critical to maintain a normal cellular physiology. The chondromodulin-I (*ChM-I*)[2] gene is a specific gene for cartilage tissue (1). We have found that the basal promoter activity of *ChM-I* is driven by a ubiquitous transcription factor, Sp3, and chondrocyte-specific expression is regulated by the methylation status of the Sp3-binding motif in the core-promoter region (2). Demethylation treatment *in vitro* restored the expression of *ChM-I* in cells of the osteogenic lineage (2, 3). A similar result was obtained with cells of the adipogenic lineage, in which the expression of an adipocyte-specific gene was restored in non-adipogenic cells by the elimination of methylated DNA in a regulatory region (4). Because DNA methylation is considered a tight epigenetic change under physiological conditions, it is a suitable mechanism for cells to inhibit the expression of unnecessary genes. It is, however, still to be investigated whether cells in tissues other than cartilage share the same inhibitory mechanism. It is also important to know how the expression of lineage-specific genes is down-regulated in tissue stem cells before differentiation is initiated. Mesenchymal stem cells (MSCs) in bone marrow are tissue stem cells, which can differentiate into multiple mesenchymal cell lineages including chondrogenic cells (5, 6). Because three-dimensional cultures supplemented with growth factors such as TGF-β can induce the chondrogenic differentiation of MSCs (6), there should be a mechanism other than DNA methylation to down-regulate the gene expression of *ChM-I* in undifferentiated MSCs. Modification of the histone tail is another mechanism regulating gene expression. The acetylation of histone H3 and H4 promotes gene expression, whereas deacetylation inhibits the expression (7). The dimethylation of histone H3 at lysine 9 (H3K9) in particular is correlated with DNA methylation and markedly inhibits gene expression (8, 9). These modifications of the histone tail and methylation status determine differentiation (10), and are regulated by several intrinsic histone modifiers including p300 and YY1 (11–13). p300 possesses intrinsic histone acetyltransferase (HAT) activity (11, 12). YY1 is a member of the polycomb group of transcription factors, which establish and maintain transcrip-

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[2] The abbreviations used are: ChM, chondromodulin; MSC, mesenchymal stem cell; HAT, histone acetyltransferase; HDAC, histone deacetylase; OND, oligonucleotides.

tional silencing by recruiting histone deacetylase (HDAC) (13, 14). These intrinsic factors regulate the epigenetic status and regulate gene expression.

Here we demonstrated that the down-regulation of *ChM-I* expression by DNA methylation is restricted in particular cell types, whereas other cells including MSCs are free from the methylation, and found that expression of the *ChM-I* gene in these cells is reversibly dependent on histone modifications, which are regulated by the net activity of intrinsic histone modifiers, YY1 and p300.

EXPERIMENTAL PROCEDURES

Tissue Specimens and Primary Cultured Cells—Mesenchymal (cartilage, bone, fat, muscle, ligament, and tendon) and non-mesenchymal tissues (nerve, artery, and skin) were obtained from the lower limb of a 56-year-old male who underwent above-knee amputation. The tissues were frozen by dry ice and kept at −80 °C until nucleic acid extraction. Human primary cultured chondrocytes (hPCs) was isolated from same patient and cultured as previously mentioned (15). MSCs were isolated from the iliac bone of healthy donor as described (16). Normal human osteoblasts (NHOSTs) and human primary pre-adipocytes (hPAs) were obtained from TaKaRa (TaKaRa Bio, Shiga, Japan). All the primary cells were maintained in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA), 100 units/ml penicillin, and 100 mg/ml streptomycin, in 5% CO_2 at 37 °C. The Ethics Committee of the Faculty of Medicine, Kyoto University, approved the procedure and informed consent was obtained.

Cell Lines and Culture Conditions—The human cell lines, Saos2, were obtained from American Type Culture Collection (ATCC; Manassas, VA). The human osteosarcoma cell lines TAKAO and ANOS were established in our laboratory (2). All the cell lines used in this study were maintained in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Thermo Fisher Scientific Inc.), 100 units/ml penicillin, and 100 mg/ml streptomycin, in 5% CO_2 at 37 °C.

Antibodies and Expression Vectors—The following antibodies were used; anti-YY1 (sc-7341, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p300 (05–257, Millipore Corp, Billerica, MA), anti-Sp3 (sc-644, Santa Cruz Biotechnology), anti-acetylated H3K9 (06–942, Millipore Corp), anti-dimethylated H3K9 (07–212, Millipore Corp), anti-pan H3 (07–690, Millipore Corp), and anti-HDAC2 (51–5100, Zymed Laboratory Inc., San Francisco, CA). Expression vectors for YY1 (pCEP-YY1) and p300 (pcDNA3-p300) were kindly provided by Drs. E. Seto and K. Miyazono, respectively. The Sp3 expression vector (pCMV-Sp3) was previously described elsewhere (17).

Reverse Transcription (RT)-PCR and Quantitative RT-PCR—RNA was isolated using the Rneasy kit (Qiagen KK, Tokyo, Japan) from frozen 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 (Invitrogen, Carlsbad, CA). The relative amount of *ChM-I* mRNA was assessed by TaqMan real-time PCR with the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) (2). A75-bp fragment from +411 (exon 4) to +485 (exon 5) of the *ChM-I* cDNA (GenBank™ accession number XM_007132) was amplified using specific primers (sense, 5′-GAAGGCTCGTATTCCTGAGGTG-3′; antisense, 5′-TGGCATGATCTTGCCTTCCAGT-3′) and labeled with a TaqMan probe (5′-FAM-CGTGACCAAACAGAGCATCTC-CTCCA-3′-TAMRA). 18 S rRNA was used as the internal control, and all reactions were run in duplicate. The ratio of *ChM-I*/18 S in each sample was calculated, and the expression level of *ChM-I* genes was demonstrated as a relative value using the *ChM-I*/18 S ratio in human articular cartilage as a standard (1.0) (2).

Drug Treatment—Cells (1×10^5) were seeded on 60-mm dishes in DMEM with 10% FBS. After they had attached to the dish, the cells were treated with either 5-*aza*-2′-deoxycytidine (5-*aza*-dC; Sigma-Aldrich) (1 μM) for 96 h or MS-275 (Nihon Scherring K.K., Chiba, Japan) (1 μM) for 24 h.

Bisulfite Genomic Sequencing—The bisulfite modification of DNA samples was performed using the EpiTect bisulfite kit (Qiagen). DNA (1 μg) was digested by BamHI for 12 h and subjected to sodium bisulfite treatment. Bisulfite-modified DNA-spanning residues −297 to −104 relative to the transcription start point (2) was amplified, cloned into the TA-vector (Invitrogen), and sequenced using an ABI 377 semiautomatic sequencer (Applied Biosystems).

Electrophoresis Mobility Shift Assay (EMSA)—Double-stranded DNA fragments corresponding to the sequence from −357 to −333 and from −86 to −44 were synthesized by annealing two single-stranded oligonucleotides (OND) (5′-CTTCACCTTCCATGAGCCATCTTC-3′ and 5′-GGGGGA-AGATGGCTCATGGAAGGT-3′; 5′-GGGCATCCGGGAG-TGCAGGACGAGCTTCCCGCGGCGGGA-3′; and 5′-TCT-CTCCCGCCGCGGGAAGCTCGTCCTGCACTCCCGGAT-3′, respectively) and filling in by DNA polymerase I (TOYOBO, Osaka, Japan). These fragments were designated GR3 and GR4 (Fig. 2*B*). For the formation of the complex, 5 μg of nuclear extract from cell lysate was incubated with ^{32}P end-labeled ONDs for 20 min at room temperature. The mixtures were electrophoresed in 5% polyacrylamide gel in 0.5× Tris borate EDTA at 45 volts for 3 h, and the gel then was dried and autoradiographed. For the competition assay, the OND-protein complex was produced in the same way in the presence of given amounts of non-labeled OND. In the supershift assay, nuclear extracts were incubated with 1 μg of anti-YY1 and anti-p300 antibody for 1 h on ice before being mixed with labeled DNA.

Luciferase Assay—The 533-bp fragment from −446 to +86 and 383-bp fragment from −296 to +86 relative to the transcription initiation site of the *ChM-I* gene was amplified by PCR, and cloned into a TA-vector using the TOPO cloning kit (Invitrogen). These fragments were subcloned into the luciferase reporter plasmid, PGV-B (Toyo Ink, Tokyo, Japan), yielding PGV-B-f1 and PGV-B-f1-del. Two tandem binding motifs of YY1 (*CC*AT) was mutated to (*TT*AT) by PCR, cloned into PGV-B, and designated PGV-B-f1-mt. One microgram of each reporter plasmid was co-transfected with 1 μg of pCEP-YY1 or pCMV-p300. Transfection efficiency was standardized by the co-transfection of 1 ng of pRL-TK control vector (Toyo Ink). Cells were harvested 24 h after transfection, and luciferase assays were performed with the

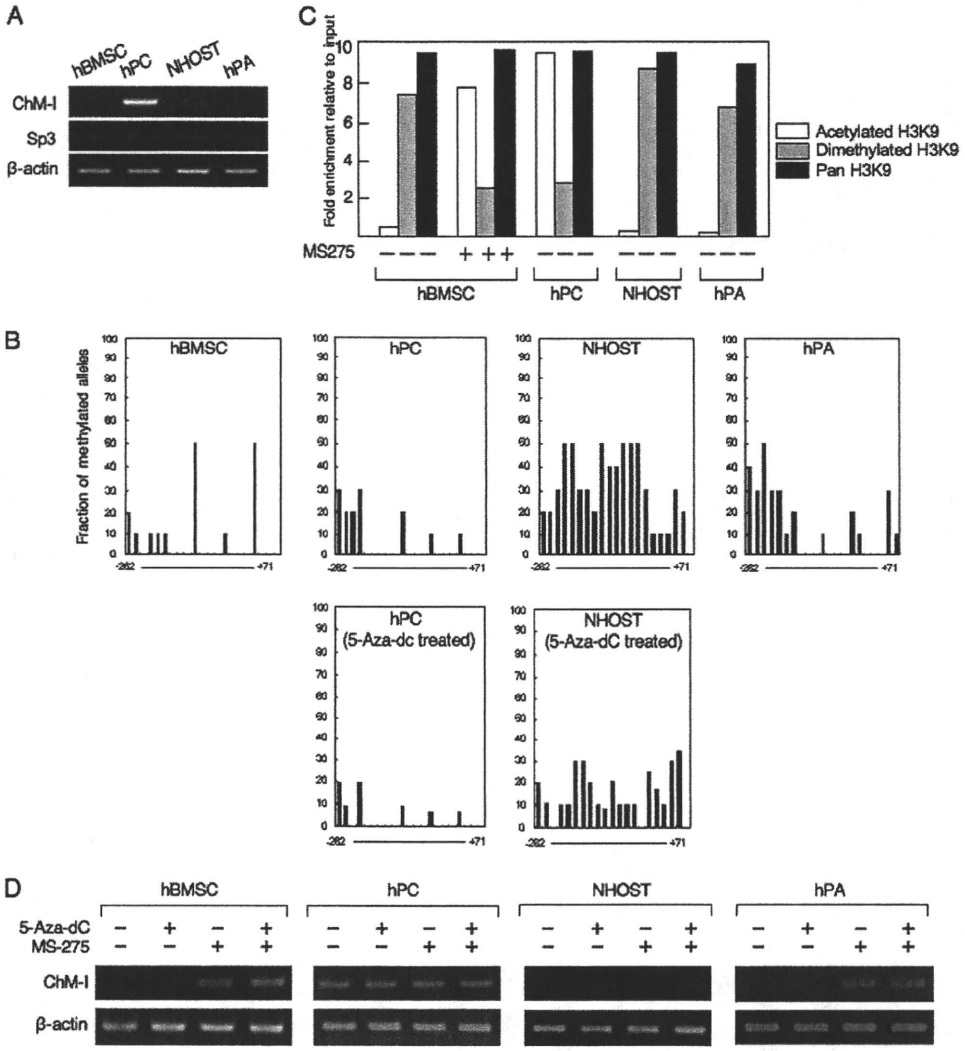

FIGURE 1. **DNA methylation and histone deacetylation down-regulate the expression of *ChM-I* in a cell type-specific manner.** *A*, expression of the *ChM-I* and *Sp3* genes in primary cultured mesenchymal cells. *B*, methylation status of the core-promoter region of *ChM-I*. The methylation of each CpG site was analyzed in 10 alleles by bisulfite genomic sequencing. The *y*-axis indicates the fraction of methylated alleles and the *x*-axis indicates the position of each CpG site relative to the transcription start site. Methylation status in hPC and NHOST treated with a demethylating reagent (5-*aza*-dC, 1 μM for 96 h) were also shown. *C*, ChIP-qPCR assay for the modification of histones in primary cultured mesenchymal cells. *Open box*, acetylated H3K9; *closed box*, dimethylated H3K9; *gray box*, Pan H3. Histone modification in hBMSC treated with an HDAC inhibitor (MS-275, 1 μM for 24 h) were also shown. The *y*-axis represents fold enrichment relative to input. *D*, expression of *ChM-I* after treatment with 5-*aza*-dC and/or MS-275.

PicaGene Dual SeaPansy system (Toyo Ink). Firefly-luciferase activity and SeaPansy-luciferase activity were measured as relative light units with a luminometer (STRATEC Biomedical Systems, Birkenfeld, Deutschland). The fold increase was calculated based on empty vector activity. Each experiment was performed in triplicate.

Chromatin Immunoprecipitation-Quantitative Polymerase Chain Reaction—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 (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 200–1,000 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 (Millipore Corp) 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 $NaHCO_3$). 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. The DNA pool from ChIP, input control and negative control was used for quantitative PCR. PCR amplification was performed on an ABI 7700 real-time PCR (Applied Biosystems). PCR amplification was performed using primers specific for the *ChM-I* regulatory region (sense, 5′-GAATGCAGGCCAGTGAGAAGGT-3′;1 antisense, 5′-GCACCCTGGGATCTGTCCCGCT-3′, Fig. 2*B*). The PCR conditions were an initial step of 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 10 s at 64 °C and 60 s at 72 °C. Primers were designed according to the selected genes for evaluating ChIP. To generate a standard curve for each amplicon, threshold cycle (CT) values of serially diluted input DNA, which were extracted in the ChIP experiment, were determined. The status of histone modification and binding of HDAC2, p300, YY1, and Sp3 changes were determined using the 2(-Delta Delta C(T)) method (18). They were demonstrated as a relative value using the enrichment of IP DNA/input DNA. A melting curve analysis was performed for each reaction to ensure a single peak. Each experiment was performed in triplicate, with the values averaged to obtain 1 datum per sample.

siRNAs—Luciferase siRNA duplex (GL2RN1, Dharmacon) was used as a negative control. 40 μM siRNA for YY1 (GeneSolution siRNA; Hs-YY1–5, Qiagen), p300 (p300 Pub. siRNA, Duplex1, Qiagen), and Sp3 (previously described in (2)) were transfected by Lipofection LTX (Invitrogen). RNA was prepared 48 h after transfection and used for the RT-PCR.

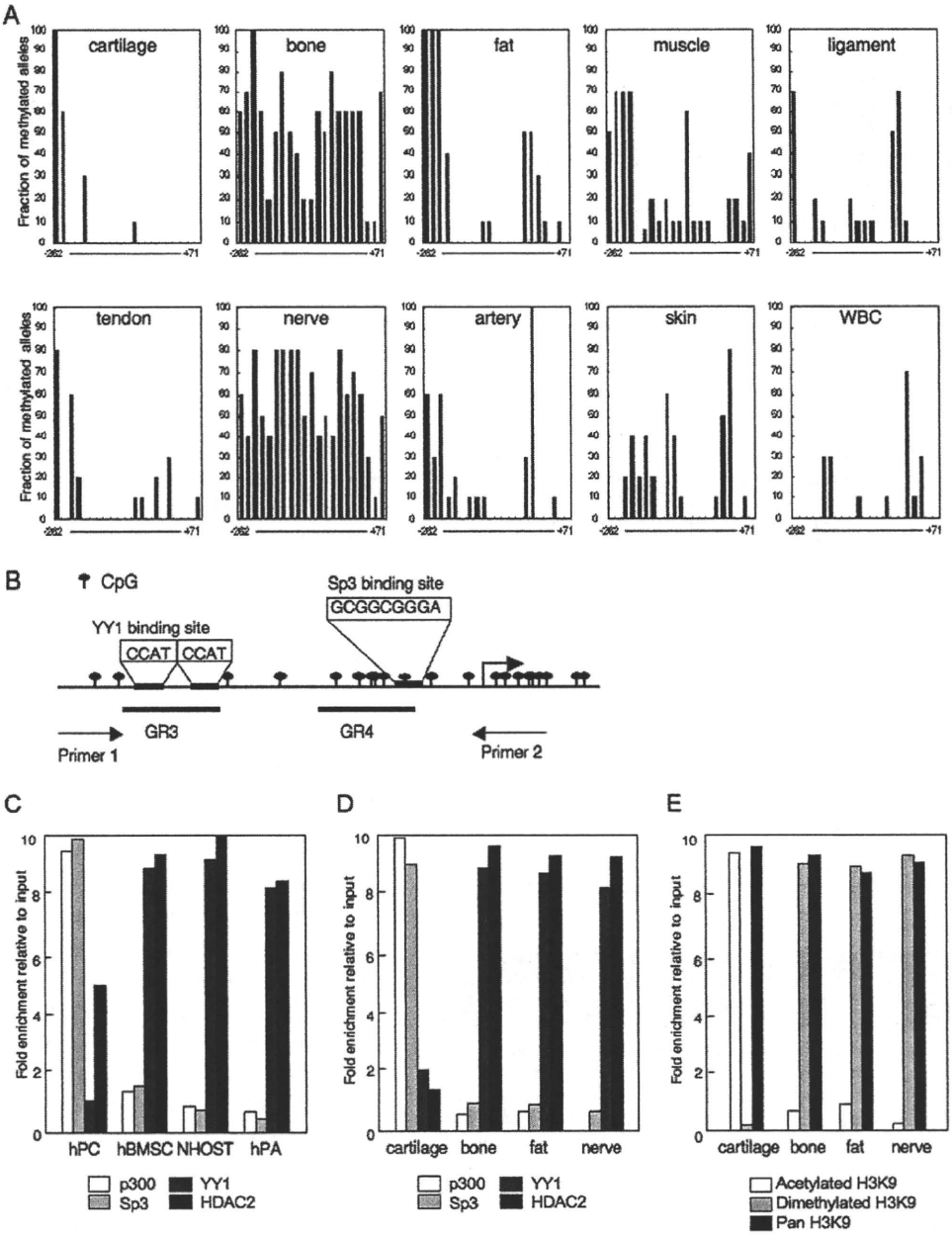

FIGURE 2. **Binding of YY1 and p300 determined the modification of H3K9 and the mRNA expression of *ChM-I* in mesenchymal tissues.** *A*, methylation status of the core-promoter region of the *ChM-I*. DNA extracted from normal tissue was analyzed by bisufite genomic sequencing. *B*, genomic structure of the core-promoter region of *ChM-I*. CpG sites from −262 to +71 were marked as indicated, and the transcription start site is indicated by an *arrow*. Two overlapping YY1-binding motifs (−344 to −347 and −342 to −346), and an Sp3-binding motifs (−56 to −48) are also indicated. A DNA fragment for the ChIP assay was amplified by primer 1 (−446 to −425) and primer 2 (+70 to +91). GR3 (−357 to −337) and GR4 (−86 to −44) were OND probes used in the EMSA for YY1 and p300, respectively. ChIP-qPCR assay for the binding of transcriptional regulators in primary cultured cells (*C*) and cells of normal tissues (*D*). The *y*-axis represents fold enrichment relative to input. *E*, ChIP-qPCR assay for the modification of histones in normal tissues.

RESULTS

DNA Methylation and Histone Deacetylation Down-regulate the Expression of ChM-I in a Cell Type-specific Manner—The expression of *ChM-I* was analyzed by RT-PCR in primary-cultured mesenchymal cells (hMSCs, hPCs, NHOSTs, and hPAs), among which only hPCs expressed the gene (Fig. 1*A*). We have previously shown the expression of *ChM-I* to be induced by the binding of Sp3, which was regulated by the methylation status of the binding motif in the core-promoter region (2). Expression levels of the *Sp3* gene did not differ among the four types of cells (Fig. 1*A*). The core-promoter region of *ChM-I* was hypomethylated in hPCs and hypermethylated in NHOSTs (Fig. 1*B*), which was consistent with the positive and negative expression of *ChM-I* in each cell. The methylation status of the core-promoter region of hMSCs or hPAs, however, was not significantly different from that of hPCs in spite that the expression of *ChM-I* was not detected in these cells (Fig. 1*B*). We have also shown that the acetylation of histone H3 at lysine 9 (H3K9) is necessary to induce the binding of Sp3 to the core-promoter region of *ChM-I*. ChIP analyses showed that H3K9 associated with the core-promoter region was acetylated in hPCs, but dimethylated in hMSCs, NHOSTs, and hPAs (Fig. 1*C*). Treatment with a demethylation reagent (5-*aza*-dC) induced the expression of *ChM-I* in NHOSTs (Fig. 1*D*), which was associated with demethylatiion in the promoter region of the *ChM-I* gene (Fig. 1*B*, *lower panel*). 5-*aza*-dC treatment, however, showed no effects in hMSCs or hPAs (Fig. 1*D*). On the other hand, treatment with a HDAC inhibitor (MS-275) induced the expression of *ChM-I* gene in hMSCs and hPAs, but not in NHOSTs (Fig. 1*D*). The induction of ChM-1 gene expression in hMSC was associated with the acetylation of H3K9 (Fig. 1*C*). These results suggested two mechanisms for the down-regulation of *ChM-I* expression in primary-cultured cells; methylation of the core-promoter region as found in NHOSTs, and histone deacetylation and methylation without DNA methylation as found in hMSCs and hPAs.

The Binding of YY1 and p300 Correlates to the Expression of ChM-I in Normal Mesenchymal Tissues—Among the normal mesenchymal tissues examined, the expression of *ChM-I* was observed only in cartilage (supplemental Fig. S1). The methylation status of the core-promoter region, however, differed significantly among tissues (Fig. 2*A*). DNA extracted from cells in cartilage and fat tissues showed a hypomethylated state in the core-promoter region, which was similar to those found in hPCs and hPAs (Fig. 1*B*). DNA extracted from cells in bone and nerve tissues showed

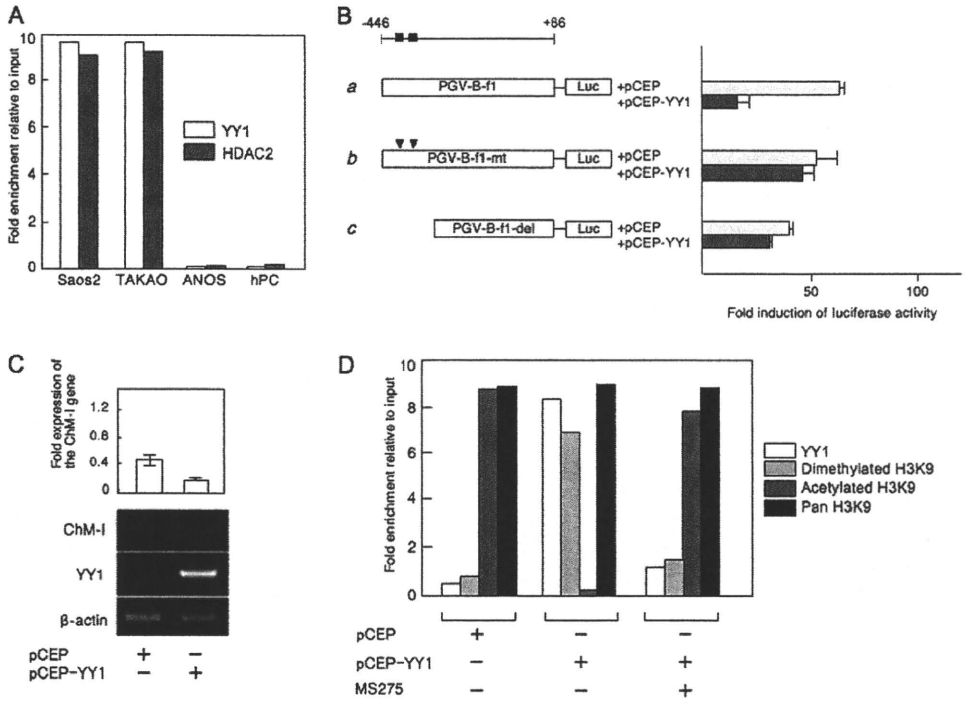

FIGURE 3. **YY1 bound to the regulatory region of *ChM-I* and decreased the promoter activity of *ChM-I*.** *A*, ChIP-qPCR assay for YY1 and HDAC2. *B*, luciferase reporter assay. *a*, DNA fragment encompassing −446 to +86 was cloned into a reporter vector containing the luciferase gene (*PGV-B-f1*). The *black box* indicates the location of the consensus sequence for the YY1-binding motif. *b*, PGV-B-f1-mt contains mutations in the YY1-binding motif (*arrowhead*), and PGV-B-f1-del lacked the YY1-binding motifs (*c*). Each reporter vector was co-transfected with empty vector (*pCEP*) or the YY1 expression vector (*pCEP-YY1*) into ANOS. The fold-increase was calculated based on empty vector activity. *C*, expression of endogenous *ChM-I* in hPCs transfected with the YY1 expression vector. The expression of *ChM-I* was semi-quantified taking the value for endogenous expression as 1.0 and is demonstrated at the *top*. *D*, ChIP-qPCR assay of hPCs transfected with the YY1 expression vector with or without MS275 treatment (1 μM for 24 h). Forced expression of YY1 in hPCs changed the modification of the H3 tail from acetylation to dimethylation.

that the core-promoter region was hypermethylated, which was similar to those that found in NHOSTs (Fig. 1*B*). In other tissues, the core-promoter region was hypomethylated. These results further suggested a mechanism other than DNA methylation to down-regulate the expression of *ChM-I*.

A search for factors regulating the chromatin structure revealed two tandem repeats (−344 to −347 and −342 to −346) of the binding motif of YY-1, which represses gene expression by recruiting HDAC to target regions (13, 14). As for factors with HAT activity, we focused on p300, which is known to relieve the transcriptional repression by YY1 (19). p300 also plays a role as a transcriptional adaptor recruiting transcription factors such as Sp3 (20). ChIP-qPCR assay showed that YY1 and HDAC2 bound to the core-promoter region in ChM-I-negative hMSCs, NHOSTs, and hPAs, whereas p300 and Sp3 bound in ChM-I-positive hPC (Fig. 2*C*). Consistent with the results obtained with primary-cultured cells, p300 and Sp3 bound to the core-promoter region in cells of cartilage, but not bone, fat or nerve tissue (Fig. 2*D*). On the other hand, the binding of YY1 and HDAC2 was observed in cells of bone, fat, and nerve, but not cartilage (Fig. 2*D*). ChIP-qPCR assay of the histone tail associated with the core-promoter region demonstrated that H3K9 was acetylated in cells of cartilage tissue, and dimethylated in those of bone, fat, and nerve tissues (Fig. 2*E*), which corresponded with the expression of *ChM-I* in each tissue. These results indicated that the expression of *ChM-I* correlated positively with the binding of p300 and negatively with that of YY1.

YY1 Binds to the Core Promoter Region and Inhibits Transcription—To further analyze the involvement of YY1 and p300 in the regulation of *ChM-I*, we used an osteosarcoma cell line, ANOS, as a ChM-I positive cell line, which we have previously investigated. The core-promoter region of the gene was hypomethylated in ANOS cells (2). As ChM-I-negative cell lines, two other osteosarcoma cell lines, Saos2 and TAKAO, were used, in which the core-promoter region was hypermethylated (2).

ChIP-qPCR assays showed that YY1and HDAC2 bound to the regulatory region of Saos2 and TAKAO cells, but not ANOS cells and hPCs (Fig. 3*A*). The binding of YY1 was further confirmed by an EMSA using an oligonucleotide (OND) (GR3) containing putative YY1-binding motifs (−342 to −347) (supplemental Fig. S2*A*). A shifted band was detected in the protein-OND complex from Saos2 cells, which disappeared on competition with unlabeled OND (supplemental Fig. S2*A*, *left panel*). The shifted band was detected also in the protein-OND complex from TAKAO cells, but not in ChM-I-positive cells (ANOS cells and hPCs) (supplemental Fig. S2*A*, *middle panel*). The shifted band was further shifted by the pretreatment with anti-YY1 antibody (supplemental Fig. S2*A*, *right panel*). These results indicated that YY1 bound to the core-promoter region of *ChM-I* in ChM-I-negative cells. To analyze the functional involvement of YY1, a reporter assay using the promoter fragment (−446 to +86) was performed (Fig. 3*B*), which contained the basal transcriptional activity of *ChM-I* (2). When the YY1 expression vector was co-transfected with the reporter plasmid, the promoter activity was significantly inhibited in ANOS cells (Fig. 3*B*, *a*). This inhibitory effect of YY1 was not observed when the reporter vector was replaced with one containing mutations in the YY1 motif (Fig. 3*B*, *b*) or lacking the motif (Fig. 3*B*, *c*). Forced expression of YY1 inhibited the expression of the endogenous *ChM-I* gene in hPCs (Fig. 3*C*), which was associated with the deacetylation and dimethylation of H3 (Fig. 3*D*). Co-treatment with MS275 inhibited the effect of forced expression of YY-1, rescuing the acetylation of H3K9 (Fig. 3*D*). These results suggested that YY1 inhibits the transcriptional activity of *ChM-I* by binding to a putative binding motif in the core-promoter region.

p300 Binds to the Core Promoter Region and Enhances Transcription—ChIP-qPCR assays showed that p300 as well as Sp3 bound to the core-promoter region of *ChM-I* in ANOS cells and hPCs, but not Saos2 and TAKAO cells (Fig. 4*A*). H3K9 associated with this region was acetylated in ANOS cells and

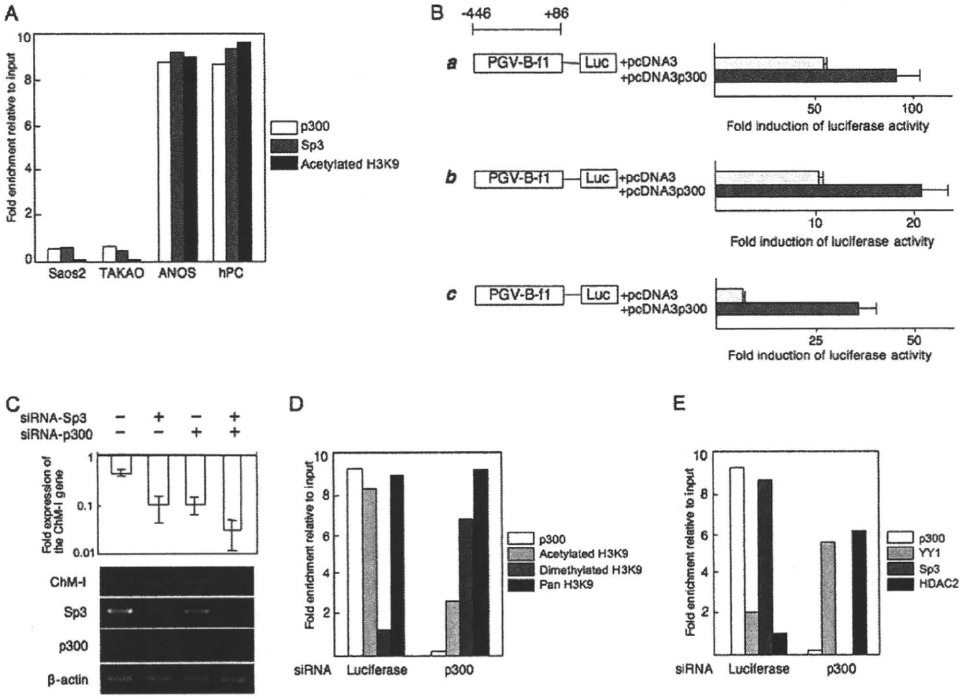

FIGURE 4. **p300 binds to the core promoter region and enhances transcription.** *A*, ChIP-qPCR assay of p300, Sp3 and acetylated H3. *B*, luciferase reporter assay. The reporter construct was described in the legend for Fig. 3*B*, and co-transfected with empty vector (pcDNA3) or the p300 expression vector (pcDNA3-p300) into ANOS (*a*), Saos2 (*b*), or TAKAO (*c*). The fold-increase was calculated based on empty vector activity. *C*, down-regulation of ChM-I gene expression by siRNA for Sp3 and/or p300. siRNAs for Sp3 and/or p300 were transfected into ANOS, and the expression of ChM-I, Sp3, and p300 was analyzed by RT-PCR. The expression of *ChM-I* was semi-quantified taking the value for endogenous expression as 1.0 and is demonstrated at the *top*. *D*, ChIP-qPCR assay for the modification of H3K9 (*D*) and for the binding of transcription regulators (*E*): cross-linked DNA-protein complexes were prepared from ANOS treated with or without siRNA for p300 and used for ChIP-qPCR assay.

hPCs, but not Saos2 or TAKAO cells (Fig. 4*A*). The binding of p300 was further confirmed by an EMSA using an OND (GR4)-containing putative p300 and Sp3-binding motifs (−56 to −48) (supplemental Fig. S2*B*). A shifted band was observed in extracts from ANOS cells, but not Saos2 or TAKAO cells (supplemental Fig. S2*B*, *middle panel*). The specificity of the band was confirmed by addition of a cold OND (supplemental Fig. S2*B*, *left panel*). The shifted band was supershifted when cell extracts were pretreated with anti-Sp3 antibody, and the same band disappeared when cell extracts were pretreated with anti-p300 antibody (supplemental Fig. S2*B*, *right panel*), suggesting that the protein-OND complex contained both Sp3 and p300. The functional involvement of p300 was analyzed with a promoter assay. Promoter activity was increased by co-transfection of the p300 expression vector in both ChM-I-positive (ANOS, Fig. 4*B*, *a*) and negative (Saos2, Fig. 4*B*, *b*; TAKAO, Fig. 4*B*, *c*) cells. Inhibition of p300 or Sp3 expression by the siRNA for each gene (siRNA-p300 and siRNA-Sp3) reduced the expression of *ChM-I* in hPCs, while the two siRNAs combined had an additive effect (Fig. 4*C*). ChIP-qPCR assays showed that the siRNA for p300 changed H3K9 from an acetylated to dimethylated form (Fig. 4*D*). These results suggest that p300 positively regulates the transcriptional activity of *ChM-I* by inducing the acetylation of H3K9 associated with this region.

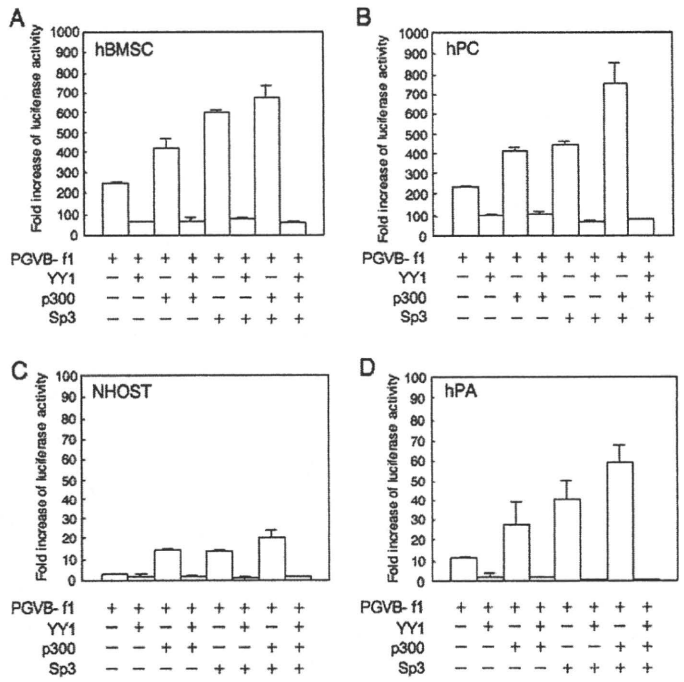

FIGURE 5. **Promoter activity of *ChM-I* was promoted by Sp3 and p300, but completely inhibited by YY1 in primary cultured cells.** The luciferase reporter vector containing the core-promoter fragment of the *ChM-I* gene (*PGV-B-f1*) was co-transfected with YY1, p300 and/or Sp3 expression vectors into hMSCs (*A*), hPCs (*B*), NHOSTs (*C*), and hPAs (*D*). The fold-increase was calculated based on empty vector activity.

Involvement of YY1 and p300 in Primary Mesenchymal Cells—The effect of YY1 and p300 on promoter activity was further analyzed in primary-cultured mesenchymal cells (Fig. 5). Surprisingly, the transcriptional activity of the basal promoter fragment in hMSCs (Fig. 5*B*) was as strong as those in hPCs (Fig. 5*A*), although the expression of endogenous *ChM-I* was weak in hMSCs. In contrast, the basal activity level was low in NHOSTs and hPAs (Fig. 5, *C* and *D*). When the YY1 expression vector was co-transfected with the reporter vector, promoter activity was significantly inhibited in all strains (Fig. 5, *A*–*D*). Co-transfection with the p300 or Sp3 expression vector enhanced the activity, and simultaneous transfection of the two vectors increased it in an additive manner (Fig. 5, *A*–*D*). The enhancement of *ChM-I* expression by the p300 and/or Sp3 expression vectors was completely inhibited by the co-transfection of YY1 in all strains (Fig. 5, *A*–*D*). These results confirmed that YY1 and p300 are involved in the regulation of *ChM-I* transcription in mesenchymal cells, and that because this luciferase reporter system has no relationship with histone modifications, YY1

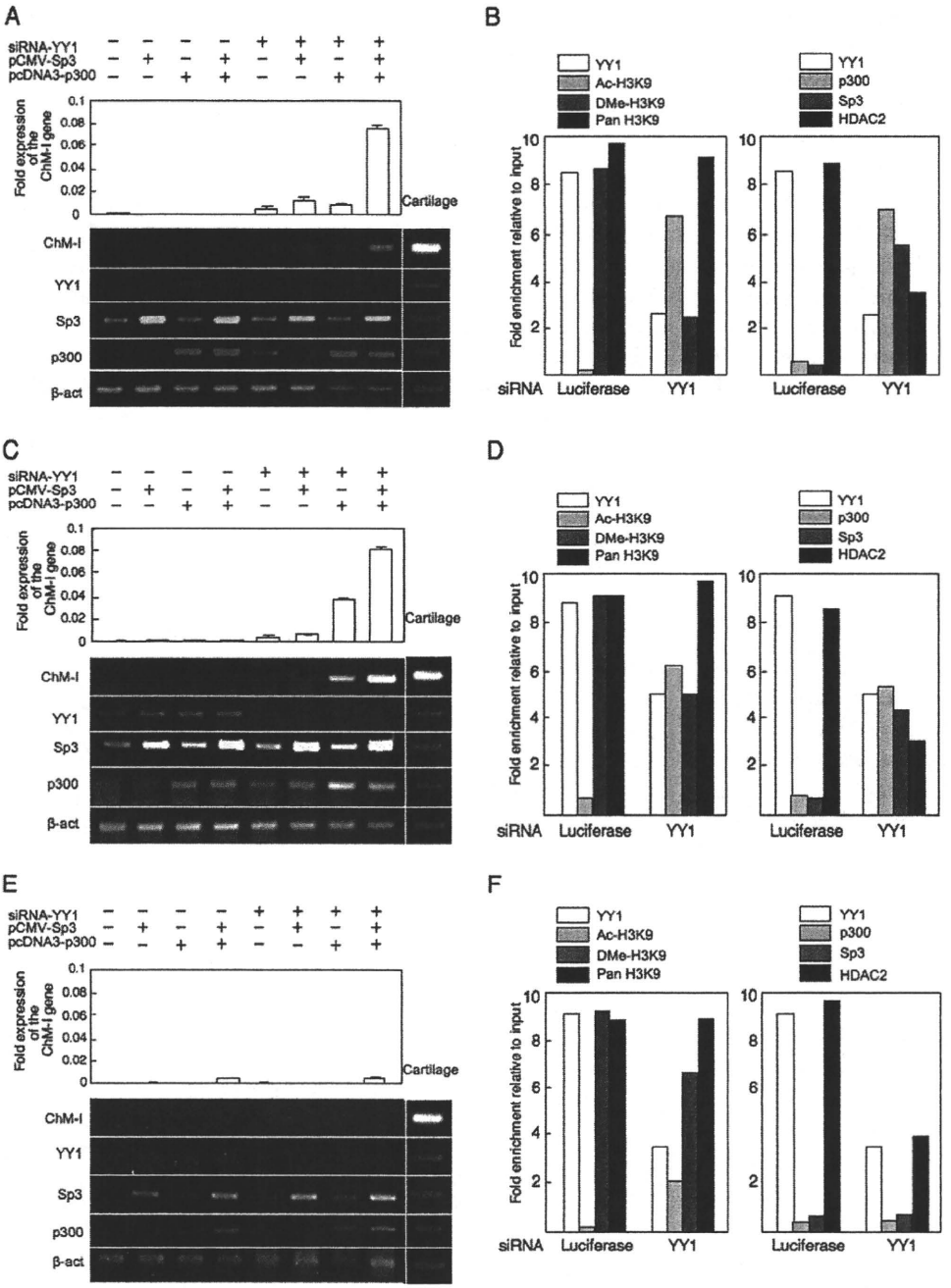

FIGURE 6. **Induction of *ChM-I* expression in ChM-I-negative primary cultured cells by modification of regulators.** *A–C*, primary-cultured cells were transfected with a combination of the siRNA for YY1, p300 expression vector, and Sp3 expression vector, and mRNA level of *ChM-1*, *YY1*, *p300*, and *Sp3* gene were analyzed by semi-quantitative RT-PCR (*lower panel*). The expression of the *ChM-I* was further analyzed by quantitative RT-PCR and digitalized (*upper panel*). *A*, hMSCs; *C*, hPAs; *E*, NHOSTs. ChIP-qPCR assay: *B*, hMSCs; *D*, hPAs; *F*, NHOSTs. Cross-linked DNA-protein complexes were prepared from primary cultured cells treated with or without siRNA for YY1 and used for ChIP-qPCR assay for the modificationf H3K9 (*left panels*) and for the binding of transcription regulators (*right panels*).

may directly inhibit the function of p300 or p300/Sp3 complex in addition to the modification of chromatin structure.

Cell-specific Effects of YY1 Inhibition on the Expression of ChM-I—Finally, the effect of YY1 expression on endogenous ChM-I expression was evaluated using siRNA for the gene (siRNA-YY1). In hMSCs (Fig. 6*A*), the inhibition of YY1 expression slightly induced the expression of *ChM-I* gene. The introduction of the p300 and/or Sp3 expression vectors had little effect on the expression of *ChM-I*, but significantly up-regulated it when combined with siRNA-YY1. Similar results were obtained in hPAs (Fig. 6*C*). In both cell types, siRNA-YY1 treatment induced the acetylation of H3K9 along with reductions in the dimetylation of H3K9 (Fig. 6, *B* and *D*, *left panels*). At the same time, siRNA-YY1 treatment dissociated HDAC2 and recruited p300 and Sp3 in the core-promoter region of *ChM-I* (Fig. 6, *B* and *D*, *right panels*). In NHOSTs (Fig. 6*E*), however, the combination the inhibition of YY1 and over-expression of p300 and Sp3 showed little effect for the induction of *ChM-I* expression. siRNA-YY1 treatment failed to induce the acetylation of H3K9 (Fig. 6*F*, *left panel*). Interestingly, although p300 was successfully recruited to the promoter region, no binding of Sp3 was observed in NHOSTs (Fig. 6*F*, *right panel*). These results suggested that Sp3 and p300 independently bind to the promoter region, and the binding of Sp3 was inhibited by the methylation of target DNA, but that of p300 was not. Therefore, the expression of a cartilage-specific gene, *ChM-I*, can be induced in some types of mesenchymal cells including MSCs by the modification of repressors (YY1) and activators (p300), but not in other cells, in which the expression is irreversibly inhibited by DNA methylation.

DISCUSSION

Numerous studies support the importance of epigenetic status for the regulation of differentiation, based on experiments involving chemical modifications of genome and the winding protein histone (21, 22). DNA methylation at CpG dinucleotides is a major epigenetic modification of the genome and associated with gene silencing (23). Because no intrinsic DNA-demethylating enzyme has been found, the inhibition by DNA methylation is tight under physiological conditions. Genomic DNA of embryonic stem (ES) cells is hypomethylated, and the total amount of methylated DNA increases with development (22, 24). Thus DNA methylation is a key mechanism to regulate and maintain the expression of cell type-specific genes. Unexpectedly, however, the methylation in the core-promoter region played a role in inhibiting the expression of *ChM-I* only

in particular types of mesenchymal cells; cells in bone (mainly osteocytes and osteoblasts) and peripheral nerve (mainly Schwann cells and perineural cells) (Fig. 2*A*). The hypomethylated status in hMSCs is reasonable considering the potential of these cells to differentiate. However, the core-promoter in terminally differentiated cells of a remote cell-lineage such as white blood cells was free from methylation and thus in a reversible state for gene expression (Fig. 2*A*). At present, we have no data to explain why some types of cells use DNA methylation to inhibit the expression of *ChM-I* and others do not. Cells of chondrogenic and osteogenic lineages are closely related, and may share a considerable proportion of transcriptional machinery. Therefore DNA methylation might be required to inhibit the expression of genes specific to chondro- or osteogenic lineages. The reporter assay gave almost identical results in hMSCs as in hPCs (Fig. 5, *A* and *B*), indicating that transcriptional machinery n hMSC to be ready to induce the expression of *ChM-I*, although there is no endogenous expression of the gene. This result strongly suggested that epigenetic machinery regulated the lineage-specific gene expression in stem cells.

The epigenetic status of each cell had been considered static, but recent a study demonstrated a dynamic nature to these modifications (25). We and others gave examples in which modification of the histone code induced a change of DNA methylation (3, 26). Notably, the modification of H3K9 strongly correlated with DNA methylation; dimethylated H3K9 correlated with hypomethylation and deacetylated H3K9 correlated with hypermethylation (9, 27). The recent discovery that the forced expression of transcription factors can reverse the epigenetic status of differentiated to that of ES cells is an extreme example of the dynamic nature of epigenetic status (28, 29). We showed that the expression of *ChM-I* was down-regulated by histone modifications in stem cells and some types of differentiated cells. The epigenetic status is induced and maintained by a number of intrinsic histone modifiers (30). In this study, we found that YY1 and p300 are main modifiers of histone associated with the core-promoter region of the ChM-I gene. YY1 is a DNA-binding zinc finger transcription factor, which has dual functions as an activator and a repressor (14). YY1 inhibits the transcription of target genes by competing for DNA-binding sites with activators, binding directly to activators, or recruiting co-repressors (14). One of these co-repressors is HDAC2, which was first identified as a binding partner of YY1 (13, 14). Previously, we demonstrated that HDAC2 bound to a histone tail associated with the core-promoter region of *ChM-I* in ChM-I-negative cells (3). In the present study, we showed that forced expression of YY1 deacetylated H3 associated with the core-promoter region in *ChM-I* (Fig. 3*E*), whereas HDAC2 was dissociated by the inhibition of YY1, causing acetylation of H3 (Fig. 6, *B*, *D*, and *F*). These results indicated that YY1 repressed the expression of *ChM-I* by recruiting HDAC2 to induce deacetylation of H3. Another repressive mechanism is the direct binding of p300, a binding partner of YY1 (14). p300 acts as an activator for target gene expression through intrinsic HAT activity (11, 12). Inhibition of p300 by siRNA resulted in the deacetylation of H3 and the repression of *ChM-I* expression (Fig. 5*C*), indicating the role of p300 as HAT for *ChM-I* expression. p300 also acts as a transcriptional co-activator for Sp3, not Sp1 (19), which is the main transcription factor of *ChM-I* (2). The exogenous expression of p300 enhanced the promoter activity in the reporter assay, suggesting the role of p300 as a co-activator. Inhibition of the promoter activity by the YYI expression vector in the reporter assay indicated that YY1 acted as a direct repressor for p300 in addition to acting as a recruiter of HDAC (Fig. 6, *A*, *C*, and *E*). These results suggested that YY1 and p300 are involved in the regulation of *ChM-I* expression through the modification of histones and also the regulation of each others function.

It remains unclear how the repression of YY1 is relieved in chondrogenic cells. One possible mechanism is a post-translational modification of the YY1 protein. YY1 is glycosylated by *O*-linked *N*-acetylglucosaminylation, and glycosylated YY1 fails to bind to DNA (31). *O*-linked glucosamine is expressed in cartilage tissue (32). Such tissue-specific modifications may determine the expression of tissue-specific genes. It is also likely that tissue-specific chromatin-remodeling factors other than YY1 and p300 are involved in the regulation. Analyzing these issues may help to elucidate how the direction of differentiation is determined in stem cells.

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DUAL-TASK WALK IS A RELIABLE PREDICTOR OF FALLS IN ROBUST ELDERLY ADULTS

To the Editor: Falls are relatively common in elderly people, with approximately 30% of individuals aged 65 and older falling at least once a year and approximately half experiencing repeated falls.[1] In daily-life situations, locomotion occurs under complicated circumstances with cognitive attention focused on a particular task, such as watching traffic or reading street signs, rather than performing the specific motor task of walking. A seminal study demonstrating that the characteristic "stops walking when talking" could serve as a predictor of falls introduced a novel method for fall prediction based on dual-task (DT) performance.[2] Recently, a number of studies have evaluated DT walking in elderly people, but one found that reliable conclusions based on DT results for fall prediction cannot be made because of the lack of standardization in DT paradigms.[3] The aim of the current study was therefore to examine prospectively whether two kinds of DT walking (cognitive task (CT) and manual task (MT)) could predict the risk of falls in a community-dwelling elderly population according to physical function.

The study population consisted of 1,038 community-dwelling elderly Japanese people aged 65 and older (401 men, 637 women, mean age 77 ± 8) in 2009. Six items of physical function were assessed: single-task (ST) 10-m walking time, DT (CT and MT) 10-m walking time, Timed Up and Go (TUG) Test,[4] functional reach, and five-chair stand test (Table 1). In CT walking, participants walked 15 m at the most comfortable speed while counting numbers aloud in reverse order starting at 100. In MT walking, participants walked 15 m at the most comfortable speed while carrying a ball (7 cm in diameter, 150 g in weight) on a tray (17 cm in diameter, 50 g in weight). The DT cost (CT and MT) was then calculated as follows:

$$\text{DT cost}[\%] = 100 \times (\text{DT walking time} - \text{ST walking time})/((\text{ST walking time} + \text{DT walking time})/2)$$

Information on the incidence of falls during the following year was collected from participants in a monthly

Table 1. Characteristics of 1,038 Individuals Aged 65 to 97 According to Quartiles of Timed Up and Go Test Results (Seconds)

	Mean ± Standard Deviation							
	Fastest (≤ 8.3) (n = 230)		Faster (8.4–11.0) (n = 258)		Slower (11.1–14.9) (n = 264)		Slowest (≥ 15) (n = 286)	
Characteristic	Faller, 46 (20.0%)	Nonfaller,	Faller, 47 (18.2%)	Nonfaller	Faller, 90 (34.1%)	Nonfaller	Faller, 126 (44.1%)	Nonfaller
Age	77.9 ± 7.9	78.4 ± 6.6	77.4 ± 7.3	78.2 ± 8.0	77.5 ± 8.1	78.2 ± 8.8	77.6 ± 9.3	77.3 ± 8.3
Height, cm	154.4 ± 8.4	153.3 ± 6.8	156.5 ± 9.5	154.7 ± 9.4	157.6 ± 8.3	156.3 ± 11.1	153.6 ± 10.2	154.2 ± 9.6
Body, kg	55.6 ± 11.0	53.6 ± 8.3	50.1 ± 22.9	48.9 ± 16.8	51.7 ± 14.7	53.3 ± 9.3	50.4 ± 17.1	49.7 ± 26.1
Locomotive function, seconds*	9.6 ± 2.0	9.2 ± 2.0	10.5 ± 1.9	10.5 ± 2.5	11.4 ± 2.7	11.2 ± 3.6	17.5 ± 7.1	16.8 ± 7.3
Balance function, cm†	27.1 ± 5.5	25.0 ± 5.4	24.3 ± 7.2	22.6 ± 6.4	21.4 ± 7.9	21.6 ± 7.6	16.6 ± 7.0	18.6 ± 7.0
Muscle power, seconds‡	7.7 ± 1.7	7.5 ± 1.9	9.7 ± 2.8	9.9 ± 2.4	12.8 ± 4.7	11.4 ± 3.5§	17.4 ± 9.8	14.9 ± 5.9§
Cognitive task costs, %	18.7 ± 29.7	16.4 ± 25.5	21.8 ± 23.6	10.6 ± 19.1§	20.2 ± 17.2	20.1 ± 22.2	20.8 ± 20.9	23.1 ± 23.6
Manual task costs, %	8.5 ± 15.8	0.2 ± 11.0§	2.2 ± 14.0	5.8 ± 14.7	12.8 ± 14.0	14.5 ± 16.5	14.5 ± 19.7	16.3 ± 20.7

*Time to complete single-task 10-m walk.
†Distance of functional reach.
‡Time to complete five-chair stand.
§Independent variable that remained in the final step of the regression model.

telephone interview. A fall was defined as any event that led to unplanned, unexpected contact with a supporting surface during walking.

For analysis, the TUG test results were divided into quartiles (fastest, faster, slower, and slowest). A multivariate analysis using logistic regression with a stepwise-forward method was performed to investigate which of the five measures of physical function (ST walking time, CT cost, MT cost, functional reach, and five-chair stand test) was independently associated with falls.

In the fastest group (n = 230), the regression analysis indicated that the MT cost (odds ratio (OR) = 1.068, 95% confidence interval (CI) = 1.04–1.10, $P<.001$) was an independent predictor of falling that remained in the final step of the regression model. In the faster group (n = 258), the regression analysis indicated that the CT cost (OR = 1.03, 95% CI = 1.01–1.04, $P<.001$) was an independent predictor of falling. In the slower (n = 264) and slowest groups (n = 286), the five-chair stand test (slower group OR = 1.11, 95% CI = 1.03–1.19, $P<.001$; slowest group OR = 1.05, CI = 1.01–1.09, $P=.045$) was found to be an independent predictor of falling.

In conclusion, this study demonstrated that DT cost is an independent and prospective predictor of falls in elderly adults with higher functional capacity (faster and fastest groups), although DT cost did not predict falls in elderly adults with lower functional capacity (slower and slowest groups). Thus, the finding that DT walking is a reliable predictor of falls is limited to the robust elderly population.

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TAURINE DIURETIC AND RENAL-REVITALIZING EFFECTS IN NONAGENARIANS

To the Editor: Congestive heart failure (CHF) is the most ominous cause of edema in older adults living in extended-care nursing homes. Despite no obvious CHF, edema resistant even to diuretic doses that cause hypotension, especially in fragile nonagenarians, often develops, and an alternative was sought.

Long-term oral taurine (OT 3 g/d) ameliorates CHF,[1] so it was desired to determine whether OT (1.0 g three times per day) relieves edema without causing hypotension in nonagenarians. Forty-nine residents of an extended-care nursing home (20 taking antihypertensive therapy) who developed edema (score ≥ 2, Appendix A) despite hospital-prescribed diuretics or excessive hypotension precluding effective diuretic usage were enrolled from March 1, 2007, to March 31, 2010.

The remarkable effects of OT on edema were apparent within the first month of treatment (Figure 1A); decreases in body weight occurred with some delay. Required doses of diuretics decreased after institution of OT in the majority of residents. Serum albumin levels increased in 32 hypoalbuminemic residents (Figure 1B).

Significant increases were observed in estimated glomerular filtration rate (eGFR) expressed as a percentage of baseline values from 6 months to 2.25 years of treatment in residents with chronic kidney disease (CKD) Stage 3 or greater (Figure 1C, lower panel); the effects of OT were distinctly greater in residents with CKD Stage 3 or greater than in those with CKD Stage 2 or less (two-way analysis of variance $P<.001$), with differences reaching significance in the third year (Figure 1C upper panel; Bonferroni***). The hyperuricemia ($\geq$ 8.6 mg/dL) observed in eight residents became normal in 6 to 9 months (Figure 1D).

Factors other than CHF play a significant pathogenic role in edema in older extended-care nursing home residents

Figure 1. (A) Effects of taurine are strongest on edema, significantly decreasing body weight. (B) Taurine increases albumin levels in patients with <3.8 g/dL at baseline. (C) Effects of taurine on renal function: Lower panel: taurine significantly increases estimated glomerular filtration rate (eGFR) in patients with chronic kidney disease (CKD) Stage 3 or greater when normalized to baseline values by the sixth month of treatment, and continues to improve significantly for up to 2.25 years. Upper panel: greater improvement of eGFR in residents with CKD Stage 3 or greater compared that in those with CKD Stage 2 of less (two-way analysis of variance $P<.001$) reaches significance after 3 years of treatment (Bonferroni***). (D) Taurine decreases hyperuricemia greater than 8.6 mg/dL to normal levels in 3 to 6 months. ANOVA = analysis of variance; SEM = standard error of the mean.