Genotype and bone mineral loss with aging

Age-related changes in bone mineral density (BMD) were examined via dual-energy x-ray absorptiometry (DXA) and a peripheral quantitative CT (pQCT) in the NILS-LSA. We found 31 genotypes that were associated with BMD (***Table 2***). These are results from association studies between genotypes of candidate genes and BMD by DXA or pQCT.

Fig. 3 shows the effects of the estrogen receptor (ERα) XbaI genotype on the relationship between BMD and lean body mass in post-menopausal women [30]. BMD tends to be higher with more muscle mass estimated as lean body mass, and the effect of lean body mass is larger in AG/GG type than in AA type of ERα XbaI genotype. We suspect that, for the purpose of preventing osteoporosis, an increase in muscle mass is more effective in people with the AG/GG type than in those with the AA type.

BMD is higher in AA type in a cohort with low muscle mass, but BMD is lower in AA type in a cohort with large muscle mass. Findings from analyses of cohorts with different muscle mass reveal that there may be an inverse association between genotype and BMD. Lack in analysis of interaction between gene and life-style would be one of the causes of poor reproducibility in genome research. Thus, comprehensive analyses of the interaction with detailed data from nutrition surveys and lifestyle examinations including smoking, alcohol drinking, and physical activity are essential in the study of Anti-Aging and disease prevention.

Gene and age-related cognitive impairment

Many genes are likely to influence cognitive function, but the associations between genetic polymorphisms and age-related cognitive impairment are unclear. There are significant differences in age-related cognitive decline among individuals.

Klotho is a type I membrane protein that shares sequence similarity with members of the glycosidase family [31], and it

Table 2 *Newly found or confirmed associations between genotypes and bone mineral density (BMD) based on NILS-LSA findings*

Genes and genotypes		Effects on BMD	Ref.
Calcium metabolism related hormones and receptors			
VDR	Vitamin D receptor (A-3731G)	Femoral neck BMD is high in men with CC type	15
ESR1	Estrogen Receptor α (PP/pp)	BMD is low in elderly women with CC type	16
ESR1	Estrogen Receptor α(XX/xx)	BMD is low in elderly women with GG type	16
OST	Osteocalcin (C298T)	BMD is low in premenopausal women with TT type	15
ADR	Androgen receptor (CAG repeat)	BMD is low in premenopausal women with frequent CAG repeat	17
CYP17A1	Cytochrome P450,family 17, subfamily A, polypeptide 1 (T-34C)	BMD is low in postmenopausal women with CC type	18
Cytokines growth hormones and receptors			
IL6	Interleukin-6 (C-634G)	Radial BMD is low in postmenopausal women with GG type	15
TGFB	Transforming growth factor-β1 (T29C)	Radial BMD is high in elderly women with CC type	19
OPG	Osteoprotegerin (T950C)	Radial BMD is low in premenopausal women with CC type	20
OPG	Osteoprotegerin (T245G)	Femoral neck BMD is low in pastmenopausal women with GG type	20
CCR	Chemokine receptor 2 (G190A)	BMD is high in postmenopausal women and middle-aged men and with AA type	21
Bone matrix related protein			
MMP1	Matrix metalloproteinase-1 (1G/2G at-1607)	Radial BMD is low in postmenopausal women with 2G/2G type	22
MMP9	Matrix metalloproteinase-9 (C-1562T)	BMD is low in men with CT/TT type	23
COL	Collagen typeI (G-1997T)	BMD is low in postmenopausal women with GG type	24
ICAM1	Intercellular adhesion molecule-1 (Lys469Glu)	BMD is low in postmenopausal women with AA type	25
PLOD1	Procollagen-lysine 2-oxyglutarate 5-dioxygenase (Ala99Thr)	BMD is low in pre and postmenopausal women with GA/AA type	25
CX37	Connexin 37 (Pro319Ser)	BMD is low in men with TT type	25
Others			
KLOT	Klotho (G-395A)	BMD is low in pre and postmenopausal women with GG type	17
MTP	Microsomal triglyceride transfer protein (G-493T)	BMD is high in premenopausal women with TT type	18
VLDLR	VLDL receptor (triplet repeat)	BMD is high in men with more than 8 CGG repeat	18
ALAP	Adipocyte-derived leucine aminopeptidase (Lys528Arg)	BMD is high in premenopausal women with GG type	25
LIPC	Hepatic lipase (C-514T)	BMD is low in postmenopausal women with TT type	25
CNR2	Cannabinoid receptor 2 gene (A/G, rs2501431)	BMD is low in pre and postmenopausal women with AA/AG type	25
PON1	Paraoxonase-1 (Gln192Arg)	BMD is low in postmenopausal women with GG type	26
PON1	Paraoxonase-1 (Met55Leu)	BMD is low in postmenopausal women with TT type	26
PON2	Paraoxonase-2 (Cys311Ser)	BMD is low in postmenopausal women with CC type	26
DRD4	Dopamine D4 Receptor (C-521T)	BMD is low in men with CC type	27
FOXC2	Forkhead box C2 (C-512T)	BMD is low in men and women with T allele	28
PLN	Perilipin (C1243T)	BMD is low in men with C allele	28
MAOA	Monoamine oxidase A (uVNTR)	BMD is low in women with repeat less than 4	29
SH2B1	Src-homology-2-B (Ala484Thr)	BMD is low in women with A allele	29

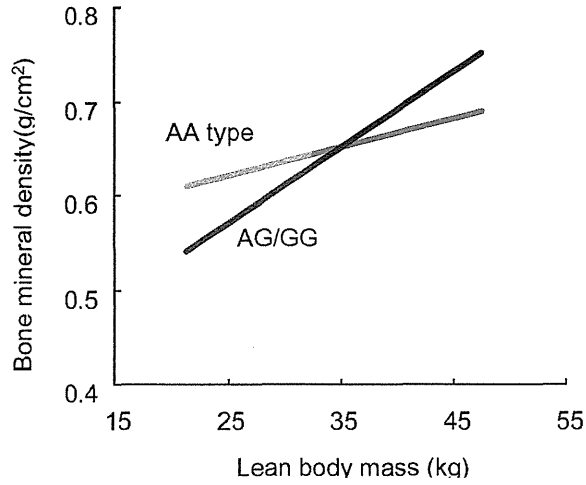

Fig. 3. **The effects of the estrogen receptor (ERα) XbaI genotype on the relationship between BMD and lean body mass in post-menopausal women. The BMD tends to be higher in women with more muscle mass as estimated as lean body mass, and the effect of lean body mass is larger in AG/GG type than in AA type of ERα XbaI genotype (modified from Kitamura *et al.*, 2007 [30]).**

is a fundamental regulator of aging in mice [32]. Mice lacking this protein exhibit multiple aging phenotypes and age-related disorders, including a shortened lifespan, reduced spontaneous activity, arteriosclerosis, infertility, skin atrophy, premature thymic involution, pulmonary emphysema, and osteopenia, although the function of klotho remains to be determined [31,33]. A human homolog of the mouse klotho gene was isolated and its structure was determined [34]. Cognitive impairment was previously shown in klotho gene mutant mice aged seven weeks or over [35]. The klotho gene may be mediate age-related changes in cognitive function in humans.

The effects of klotho gene genotype on cognition were examined in the NILS-LSA [36]. The subjects comprised 2,234 participants in the NILS-LSA aged 40 to 79 years. The klotho gene promoter polymorphism G-395A was identified, and cognitive function was assessed using the Japanese Wechsler Adult Intelligence Scales - Revised Short Forms (JWAIS-R SF) and Mini Mental State Examination (MMSE). The differences in cognitive function were compared between the GG type and GA/AA type of the klotho gene G-395A polymorphism. There was no significant difference in IQ between the GG type and GA/AA type in the subjects aged 40 to 59 years. However, the IQ level was significantly different in terms of the klotho genotype for subjects aged 60 to 79 years (p=0.004). The mean and SE of IQ levels of the subjects with the GG type and the GA/AA type at nucleotide –395 were 99.8 ± 0.5 and 102.6 ± 0.8, respectively. There were also significant differences in three subtests within the JWAIS-R SF: Information, Similarities, and Picture Completion for subjects aged 60 to 79 years. Also, the MMSE score was slightly lower for the GG type than for the GA/AA type (p=0.099).

There were statistically significant differences in cognitive function for klotho gene promoter polymorphism G-395A only in subjects aged 60 or over. This polymorphism may be associated with age-related cognitive impairment, and not associated with cognitive development during childhood to adolescence.

A new genetic strategy for Anti-Aging and prevention of age-related disease

The impact of genetic surveys could be enormously helpful for preventive treatments of geriatric disease as well as Anti-Aging. Previously, associations between disease and genotype were usually investigated by association studies of a specific genotype and a specific disease in molecular epidemiology research. However, we should clarify the following to apply results of epidemiological study to Anti-Aging medicine and preventive medicine: 1) the penetration rates of the genotypes in Japanese; 2) contribution rate to incidence of disease by each susceptibility genotype; 3) factors associated with development of disease in carriers of disease susceptibility genotype; 4) interactive effects with other genotypes; and 5) other physiological effects of the genotype.

These can be investigated in community-dwelling populations and patient cohorts that have detailed background data. Risk of disease can be estimated with the aid of accumulated data. The best-suited education and modification of lifestyles and the content and frequency of examinations for each individual can be determined based on the risk estimation can be applied for disease prevention and Anti-Aging.

Conflict of interest statement

The authors declare no financial or other conflicts of interest in the writing of this paper.

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EPIDEMIOLOGY

Relationship between Physical Activity and Brain Atrophy Progression

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[1]*Department for Development of Preventive Medicine, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Obu City, Aichi Prefecture, JAPAN;* [2]*Department of Human Wellness, Tokaigakuen University, Miyoshi, Aichi Prefecture, JAPAN;* [3]*Gerontology Research Center, University of Jyvaskyla, Jyvaskyla, FINLAND; and* [4]*Department of Health and Medical Sciences, Aichi Shukutoku University, Nagakute, Aichi Prefecture, JAPAN*

ABSTRACT

YUKI, A., S. LEE, H. KIM, R. KOZAKAI, F. ANDO, and H. SHIMOKATA. Relationship between Physical Activity and Brain Atrophy Progression. *Med. Sci. Sports Exerc.*, Vol. 44, No. 12, pp. 2362–2368, 2012. **Introduction**: Brain atrophy is associated with impairment in cognitive function and learning function. The aim of this study was to determine whether daily physical activity prevents age-related brain atrophy progression. **Methods**: The participants were 381 men and 393 women who had participated in both the baseline and the follow-up surveys (mean duration = 8.2 yr). Magnetic resonance imaging of the frontal and temporal lobes was performed at the time of the baseline and follow-up surveys. The daily physical activities and total energy expenditures of the participants were recorded at baseline with uniaxial accelerometry sensors. Multiple logistic regression models were fit to determine the association between activity energy expenditure, number of steps, and total energy expenditure variables and frontal and temporal lobe atrophy progression while controlling for possible confounders. **Results**: In male participants, the odds ratio of frontal lobe atrophy progression for the fifth quintile compared with the first quintile in activity energy expenditure was 3.408 (95% confidence interval = 1.205–9.643) and for the number of steps was 3.651 (95% confidence interval = 1.304–10.219). Men and women with low total energy expenditure were at risk for frontal lobe atrophy progression. There were no significant differences between temporal lobe atrophy progression and physical activity or total energy expenditure. **Conclusion**: The results indicate that physical activity and total energy expenditure are significant predictors of frontal lobe atrophy progression during an 8-yr period. Promoting participation in activities may be beneficial for attenuating age-related frontal lobe atrophy and for preventing dementia.
Key Words: LONGITUDINAL STUDY, MIDDLE AGED AND ELDERLY, ACCELEROMETRY SENSORS, MRI

Atrophy of brain structures is associated with impairment in cognitive function and learning function (the extreme case is Alzheimer disease) (21). Brain atrophy progresses with aging (17). The gray matter volume decreases by approximately 15%, from the 20s through the 70s (38). A previous study reported that a decline in cognitive function is associated with the progression rate of brain atrophy for 6 yr in normal elderly people (33). Thus, preventing brain atrophy may be a promising strategy for preventing cognitive impairment and decline.

Physical exercise appears to induce neurogenesis in the brain not only in animals but also in humans (11). The practice of juggling for 3 months increases the volume of gray matter in the bilateral midtemporal area and in the left posterior intraparietal sulcus in young people (10). Similarly, the increase in brain volume in the anterior cingulate gyrus and frontal pole caused by juggling occurs in elderly people (3). In particular, aerobic exercise appears to suppress global and regional brain atrophy to effectively increase brain volume (14). Relatively little brain structural atrophy is seen in elderly people with high aerobic capacity (7). Six months of aerobic exercise increases the volume of the frontal lobe, temporal lobe, and hippocampus (8). Aerobic capacity is correlated with the preservation of gray matter in the medial–temporal, parietal, and frontal areas in elderly people (18). Aerobic quick-step walking suppresses hippocampal atrophy and improves cognitive function in elderly people (15). These reports suggest the possibility that aerobic exercise prevents brain atrophy.

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We hypothesized that brain atrophy progression can be prevented in middle-age and elderly people with a high level of daily physical activity. Daily physical activities are correlated with aerobic capacity in middle-age and elderly people (2,6). In cross-sectional studies, high physical activity levels are related to larger superior frontal volumes (5). Increased physical activity is associated with greater average brain tissue volumes in the white matter of the corona radiata, extending into the parietal–occipital junction (19). Although daily physical activities may prevent brain atrophy progression, there has been no specific longitudinal analysis showing that daily physical activity maintained at a high level prevents brain atrophy. Recent longitudinal studies have reported that elderly people with high levels of daily physical activity have a low risk of decline in cognitive function (26,34). A demonstration of the prevention of brain atrophy progression by high levels of physical activity in a longitudinal study may support the association between daily physical activity and cognitive function.

The aim of this study was to determine whether high levels of daily physical activity prevent brain atrophy progression with aging. We assessed the progression of frontal and temporal lobe atrophy with aging using 8-yr follow-up surveys and magnetic resonance imaging (MRI) of middle-age and elderly people. We also recorded the amount of physical activity (activity energy expenditure and number of steps) and total energy expenditure using a uniaxial accelerometry sensor. We evaluated the association between brain atrophy progression and daily physical activity and total energy expenditure in 774 community-living, middle-age, and elderly Japanese people using longitudinal analysis.

METHODS

Participants. The participants in this study were derived from the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA), which involves ongoing population-based biennial examinations of a cohort of approximately 2300 persons. The participants in the NILS-LSA were randomly selected from resident registrations and stratified by both decade of age and sex. The NILS-LSA is a comprehensive and interdisciplinary study to observe age-related changes and consists of various gerontological and geriatric measurements, including medical examinations, blood chemical analysis, body composition, anthropometry, nutritional analysis, psychological tests, physical function, and physical activity. Details of the NILS-LSA have been described elsewhere (35).

The baseline participants of this study were 1526 middle-age and elderly people (773 men and 753 women) who completed the second wave examinations of NILS-LSA between April 2000 and May 2002. Of these, 942 (61.6%, 481 men and 461 women) participated in the 8-yr follow-up surveys (NILS-LSA sixth wave examination from July 2008 to July 2010). The dropouts were 584 participants (292 men and 292 women). In male and female participants, the age at baseline of the dropouts was significantly higher than that of the participants who completed both examinations (t-test, $P < 0.0001$). In male participants, the ratios of stroke and ischemic heart disease histories in dropouts were significantly higher than those in participants who completed both examinations (chi-square test: stroke, $P = 0.0002$; ischemic heart disease, $P = 0.0019$). In female participants, there were no differences in the ratios of stroke and ischemic heart disease histories between the dropouts and the participants who completed both examinations. In male and female participants, the ratio of diabetes histories in dropouts was significantly higher than that in participants who completed both examinations (chi-square test: men, $P = 0.0077$; women, $P = 0.0369$). There were no differences in the ratios of hypertension and hyperlipidemia histories between the dropouts and the participants who completed both examinations in men or women. There were no differences in the ratios of severe atrophy in the frontal and temporal lobe between the dropouts and the participants who completed both examinations in men or women.

Participants with severe atrophy in the second wave examination were excluded because severe atrophy was of a high-end grade that cannot be used to determine further atrophy progression. Participants in their 40s were also excluded because few participants of this age show brain atrophy progression. Participants with a current medical history of Parkinson disease, dementia, or open head surgery were also excluded. Finally, the participants for this study were 381 men and 393 women.

The study protocol was approved by the Ethics Committee of the National Center for Geriatrics and Gerontology, and written informed consent was obtained from all participants.

Brain MRI examination. Brain MRI was performed on participants at the second and sixth wave examinations using a 1.5-T scanner (Toshiba Visart, Tokyo, Japan) at the National Center for Geriatrics and Gerontology. Each participant's head was oriented in the scanner and stabilized during the scanning procedure by a head support. To establish slice orientation, the first scanning sequence consisted of a T1-weighted sagittal series (repetition time (TR) = 500 ms, echo time (TE) = 15 ms, 256 × 256 matrix) centered along the midline to define the orbitomeatal line. The second series of T1-weighted axial images (TR = 500 ms, TE = 15 ms, thickness = 8 mm, gap = 1.5 mm, 256 × 256 matrix) and T2-weighted axial images (TR = 4000 ms, TE = 120 ms, thickness = 8 mm, gap = 1.5 mm, 320 × 320 matrix) were oriented parallel to the orbitomeatal line. Fourteen slices were taken during each examination.

The presence and the degree of brain atrophy in the frontal and temporal lobes were assessed as no atrophy (I), mild atrophy (II), moderate atrophy (III), and severe atrophy (IV) (25,36). The participants were divided into two groups on the basis of results from the MRI in the second wave examination and sixth wave examination: the brain atrophy progression group (progress: degree of brain atrophy in the second wave < sixth wave) and the brain atrophy nonprogression group.

Daily physical activities and total energy expenditure assessments. We recorded the daily physical activities and total energy expenditures of the participants at the second wave examinations using a uniaxial accelerometry sensor (Lifecorder; Suzuken, Aichi, Japan). Lifecorder can assess two types of activity energy expenditure by activity level: energy expenditure of activities (with body movements) and energy expenditure of minor activities (working at a desk or reading a book). In this study, the activity energy expenditure was estimated as the energy expenditure of both types of activities. The total energy expenditure was determined as the sum of basal metabolism, energy expenditure of activities, energy expenditure of minor activities, and thermic effects of food. Participants wore the Lifecorder constantly (except while sleeping or bathing) for a 7-d period. We calculated the mean activity energy expenditure, the number of steps, and the total energy expenditure from 5 d of records (the maximum and the minimum records were excluded).

Other parameters. Body height and weight were measured using a digital scale. Body mass index (BMI) was calculated as weight divided by height squared ($kg \cdot m^{-2}$). Body fat mass was assessed by dual x-ray absorptiometry (QDR-4500A; Hologic, Bedford, MA). Lifestyle factors (including alcohol intake, smoking habit, and education levels), medical history, and use of medications were assessed with questionnaires. These questionnaires were confirmed by a physician at the medical examinations. All prescribed and nonprescribed medications used during the previous 2 wk were documented and brought by the participants; the physicians confirmed and coded them. Users of antihypertensive, antilipemic, or hypoglycemic medications were considered participants with hypertension, hyperlipidemia, and diabetes histories, respectively.

Statistical analysis. The results are shown as the mean ± SD or mean ± SE. Differences in continuous and class variables between the progression and the nonprogression groups were assessed with *t*-tests and chi-square tests, respectively. Cochran–Mantel–Haenszel statistics were used to examine the relationship between the age group and the brain atrophy progression. Multiple logistic regression models were fit to determine the associations of activity energy expenditure, number of steps, and total energy expenditure variables with frontal and temporal lobe atrophy progression while controlling for the baseline decade of age group (38), BMI (19), education history (19), medical history (stroke, ischemic heart disease, hypertension, hyperlipidemia, and diabetes) (4,12,24), and current smoking and alcohol intake as possible confounders (9,37). Activity energy expenditure, number of steps, and total energy expenditure were modeled as sex-specific quintiles. Statistical testing was performed using the Statistical Analysis System release 9.1.3 (SAS Institute Inc., Cary, NC). Significant probability levels were considered to be less than 0.05.

RESULTS

Characteristics of the participants. Table 1 shows elementary statistics of the study variables in male and female participants. The mean follow-up durations of all participants were 8.2 ± 0.3 yr. There were no significant differences in baseline age, BMI, or number of steps between male and female participants. Body height and weight, alcohol intake, and education history were significantly higher in male participants than those in female participants (each, $P < 0.0001$). The percentage of body fat in female participants was significantly higher than that in male participants ($P = 0.0126$). The activity and total energy expenditures in men were significantly higher than those in women (each, $P < 0.0001$). There were no sex differences in the ratios of stroke, ischemic heart disease, and hypertension histories. The ratio of hyperlipidemia history in female participants was significantly higher than that in male participants ($P = 0.0060$). The ratios of diabetes history and smoking habits in male participants were significantly higher than that in female participants (diabetes history, $P = 0.0126$; smoking habits, $P < 0.0001$).

TABLE 1. The characteristics of participants at the time of the second wave examination of the NILS-LSA, 2000–2002.

	Male ($n = 381$)	Female ($n = 393$)	*P*
Mean follow-up (yr)	8.2 ± 0.3	8.2 ± 0.3	0.5777
Age (yr)	60.4 ± 7.3	60.8 ± 7.6	0.5421
Body height (cm)	164.7 ± 5.4	152.2 ± 5.2	<0.0001
Body weight (kg)	62.5 ± 7.1	52.7 ± 7.0	<0.0001
BMI ($kg \cdot m^{-2}$)	23.0 ± 2.4	22.7 ± 2.9	0.1279
% body fat	21.0 ± 4.0	31.3 ± 4.9	<0.0001
Alcohol intake ($g \cdot d^{-1}$)	16.6 ± 20.9	2.7 ± 6.1	<0.0001
Education (yr)	12.3 ± 2.7	11.4 ± 2.3	<0.0001
Activity energy expenditure ($kcal \cdot d^{-1}$)	215.1 ± 78.5	175.1 ± 64.8	<0.0001
No. of steps per day	7993.2 ± 2588.0	7925.6 ± 2297.1	0.7011
Total energy expenditure ($kcal \cdot d^{-1}$)	1932.3 ± 168.5	1607.5 ± 150.0	<0.0001
With medical history, *n* (%)			
Stroke	14 (3.7%)	7 (1.8%)	0.1050
Ischemic heart disease	13 (3.5%)	19 (4.8%)	0.3203
Hypertension	40 (10.5%)	40 (10.2%)	0.8836
Hyperlipidemia	61 (16.0%)	94 (23.9%)	0.0060
Diabetes	32 (8.4%)	16 (4.1%)	0.0126
Smoking habit	102 (26.8%)	27 (6.9%)	<0.0001

Values are presented as mean ± SD. *P* values were obtained using the *t*-test for continuous data and the chi-square test for categorical data.

TABLE 2. The ratio of frontal and temporal lobe atrophy progression in participants from the second (2000–2002) to the sixth (2008–2010) wave examination of the NILS-LSA.

	Frontal Lobe Atrophy			Temporal Lobe Atrophy		
	Nonprogression	Progress	Trend *P*	Nonprogression	Progress	Trend *P*
Male, *n* (%)						
Age group						
50s	176 (95.1%)	9 (4.9%)	<0.001	156 (84.3%)	29 (15.7%)	<0.001
60s	112 (79.4%)	29 (20.6%)		87 (61.7%)	54 (38.3%)	
70s	38 (69.1%)	17 (30.9%)		38 (69.1%)	17 (30.9%)	
Total	326 (85.6%)	55 (14.4%)		281 (73.8%)	100 (26.3%)	
Female, *n* (%)						
Age group						
50s	191 (96.0%)	8 (4.0%)	<0.001	188 (94.5%)	11 (5.5%)	<0.001
60s	117 (90.0%)	13 (10.0%)		92 (70.8%)	38 (29.2%)	
70s	50 (78.1%)	14 (21.9%)		35 (54.7%)	29 (45.3%)	
Total	358 (91.1%)	35 (8.9%)		315 (80.2%)	78 (19.8%)	

The trend *P* values were obtained using the Cochran–Mantel–Haenszel test.

Progress of frontal and temporal lobe atrophy. Table 2 shows comparisons of the incidence of frontal and temporal lobe atrophy progression in each age group. Frontal lobe atrophy progression from the second wave examination to the sixth wave examination was present in 55 (14.4%) of 381 male participants and 35 (8.9%) of 393 female participants. The ratio of participants with frontal lobe atrophy progression in male participants was significantly higher than that in female participants ($P = 0.0213$). Aging raised the percentage of participants with frontal lobe atrophy progression in men and women (*P* trend <0.0001).

Temporal lobe atrophy progression from the second wave examination to the sixth wave examination was present in 100 (26.3%) of 381 male participants and 78 (19.8%) of 393 female participants. The ratio of participants with temporal lobe atrophy progression in male participants was significantly higher than that in female participants ($P = 0.0344$). Aging raised the percentage of participants with temporal lobe atrophy progression in men and women (*P* trend <0.0001).

Brain atrophy progression and physical activity level. Table 3 shows the activity energy expenditure, number of steps, and total energy expenditure in the frontal and temporal lobe atrophy progression and nonprogression groups. In the frontal lobe, activity energy expenditure ($P = 0.0095$), number of steps ($P = 0.0131$), and total energy expenditure ($P < 0.0001$) were significantly higher in the male nonprogression group than the progression group. In female participants, total energy expenditure was significantly higher in the nonprogression group than that in the progression group ($P = 0.0097$). There were no differences in the activity energy expenditure or number of steps between the female nonprogression and progression groups.

In the temporal lobe, there were no differences in the activity energy expenditure or number of steps between the nonprogression and the progression groups in male or female participants. The total energy expenditure was significantly higher in the nonprogression group than that in the progression group in male ($P = 0.0028$) and female ($P = 0.0096$) participants.

Risk of brain atrophy progression according to physical activity level differences. The results of multiple logistic regression analyses for risk of brain atrophy progression according to differences in the physical activity level in men and women are shown in Tables 4 and 5, respectively. In male participants, the odds ratio of frontal lobe atrophy progression for the comparison between the fifth quintile in activity energy expenditure and the first quintile was 3.408 (95% confidence interval (CI) = 1.205–9.643). The odds ratio of frontal lobe atrophy progression for the comparison between the fifth quintile in number of steps and the first quintile was 3.651 (95% CI = 1.304–10.219). The odds ratios of frontal lobe atrophy progression for the comparison between the fifth quintile in total energy expenditure and the first and third quintiles were 4.816 (95% CI = 1.037–22.376) and 4.639 (95% CI = 1.191–18.067), respectively.

In female participants, there were no significant differences between frontal lobe atrophy progression and physical activity parameters. The odds ratios of frontal lobe atrophy progression for the comparison between the fifth quintile in total energy expenditure and the first to the third quintiles

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TABLE 3. Mean activity energy expenditure, number of steps, and total energy expenditure per day in each group.

	Frontal Lobe Atrophy			Temporal Lobe Atrophy		
	Nonprogression	Progress	*P*	Nonprogression	Progress	*P*
Male (*n*)	326	55		281	100	
Activity energy expenditure (kcal·d^{-1})	219.3 ± 4.4	189.7 ± 9.9	0.0095	217.3 ± 4.6	208.8 ± 8.1	0.3503
No. of steps per day	8128.0 ± 143.6	7194.3 ± 327.4	0.0131	7983.1 ± 155.1	8021.8 ± 256.6	0.8979
Total energy expenditure (kcal·d^{-1})	1947.0 ± 9.2	1845.22 ± 1.2	<0.0001	1945.6 ± 10.1	1895.0 ± 15.9	0.0097
Female (*n*)	358	35		315	78	
Activity energy expenditure (kcal·d^{-1})	176.4 ± 3.4	161.6 ± 10.1	0.1965	176.7 ± 3.7	169.4 ± 6.9	0.3664
No. of steps per day	7984.9 ± 121.8	7318.7 ± 365.6	0.1016	7997.4 ± 130.1	7699.5 ± 254.6	0.3043
Total energy expenditure (kcal·d^{-1})	1614.5 ± 7.9	1535.4 ± 21.8	0.0028	1616.5 ± 8.3	1567.7 ± 17.8	0.0096

Values are presented as means ± SE. The *P* values were obtained using the *t*-test.

TABLE 4. Adjusted odds ratios of frontal and temporal lobe atrophy progression in male participants distributed into quintiles of physical activity and total energy expenditure data.

	Odds Ratio, 95% CI				
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5
Frontal lobe (*n*)	76	76	76	76	77
Activity energy expenditure (kcal·d^{-1})	3.408, 1.205–9.643 (<143.2)	1.054, 0.321–3.462 (143.2 to <184.4)	1.623, 0.523–5.035 (184.4 to <226.2)	2.054, 0.691–6.904 (226.2 to <284.4)	1.00, referent (≥284.4)
No. of step per day	3.651, 1.304–10.219 (<5736.0)	1.216, 0.383–3.863 (5736.0 to <6955.0)	1.487, 0.471–4.689 (6955.0 to <8261.4)	2.403, 0.819–7.052 (8261.4 to <10,407.4)	1.00, referent (≥10,407.4)
Total energy expenditure (kcal·d^{-1})	4.816, 1.037–22.376 (<1771.4)	2.758, 0.652–11.672 (1771.4 to <1897.4)	4.639, 1.191–18.067 (1897.4 to <1983.4)	2.275, 0.553–9.358 (1983.4 to <2091.2)	1.00, referent (≥2091.2)
Temporal lobe (*n*)	76	76	76	76	77
Activity energy expenditure (kcal·d^{-1})	1.015, 0.473–2.178 (<143.2)	1.293, 0.617–2.708 (143.2 to <184.4)	0.800, 0.364–1.756 (184.4 to <226.2)	0.845, 0.390–1.833 (226.2 to <284.4)	1.00, referent (≥284.4)
No. of step per day	0.938, 0.435–2.024 (<5736.0)	1.100, 0.519–2.330 (5736.0 to <6955.0)	1.142, 0.538–2.425 (6955.0 to <8261.4)	1.123, 0.528–2.389 (8261.4 to <10,407.4)	1.00, referent (≥10,407.4)
Total energy expenditure (kcal·d^{-1})	1.045, 0.388–2.816 (<1771.4)	1.303, 0.554–3.065 (1771.4 to <1897.4)	1.229, 0.537–2.810 (1897.4 to <1983.4)	1.006, 0.439–2.307 (1983.4 to <2091.2)	1.00, referent (≥2091.2)

Odds ratios were controlled for age, BMI, education history, medical history (stroke, ischemic heart disease, hypertension, hyperlipidemia, and diabetes), current smoking, and alcohol intake in a multinomial logistic regression model.

were 12.363 (95% CI = 1.029–148.594), 12.743 (95% CI = 1.292–125.792), and 21.539 (95% CI = 2.381–194.839), respectively.

We also evaluated temporal lobe atrophy progression using the adjustment model, similar to the frontal lobe atrophy progression analysis. There were no significant differences between temporal lobe atrophy progression and physical activities or total energy expenditure (Tables 4 and 5) in any groups of participants.

DISCUSSION

Using longitudinal analyses, we showed that a high level of physical activity and total energy expenditure suppressed the frontal lobe atrophy progression that is induced by aging.

An inactive daily life appears to be a risk factor for frontal lobe atrophy progression. In male participants, those with the lowest activity energy expenditure (first quintile, <143.2 kcal) had a 3.408-fold risk of frontal lobe atrophy progression compared with those with the highest activity energy expenditure (fifth quintile, ≥284.4 kcal) (Table 4). Similarly, men with the fewest number of steps (first quintile, <5736.0 steps) had a 3.651-fold risk of frontal lobe atrophy progression compared with those with the most number of steps (fifth quintile, ≥10,407.4 steps) (Table 4). An activity energy expenditure of 143.2 kcal is equivalent to activity in 4 METs (e.g., raking the lawn and table tennis) for 33 min in 62.5-kg men (1). Thirty minutes of middle-intensity or greater activities per day, such as 5700 steps or more walking per day, may be necessary to reduce the risk of frontal lobe atrophy progression. In addition, daily physical activity decreases with aging (27). An increase in planned physical activities may be necessary to prevent frontal lobe atrophy progression in older people.

Not only the expenditure of energy with physical activity but also the energy metabolic rate of the whole body appears to be associated with frontal lobe atrophy. Low total energy expenditure tended to be a risk for frontal lobe atrophy in male and female participants (Tables 4 and 5). In a study of prosimians and anthropoid apes and humans, brain volume is correlated with basal metabolism (23). The amount of basal metabolism may determine frontal lobe atrophy progression. It is well known that basal metabolism decreases with aging (32). Age-related skeletal muscle loss (sarcopenia) may be a risk factor for frontal lobe atrophy progression due to decreasing basal metabolism. Physical activity may compensate for a reduction in basal metabolism in the elderly.

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TABLE 5. Adjusted odds ratios of frontal and temporal lobe atrophy progression in female participants distributed into quintiles of physical activity and total energy expenditure data.

	Odds Ratio, 95% CI				
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5
Frontal lobe (*n*)	78	79	78	79	79
Activity energy expenditure (kcal·d^{-1})	1.442, 0.421–4.945 (<119.6)	1.422, 0.435–4.644 (119.6 to <148.4)	0.610, 0.148–2.520 (148.4 to <182.8)	1.233, 0.362–4.199 (182.8 to <226.4)	1.00, referent (≥226.4)
No. of step per day	1.559, 0.420–5.791 (<5825.2)	2.269, 0.627–8.209 (5825.2 to <7090.0)	0.826, 0.181–3.769 (7090.0 to <8374.0)	1.887, 0.505–7.053 (8374.0 to <9910.4)	1.00, referent (≥9910.4)
Total energy expenditure (kcal·d^{-1})	12.363, 1.029–148.594 (<1495.6)	12.743, 1.292–125.792 (1495.6 to <1570.2)	21.539, 2.381–194.839 (1570.2 to <1639.6)	4.261, 0.430–42.214 (1639.6 to <1722.0)	1.00, referent (≥1722.0)
Temporal lobe (*n*)	78	79	78	79	79
Activity energy expenditure (kcal·d^{-1})	0.978, 0.362–2.645 (<119.6)	1.023, 0.400–2.614 (119.6 to <148.4)	1.569, 0.591–4.162 (148.4 to <182.8)	1.547, 0.617–3.876 (182.8 to <226.4)	1.00, referent (≥226.4)
No. of step per day	0.879, 0.355–2.178 (<5825.2)	0.789, 0.311–2.005 (5825.2 to <7090.0)	0.825, 0.317–2.147 (7090.0 to <8374.0)	1.206, 0.489–2.974 (8374.0 to <9910.4)	1.00, referent (≥9910.4)
Total energy expenditure (kcal·d^{-1})	0.881, 0.260–2.984 (<1495.6)	1.127, 0.405–3.138 (1495.6 to <1570.2)	0.948, 0.337–2.668 (1570.2 to <1639.6)	1.285, 0.499–3.305 (1639.6 to <1722.0)	1.00, referent (≥1722.0)

Odds ratios were controlled for age, BMI, education history, medical history (stroke, ischemic heart disease, hypertension, hyperlipidemia, and diabetes), current smoking, and alcohol intake in a multinomial logistic regression model.

Although a low-activity energy expenditure and a low number of steps were risk factors for frontal lobe atrophy progression in male participants, they were not risk factors in female participants (Tables 4 and 5). Generally, there are many more men with brain atrophy than women (38). In this study, the ratios of frontal lobe atrophy progression were different between male and female participants (Table 2). Sex hormones may also affect the relationship between physical activity and frontal lobe atrophy. Androgens and estrogens are associated with brain volume (13,24), and the adaptability of the brain to physical activity may be higher in men than that in women.

In contrast to activity energy expenditure, total energy expenditure was associated with frontal lobe atrophy progression in both men and women. Basal metabolism is the maximal occupation ratio in total energy expenditure. The brain metabolic rate is included in the basal metabolism. In women, total energy expenditure including basal metabolism appears to be a better index of the risks for frontal lobe atrophy progression compared with physical activity parameters. However, because some of the odds ratios were exceedingly large in female participants, our logistic regression model may not have precisely estimated the risk of frontal lobe atrophy. There were 55 male participants with frontal lobe atrophy progression (Table 2), but only 35 female participants had frontal lobe atrophy progression (Table 2). These sex differences in the brain atrophy progression rate may have influenced estimation of the odds ratio. In women in particular, further investigations may be needed to determine the association of frontal lobe atrophy progression with total energy expenditure.

Brain atrophy is caused in part by obesity (19), metabolic syndrome, and its components (4,12). A high level of physical activity improves obesity and metabolic syndrome (29). Cross-sectional research suggests that prevention of obesity by physical activity causes the relationship between physical activity and brain volume (19). However, in this study, frontal lobe atrophy progression was associated with the physical activity level in logistic regression models that controlled for BMI. Physical activity or the total energy expenditure may be independent factors for preventing frontal lobe atrophy progression, regardless of obesity.

In this study, the activity energy expenditure, the number of steps, and the total energy expenditure were quantitative data collected by an accelerometer. The objectivity of our study is higher than that of past studies that estimated the physical activity level with a questionnaire (5,19).

A limitation of this study is the noninvasive approach using MRI. We could not elucidate the mechanism of frontal lobe atrophy progression induced by a low level of physical activity or total energy expenditure. In an animal study, the beta amyloid cumulative dose is active mass dependent in mouse brain (22). The death of neurons may be inhibited by physical activity. Some growth factors, such as nerve growth factor or brain-derived neurotrophic factor, contribute to neuronal survival or neurogenesis (31,39). The serum level of nerve growth factors fluctuates with physical exercise (16), and thus, exercise stimulus with physical activity may modify expression of nerve growth factors.

Exercise and physical activity have been reported to change the volume of every region of the brain, including the frontal lobe, the temporal lobe, the parietal lobe, and the hippocampus (3,5,8,19). Interestingly, our results showed associations between brain atrophy progression and physical activity or total energy expenditure only in the frontal lobe, but not in the temporal lobe. We hypothesize that the regional differences in brain atrophy progression were due to differences in the patterns of physical activities (including types, intensities, or frequencies). A previous study suggests that increased blood flow in the brain due to physical exercise promotes neurogenesis (30). Blood flow in the brain varies with exercise type and intensity (20,28). In this study, because the activity energy expenditure, the number of steps, and the total energy expenditure data were collected as the total amount per day with accelerometer sensors, the differences in the patterns of physical activities between participants were not determined. Further investigations that define these details may clearly uncover an association between physical activities and regional differences in brain atrophy progression.

In summary, using the longitudinal design of the NILS-LSA cohort, we evaluated the association between brain atrophy progression and daily physical activity and total energy expenditure in 774 community-living, middle-age, and elderly Japanese people with an 8-yr follow-up duration. Our data confirm that low levels of physical activity and total energy consumption are significant predictors of the risk for brain atrophy, and the effect of atrophy suppression is seen only in the frontal lobe. Promoting participation in physical activities may be beneficial in attenuating age-related frontal lobe atrophy and in preventing dementia.

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Osteoporosis Japan

地域在住中高齢者の膝関節変形と膝伸展筋力との関連

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はじめに

変形性膝関節症（以下，膝OA）の保存的療法において大腿四頭筋筋力訓練の有効性が確かめられている[1)]。一方，膝関節変形と同筋力との関連を調べた疫学的研究は限られている。そこで，地域在住中高年者対象の大規模コホートにおいて，膝関節変形の有無や程度と膝伸展筋力との関連についての検討を行った。

1　対象と方法

「国立長寿医療研究センター・老化に関する長期縦断疫学研究（NILS-LSA）[2)]」の第5次調査に参加した，50歳以上の1,653名（平均年齢65.4±9.3歳）（男性862名，女性791名），3,299膝を対象とした。膝伸展筋力は，竹井機器工業（新潟市）製の測定装置で，左右別に3回ずつ座位にて膝関節90度屈曲位における等尺性筋力を測定し，最大値を採用した。膝関節変形の程度は，膝関節レントゲン荷重位正面像を左右別々に撮影し，Kellgren-Lawrence（KL）分類をもとに5段階に分類し，Ⅱ度以上を膝OAありとし，また変形程度により0～Ⅰ度を正常，Ⅱ度を軽度変形，Ⅲ～Ⅳ度を重度変形と，3段階に分類した。そしてOAの有無，変形程度により，膝伸展筋力に差があるか否かを，一般線形モデルを用い，年齢，BMIを調整した多重比較（Tukey-Kramer法）にて，左右の膝を合わせ，男女別に検討した。統計解析にはSAS ver9.1.3を用いた。

2　結　　果

年代別の膝関節変形のある割合は図1のごとくで，男女とも，また軽度変形，重度変形例ともに，年代が高くなるほど有意に増加していた（$p<0.0001$）。膝OAの有無により膝伸展筋力を比較した結果においては，男女とも，膝OAの有無では膝伸展筋力に有意差を認めなかったが，変形の程度を3段階別に分けた場合の同筋力（平均±SE）は，女性では正常例25.4±0.26kg，軽度変形例26.0±0.26kg，重度変形例24.4±0.57kgであり，軽度変形と重度変形例の間に有意差を認めた（p

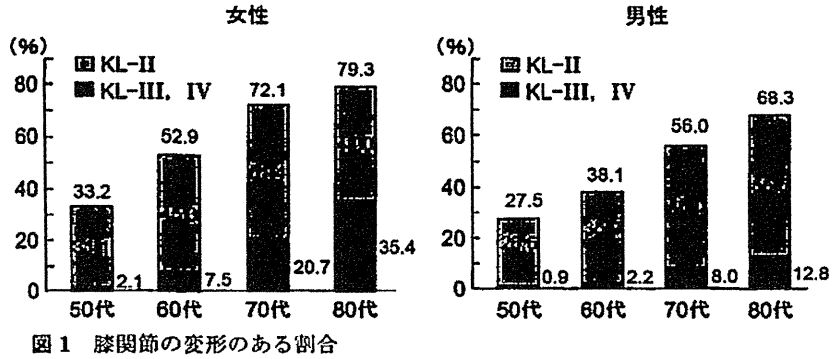

図1　膝関節の変形のある割合

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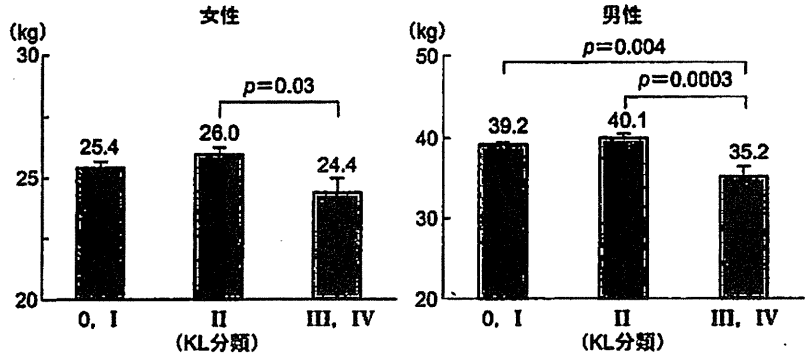

図2　変形の程度による膝伸展筋力の比較
年齢，BMIを調整（平均値±SE）

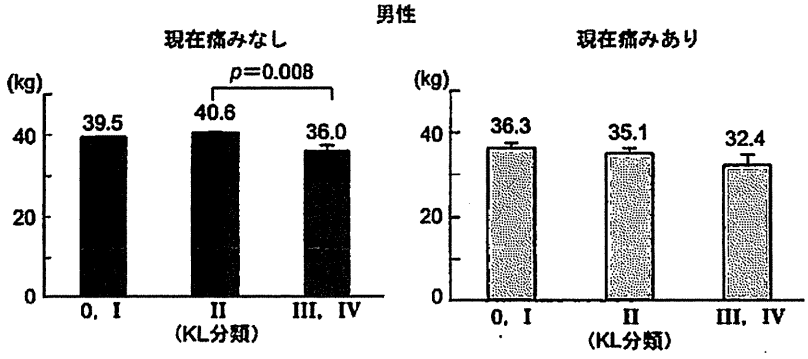

図3　痛みの有無別での変形の程度による膝伸展筋力の比較
年齢，BMIを調整（平均値±SE）

=0.03）（図2）。また男性においては，正常例39.2±0.29kg，軽度変形例40.1±0.36kg，重度変形例35.2±1.21kgであり，正常と重度変形例，軽度変形と重度変形例の間に有意差を認めた（各 $p=0.004$，$p=0.0003$）（図2）。さらに，現在の痛みの有無別での検討においては，女性では，現在痛みのない群，ある群とも，膝伸展筋力は変形の程度による差を認めなかったが，男性では，現在痛みのない群で，軽度変形と重度変形例の間に有意差を認めた（$p=0.008$）（図3）。

3　考　　察

膝OAの症状改善に大腿四頭筋筋力訓練が有効であることが知られているが，膝関節の変形の程度と同筋力の関連を調べた疫学調査は限られており，一般住民における実態はいまだ不明な点が多い。過去の主な横断的な疫学研究においては，欧米の報告ではSlemandaらはKL分類Ⅱ度以上は，膝伸展筋力（体重比）が関節変形のない群より約20%低下しており，同筋力が10lb-ft増加するごとの膝OAありのオッズ比は0.8であったと報告している[2]。Bakerらは，大腿四頭筋筋力低下は，女性では脛骨大腿関節型膝OA，膝蓋大腿関節型膝OA，両混合型膝OAの有病率と関連し，男性では両混合型膝OAでのみ関連していたとしている[3]。わが国においては，松代膝検診ではKL分類進行に伴い男女ともに大腿四頭筋筋力は低下し，女性はKL分類Ⅱ度とⅢ度の間において，男性はKL分類Ⅲ度とⅣ度の間に有意差を認めたと報告されている[4]。ROAD（Research on Osteoarthritis Against Disability）studyの漁村コホートでは，男性はKL分類Ⅳ度

が0度と比べ，女性はKL分類Ⅲ度とⅣ度が0度と比べ膝伸展筋力が有意に低下しており[5]，また，下肢筋力の高い群はKL分類Ⅲ度以上の有病率が男性13.6%，女性14.5%であったのに対し，筋力の低い群では男性28.4%，女性36.1%と，筋力が低い群で有意に高かったと報告されている[6]。これらわが国の報告は，膝関節軽度屈曲位にて等尺性の膝伸展筋力を測定したものである。しかしながら変形性膝関節症では，通常歩行時より椅子からの立ち上がり動作時などのような，膝をより深く曲げた状態での症状が多く，今回の膝関節90度屈曲位での膝伸展筋力と膝関節変形との関連の検討はさらに意義深いと考えられる。結果として，男女ともに，軽度変形例では筋力低下を認めずに重度変形でのみ筋力低下を認め，今回検討した範囲では軽度屈曲位で測定した結果とおおむね同様であった。また痛みの有無別の検討にて男女で違いがあり，今後その意義などの検討が必要である。

結　　語

地域在住中高年者を対象とした大規模コホートにて，膝関節変形の有無や程度と膝伸展筋力との関連を検討した結果，軽度変形例と比べて重度変形例で男女ともに膝伸展筋力の低下をきたしていた。

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表Ⅶ-5　性・年齢階級別、飲酒習慣

		30-39歳		40-49歳		50-59歳		60-69歳		70歳-		計	
		実数	%	実数	%	実数	%	実数	%	実数	%	実数	%
性　別	--飲酒習慣--												
男　性	以前から（ほとんど）飲んでいない	174	43.6	191	41.1	203	33.4	210	31.8	208	42.4	986	37.6
	以前は飲酒習慣があったが現在なし	12	3.0	17	3.7	33	5.4	74	11.2	95	19.3	231	8.8
	現在飲酒の習慣有り	213	53.4	257	55.3	371	61.1	376	57.0	188	38.3	1405	53.6
	計	399	100.0	465	100.0	607	100.0	660	100.0	491	100.0	2622	100.0
女　性	--飲酒習慣--												
	以前から（ほとんど）飲んでいない	499	83.3	573	87.1	752	88.2	672	92.1	641	95.2	3137	89.3
	以前は飲酒習慣があったが現在なし	14	2.3	9	1.4	16	1.9	4	0.5	8	1.2	51	1.5
	現在飲酒の習慣有り	86	14.4	76	11.6	85	10.0	54	7.4	24	3.6	325	9.3
	計	599	100.0	658	100.0	853	100.0	730	100.0	673	100.0	3513	100.0
男女計	--飲酒習慣--												
	以前から（ほとんど）飲んでいない	673	67.4	764	68.0	955	65.4	882	63.5	849	72.9	4123	67.2
	以前は飲酒習慣があったが現在なし	26	2.6	26	2.3	49	3.4	78	5.6	103	8.8	282	4.6
	現在飲酒の習慣有り	299	30.0	333	29.7	456	31.2	430	30.9	212	18.2	1730	28.2
	計	998	100.0	1123	100.0	1460	100.0	1390	100.0	1164	100.0	6135	100.0

表Ⅶ-6　性・年齢階級別、飲酒習慣・量

		30-39歳		40-49歳		50-59歳		60-69歳		70歳-		計	
		実数	%	実数	%	実数	%	実数	%	実数	%	実数	%
性　別	--飲酒習慣・量--												
男　性	現飲酒３合以上	38	9.5	39	8.4	64	10.6	48	7.3	18	3.7	207	7.9
	現飲酒２合	67	16.8	76	16.4	139	22.9	130	19.7	53	10.8	465	17.8
	現飲酒１合	107	26.8	140	30.2	165	27.2	197	29.8	116	23.7	725	27.7
	現飲酒　量不明	1	0.3	1	0.2	2	0.3	1	0.2	0	0	5	0.2
	過去飲酒	12	3.0	17	3.7	33	5.4	74	11.2	95	19.4	231	8.8
	飲酒歴無し	174	43.6	191	41.2	203	33.5	210	31.8	208	42.4	986	37.6
	計	399	100.0	464	100.0	606	100.0	660	100.0	490	100.0	2619	100.0
女　性	--飲酒習慣・量--												
	現飲酒３合以上	5	0.8	6	0.9	7	0.8	6	0.8	2	0.3	26	0.7
	現飲酒２合	16	2.7	10	1.5	18	2.1	7	1.0	0	0	51	1.5
	現飲酒１合	63	10.5	60	9.1	56	6.6	39	5.4	21	3.1	239	6.8
	現飲酒　量不明	2	0.3	0	0	4	0.5	0	0	1	0.1	7	0.2
	過去飲酒	14	2.3	9	1.4	16	1.9	4	0.5	8	1.2	51	1.5
	飲酒歴無し	499	83.3	573	87.1	752	88.2	672	92.3	641	95.2	3137	89.3
	計	599	100.0	658	100.0	853	100.0	728	100.0	673	100.0	3511	100.0
男女計	--飲酒習慣・量--												
	現飲酒３合以上	43	4.3	45	4.0	71	4.9	54	3.9	20	1.7	233	3.8
	現飲酒２合	83	8.3	86	7.7	157	10.8	137	9.9	53	4.6	516	8.4
	現飲酒１合	170	17.0	200	17.8	221	15.1	236	17.0	137	11.8	964	15.7
	現飲酒　量不明	3	0.3	1	0.1	6	0.4	1	0.1	1	0.1	12	0.2
	過去飲酒	26	2.6	26	2.3	49	3.4	78	5.6	103	8.9	282	4.6
	飲酒歴無し	673	67.4	764	68.1	955	65.5	882	63.5	849	73.0	4123	67.3
	計	998	100.0	1122	100.0	1459	100.0	1388	100.0	1163	100.0	6130	100.0

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Cryopreservation of Induced Pluripotent Stem Cells

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Induced pluripotent stem (iPS) cells have attracted attention as a promising cell source for medical treatment that could replace marrow stromal cells (MSCs) and adipose tissue-derived stem cells (ASCs). These pluripotent cells can be induced in vitro and in vivo to differentiate into various tissues and organs. The cells will be useful for regenerative medicine, cell therapy, and drug screening. Vitrification is used, as well as a rapid-freeze method, for colony-forming iPS cells. However, the method requires a high degree of technical skill. We herein report a more convenient method for freezing iPS cells in suspension. We examined the proliferation potency of cryopreserved mouse iPS cells using culture medium, 10% DMSO, 10% glycerol, 5% DMSO, 5% glycerol, 5% DMSO+5% glycerol, cell-freezing medium-DMSO, cell-freezing medium-glycerol, Cell Banker 1, Cell Banker 1+, Cell Banker 2, and Cell Banker 3 as cryopreservation solutions. Among them, Cell Banker 3 showed the highest efficacy in terms of the proliferation of mouse iPS cells. The mouse iPS cells cryopreserved in Cell Banker 3 at –80°C for 12 months maintained a high proliferation rate and an undifferentiated status. The formation of teratomas was also examined. In conclusion, Cell Banker 3 allows for freezing of iPS cells in suspension.

Key words: Induced pluripotent stem (iPS) cells; Pluripotency; Cryopreservation; Slow freezing

INTRODUCTION

Induced pluripotent stem (iPS) cells (14,17,23,24) are pluripotent, thus allowing them to differentiate into various cell types, and these cells have a self-renewal capability similar to that of embryonic stem (ES) cells (20,26). The use of ES cells poses the ethical dilemma of requiring the breakdown of fertilized eggs and also is associated with the potential for immune rejection during cell transplantation. These problems can be solved by using iPS cells. Therefore, iPS cells will be useful for regenerative medicine, cell therapy, and drug screening.

Cryopreservation of cells is an essential technique in basic research on cell biology and in clinical use for cell transplantation. Most cell lines and primary cells are provided as frozen cells. A large number of high-quality cells can be supplied to patients at any time by using this technology. Cryopreservation of sperm, ova, and fertilized eggs is currently performed in clinical practice throughout the world. The iPS cells are an alternative promising cell source of pluripotent cells that can be used in place of marrow stromal cells (MSCs) and adipose tissue-derived stem cells (ASCs). However, the viability of human iPS cells decreases significantly during cryopreservation, as does that of human ES cells. To solve this problem, the vitrification technique used for cryopreservation of fertilized eggs, embryos, and oocytes (5,8,18,19) was examined to freeze ES and iPS cells (7,15,21). Vitrification is an effective cryopreservation technique, but the cells are damaged if there is an increase in osmotic pressure. Therefore, more effective and less cell toxic solutions, as well as more convenient techniques, are strongly desired.

The authors have previously reported a new cryopreservation technique in which ES cells and mouse embryonic fibroblast (MEF) feeder cells were seeded on collagen vitrigel (10). The morphology of these ES cells was good, and the survival rate was high after thawing. In contrast to vitrification, slow freezing in suspension is very convenient

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to perform and is frequently used for the cryopreservation of various other types of cells. However, when this convenient method is used for ES and iPS cells, it is necessary to examine their condition, because such a freezing process can result in low cell viability or a loss of their pluripotency.

In the present study, the effects of cryopreservation solutions containing dimethyl sulfoxide (DMSO) or glycerol and commercially available cryopreservation solutions were compared for mouse iPS cells. We confirmed that mouse iPS cells could be preserved at –80°C for at least a year and that the cells were maintained in an undifferentiated state.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotics (penicillin, streptomycin), and MEM nonessential amino acids solution (NEAA) were purchased from GIBCO BRL, Life Technologies (Grand Island, NY). Fetal bovine serum (FBS, BIO-WEST) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Phosphate-buffered saline (PBS), 2-mercaptoethanol (M6250), glycerol, DMSO (D2650), and formaldehyde were obtained from Sigma-Aldrich (St. Louis, MO). Leukemia inhibitory factor (LIF, Chemicon) was from Dainippon Sumitomo Pharma (Osaka, Japan). Hematoxylin and eosin were purchased from Muto Pure Chemicals (Tokyo, Japan). All chemicals were reagent grade and used as received without further purification.

Mice

Nude mice (BALB/cA Jcl-nu) were purchased from Clea Japan (Osaka, Japan). These mouse studies were approved by the institutional animal care and use committees (IACUC).

Cells

The iPS cell line (iPS-MEF-Ng-20D-17) established by Professor S. Yamanaka at Kyoto University was obtained from the Cell Bank of Riken Bioresource Center (22). MEF feeder cells (Chemicon) were purchased from Dainippon Sumitomo Pharma (Osaka, Japan).

iPS Cell and MEF Feeder Cell Culture

Mouse iPS cells were cultured with mitomycin C-treated feeder cell layers as follows. First, MEF cells were cultured at 37°C with 5% CO_2 in MEF culture medium (DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin) and maintained until the cells reached confluence. The cells were then treated with 50 µl of 100×mitomycin C and incubated at 37°C for at least 2 h. The cells were then cultured on 0.1% gelatin-coated plate for more than 5 h. The iPS cells were maintained at 37°C with 5% CO_2 in ES cell culture medium (DMEM containing 15% FBS, 1×NEAA, 1 mM sodium pyruvate, 2 mM 2-mercaptoethanol, 1% penicillin–streptomycin and 1,000 U/ml LIF) on feeder layers of mitomycin-treated MEF cells. The medium was changed to fresh ES cell culture medium everyday, and the cells were passaged every third day (17,22,25).

Cell Freezing and Thawing Procedures

The basal composition of the cryopreservation medium in this study was the same as that of the ES cell culture medium. Cryoprotectant agents (glycerol and DMSO) were added to the ES cell culture medium at concentrations of 5–10%. The other cryopreservation solutions used were the cell-freezing medium-DMSO, cell-freezing medium-glycerol, Cell Banker 1, Cell Banker 1$^+$, Cell Banker 2, and Cell Banker 3. One milliliter of cell suspension containing 5×10^6 cells was quickly transferred to a 2.0-ml freezing tube and frozen at a cooling rate of 1°C/min. After cooling to –80°C, the cells were stored until use (typically from 1 to 12 months). Frozen tubes were placed in a 37°C water bath to thaw until most ice crystals were melted. The cell suspension was then diluted 1:9 with ES cell culture medium and was centrifuged at 100 *g* for 1 min. The supernatant was removed, and the cells were resuspended in fresh medium. Cell viability was assessed using the trypan blue exclusion test. The final concentration of trypan blue (GIBCO BRL, Grand Island, NY) was 0.2% in the experiments.

Proliferation Assay of iPS Cells

The mitomycin-treated MEF cells (2×10^4 cells) were seeded on 0.1% gelatin-coated 24-well plates (BD Biosciences) with 0.5 ml of MEF culture medium. After culture for 24 h, the cryopreserved iPS and MEF cells (3×10^4 cells) were cultured on mitomycin C-treated feeder cell layers. The cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). The CCK-8 reagent (30 µl) was added to each well (300 µl), and the reaction was allowed to proceed for up to 15 min. The absorbance of the sample at 450 nm was measured against a background control using a microplate reader. The cell proliferation was evaluated after 0–72 h.

Teratoma Formation and Histological Analysis

Mouse iPS cells were suspended at 1×10^7 cells/ml in PBS. Nude mice were anesthetized with diethyl ether. A total of 100 µl of the cell suspension (1×10^6 cells) was injected subcutaneously into the dorsal flank of nude mice. Four weeks after the injection, the tumors were surgically dissected from the mice. The samples were weighed, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin.

RESULTS

Cryopreservation of Mouse iPS Cells and MEF Feeder Cells in Various Solutions

The iPS cells and MEF feeder cells were frozen and preserved at –80°C for 3 months. These cells were cryopreserved in the following solutions: ES cell culture medium, ES cell culture medium containing 10% DMSO, ES cell culture medium +10% glycerol, ES cell culture medium +5% DMSO, ES cell culture medium +5% glycerol, ES cell culture medium +5% DMSO, 5% glycerol, cell-freezing medium-DMSO, cell-freezing medium-glycerol, Cell Banker 1, Cell Banker 1+, Cell Banker 2, and Cell Banker 3. In order to investigate the effects of cryopreservation on cell functions, we determined cell viability immediately after thawing (Fig. 1A) and also examined

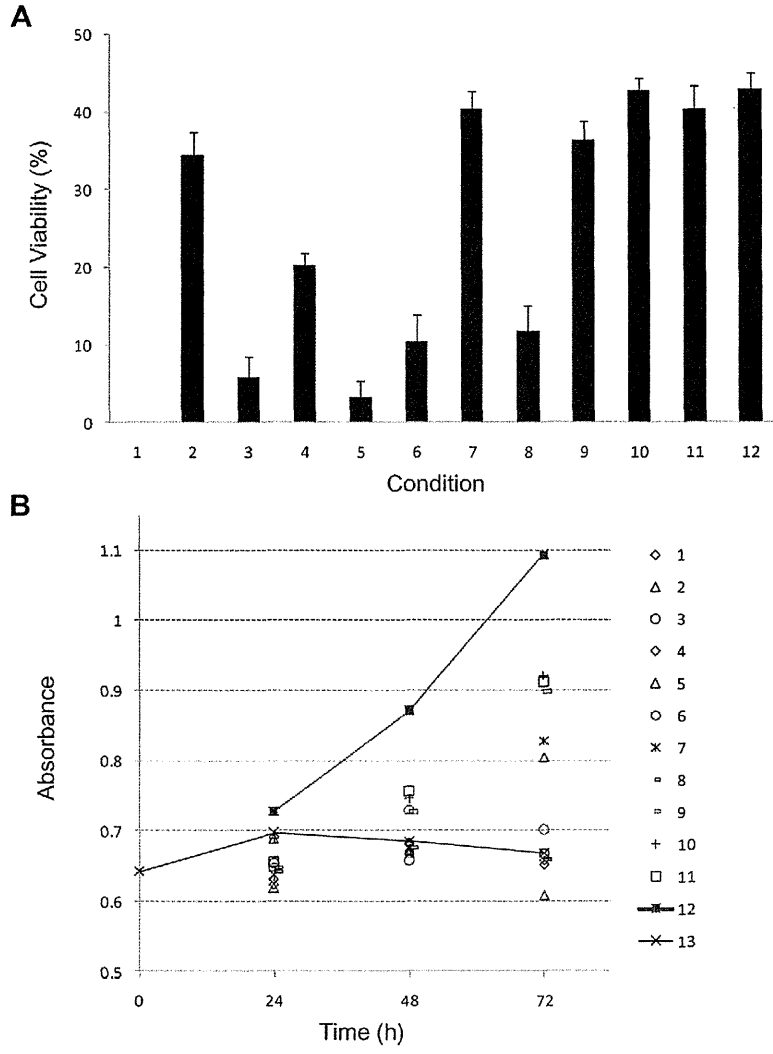

Figure 1. The viability (A) and proliferation (B) of the cryopreserved induced pluripotent stem (iPS) cells frozen using different preservation solutions. 1, Embryonic stem (ES) cell culture medium; 2, ES cell culture medium containing 10% dimethyl sulfoxide (DMSO); 3, ES cell culture medium +10% glycerol; 4, ES cell culture medium +5% DMSO; 5, ES cell culture medium +5% glycerol; 6, ES cell culture medium +5% DMSO, 5% glycerol; 7, cell-freezing medium-DMSO; 8, cell-freezing medium-glycerol; 9, Cell Banker 1; 10, Cell Banker 1+; 11, Cell Banker 2; 12, Cell Banker 3; 13, only mouse embryonic fibroblast (MEF) feeder cell. The data are the means and SD of three independent experiments.

cell proliferation (Fig. 1B). The viability of iPS cells and MEF feeder cells in 10% DMSO, cell-freezing medium DMSO, Cell Banker 1, Cell Banker 1+, Cell Banker 2, and Cell Banker 3 was shown to be over 30% (Fig. 1A). It was difficult to evaluate the cell viability (10–32%) of only the MEF feeder cells (data not shown). The proliferation of the cells was monitored for 72 h using a commercially available cell-counting reagent (Fig. 1B). The cryopreserved iPS cells in 10% DMSO, cell-freezing medium-DMSO, Cell Banker 1, Cell Banker 1+, Cell Banker 2, and Cell Banker 3 showed higher potency than the MEF feeder cells. The proliferation of the iPS cells frozen in Cell Banker 3 showed the highest proliferation among the 12 cryopreserved solutions. Three days after the inoculation, both iPS and MEF feeder cells adhered and grew well on MEF feeder cell layers (Fig. 2A and B). The cells cryopreserved in 10% DMSO, cell-freezing medium-DMSO, Cell Banker 1, Cell Banker 1+, Cell Banker 2, and Cell Banker 3 were identified as iPS cells. The iPS cells frozen in Cell Banker 1, Cell Banker 1+, Cell Banker 2, and Cell Banker 3 had a morphology similar to that of undifferentiated cells (Fig. 2B; 9–12).

Long-Term Cryopreservation of Mouse iPS Cells and MEF Feeder Cells

To examine the quality of cryopreserved iPS cells in Cell Banker 3 at –80°C for 12 months, we investigated the viability, proliferation, and morphology of the cryopreserved cells (Fig. 3). No significant difference in cell viability or the proliferation rate was observed from the results for iPS cells and MEF feeder cells cryopreserved for 3–12 months (Fig. 3A and B). The cells cryopreserved in Cell Banker 3 were identified as undifferentiated iPS cells (Fig. 3C, 1). Fluorescent microscopy demonstrated that green fluorescent protein genes derived from undifferentiated iPS cells were expressed in these cultures (Fig. 3C, 2).

Teratoma Formation by Cryopreserved Mouse iPS Cells

To evaluate the pluripotency of cryopreserved iPS cells, we transplanted the cells into the dorsal flank of nude mice. The iPS cells produced teratomas after transplantation (Fig. 4A). The teratomas contained various tissues, such as arteries, nerves, cartilage, adipose, and gut epithelium belonging to the three germ layers (endoderm, mesoderm, and ectoderm) (Fig. 4B–F).

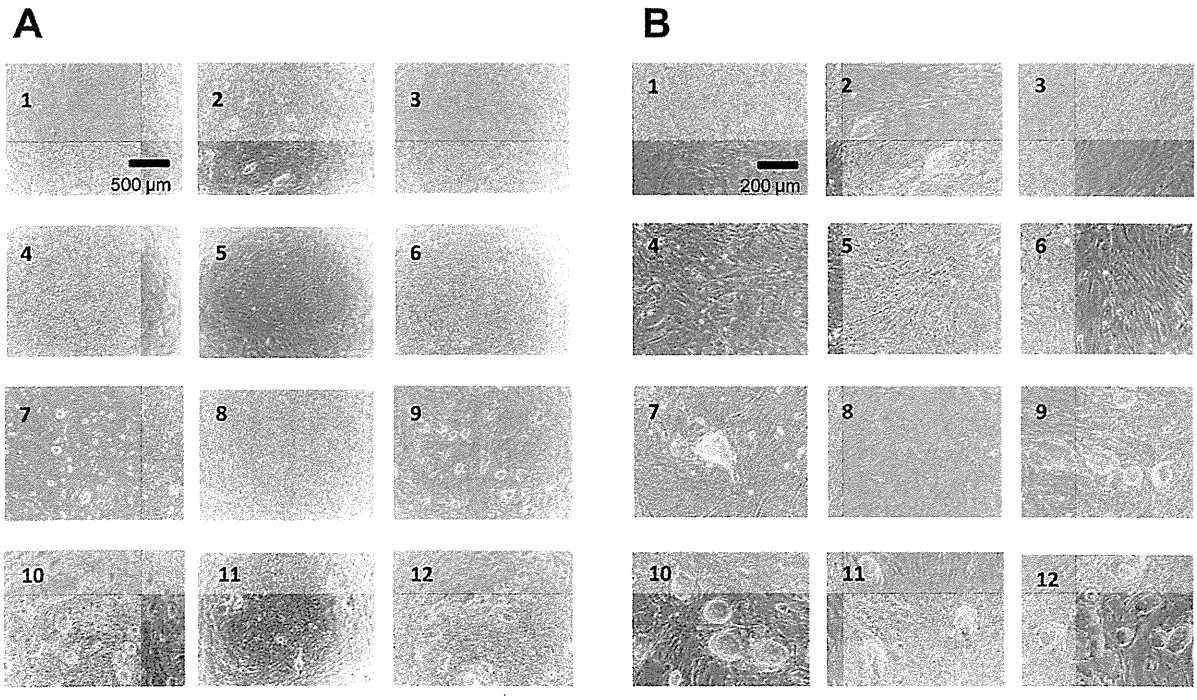

Figure 2. The phase-contrast photomicrographs of iPS cells after cryopreservation (1)–(12). 1, ES cell culture medium; 2, ES cell culture medium containing 10% DMSO; 3, ES cell culture medium + 10% glycerol; 4, ES cell culture medium +5% DMSO; 5, ES cell culture medium +5% glycerol; 6, ES cell culture medium +5% DMSO; 5% glycerol; 7, cell-freezing medium-DMSO; 8, cell-freezing medium-glycerol; 9, Cell Banker 1; 10, Cell Banker 1+; 11, Cell Banker 2; 12, Cell Banker 3. The photomicrographs were taken with ×40 (A) and ×100 (B) objectives. The iPS cells were cultured on mitomycin-treated MEF cells for 3 days after inoculation. Scale bars: 500 µm (A) and 200 µm (B).

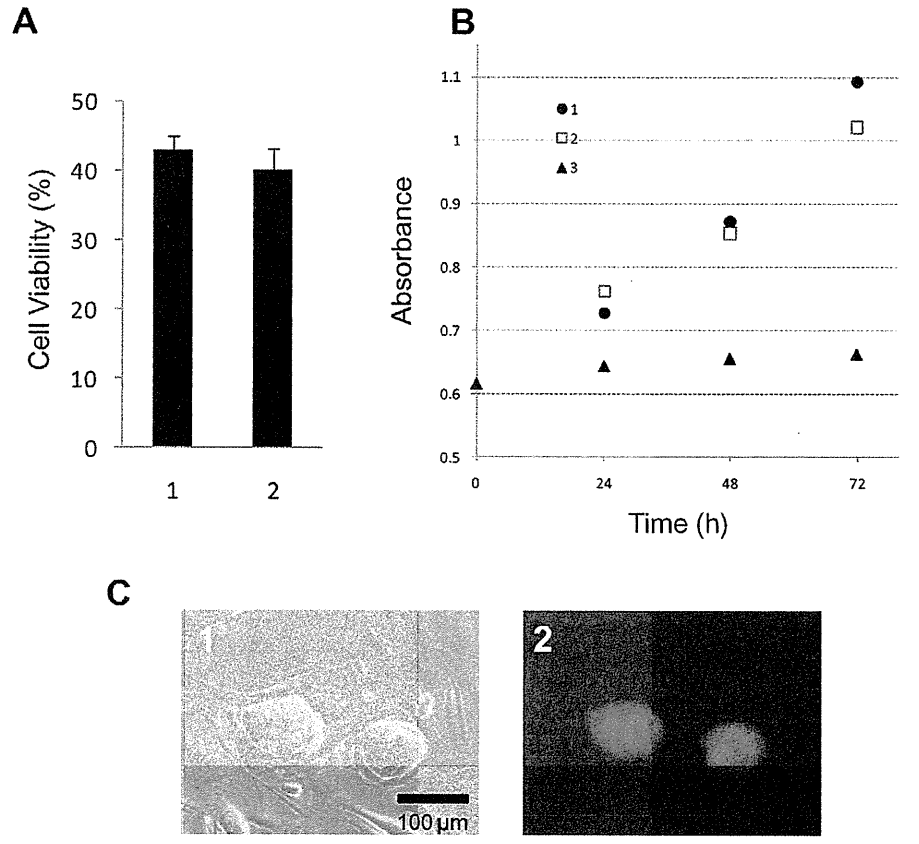

Figure 3. Evaluation of the iPS cells cryopreserved with Cell Banker 3 after 3 and 12 months of storage. (A) The viability of the cryopreserved iPS cells after 3 and 12 months of storage at –80°C. 1, After 3 months; 2, after 12 months. (B) The proliferation of cryopreserved iPS cells after 3 and 12 months of storage. 1, After 3 months; 2, after 12 months; 3, only the MEF feeder cell layer. The data are the means and SD of three independent experiments. (C) The phase-contrast photomicrographs of (1) cryopreserved iPS cells after 12 months. The fluorescent photomicrograph (2) exhibited the expression of green fluorescent protein derived from undifferentiated iPS cells. (C) Scale bar: 100 μm.

DISCUSSION

The iPS cells are a promising cell source for regenerative medicine and cell transplantation therapy. The vitrification process has been used as a rapid-freezing method for colony-forming iPS and ES cells (7,15,21). However, this method requires a high degree of technical skill. We herein report a convenient method for freezing iPS cells in suspension.

In recent years, a wide variety of cryopreservation solutions have been examined to preserve cells without compromising their viability. DMSO and glycerol are conventionally used for the cryopreservation of cells, but they may affect cell functions because of their cytotoxicity (1,9). Either disaccharide trehalose (2–4), oligosaccharide (12), sericin (11,13), or antifreeze proteins (AFP) (6) can be used as a cryopreservation reagent.

In the present study, the functions of cryopreserved iPS cells were investigated using the 12 kinds of newly prepared and commercially available cryopreservation solutions. The iPS cells cryopreserved at –80°C using Cell Banker 3 showed the highest cell viability and proliferation of all the solutions examined in this study (Fig. 1). In addition, the iPS cells cryopreserved in Cell Banker 3 at –80°C for 12 months maintained a high proliferation rate and remained undifferentiated (Fig. 3), and these cells formed teratomas when injected into nude mice (Fig. 4). The cryopreservation technique using Cell Banker 3 can be widely used because it does not require any special technical skills.

The other serum-containing cryopreservation solutions, such as the prepared solutions (DMSO), Cell Banker 1, and Cell Banker 1$^+$, and serum-free Cell Banker 2, were

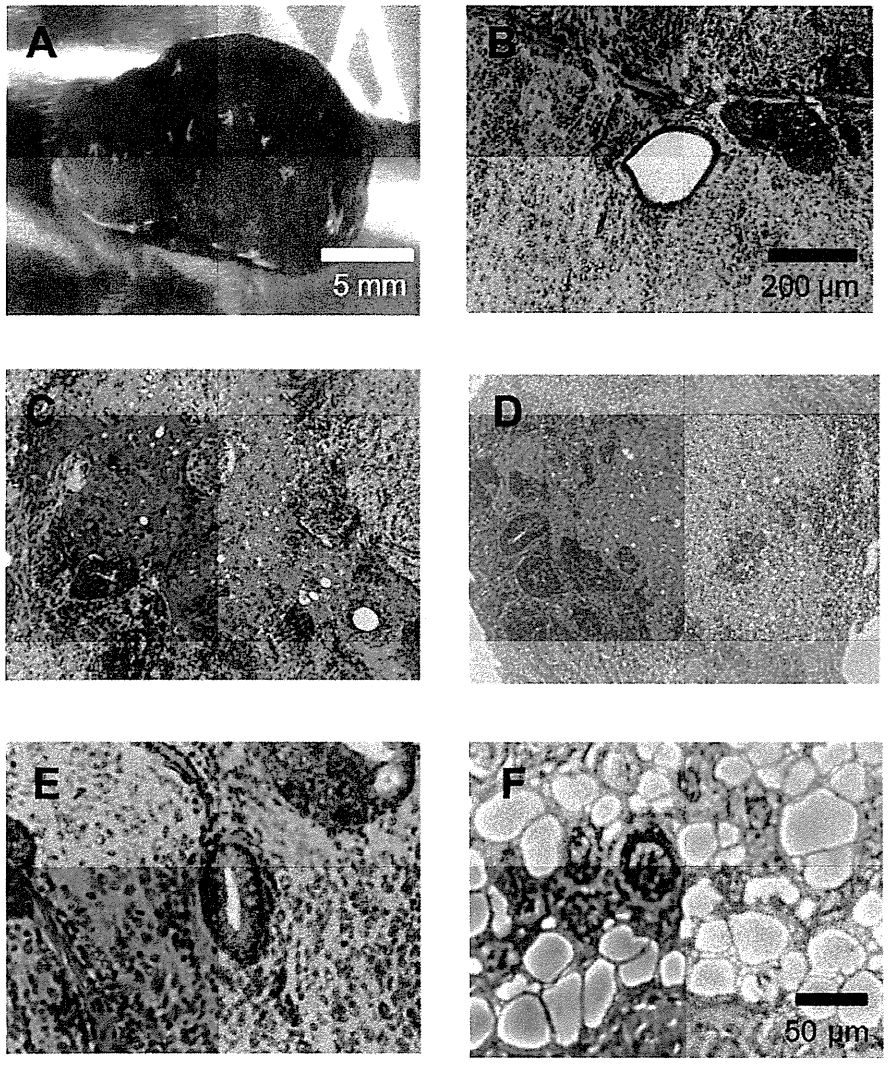

Figure 4. Teratoma formation by iPS cells. Various tissues were present in teratomas derived from iPS cells. (A) Four weeks after the injection, the teratoma was surgically dissected from the mice. (B–F) Slides were stained with hematoxylin and eosin. (B) Artery-like structures. (C) Nerve-like structures. (D) Cartilage-like structures. (E) Gut epithelium-like structures. (F) Adipose-like structures.

also effective for the preservation of iPS cells. In particular, serum-free cryopreservation solutions (Cell Banker 2 and Cell Banker 3) will be useful for regenerative medicine and transplantation.

We previously reported that Cell Banker 2 was effective for the cryopreservation of mouse and human ASCs (11,16). Comparison of the viability, proliferation, and multipotency of mouse ASCs between Cell Banker 2 and Cell Banker 3 showed that Cell Banker 3 led to better preservation of the cells (data not shown). Cell Banker 3 had a similar effect on the preservation of mouse iPS cells as on mouse ASCs and is being used for the preservation of embryos and tissue stem cells, as well as primary hepatocytes, etc.

In conclusion, Cell Banker 3 allows for the effective freezing of iPS cells in suspension.

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