ductivity and osseointegration properties. However, they do not induce osteogenic differentiation, i.e., they lack osteoinductivity. Moreover, problems arise associated with their slow biodegradability and their association with inflammation because of immunologic reactions.

Further modification of PLGA/COL sheets for bone tissue engineering

To circumvent these limitations, natural or synthetic materials and composite scaffolds based on poly(lactic acid), poly(glycolic acid), and their co-polymer, PLGA, have been developed to increase biodegradability (Ishaug et al. 1997; Ishaug-Riley et al. 1998) and decrease immunological reactions (Mikos et al. 1998). These synthetic polymers are mechanically stronger (Boyan et al. 1999) than naturally derived polymers, such as collagen, and the scaffold can be used either alone, in combination with osteoinductive growth factors, or with osteoconductive inorganic materials (Chen et al. 2001b; Kikuchi et al. 2002). Growth factors, such as bone morphogenetic proteins (Lane et al. 1999; Oldham et al. 2000; Peter et al. 2000) and vascular endothelial growth factor (Murphy et al. 2000; Tabata et al. 2000), can be incorporated into these synthetic polymers, and small hydroxyapatite particles can also be coated onto the polymers. Thus, the PLGA/COL hybrid sheet can be endowed with osteoinductivity and osteoconductivity to shorten the osteogenesis period after implantation and to obtain mechanical strength with plasticity. However, even after the addition of hydroxyapatite, the mechanical strength of the scaffold may be insufficient to maintain its original shape when the scaffold is used to treat long bone defects, and greater strength may be required to resist excessive mechanical overload or to support body weight. There is also concern that the acidic milieu associated with PLGA degradation may be toxic to cells and may induce inflammation (Bostman 1991; Wake et al. 1998), but we have found no evidence of such adverse reactions in our study.

Synthetic polymers are currently used for a number of orthopedic devices, including suture anchors and interference screws. Collagraft (Zimmer, Warsaw, Ind.), a composite of porous calcium phosphate granules and bovine-derived fibrillar collagen for bone regeneration (Cornell et al. 1991), was approved by the US Food and Drug Administration (FDA) in 1993 (Naughton 2002). Our hybrid sheet also consists of matrices that have been approved by the FDA. Some tissue-engineered skin replacement products are on the market, and the technology of tissue engineering for sheet materials is well-established. The scaffold used in this study retains large numbers of osteoprogenitors or osteoblasts derived from bone marrow, and because of its flexibility, assembles them into tissue of the desired shape in mice. The hybrid sheet is expected to become a useful scaffold for bone tissue engineering, and by taking advantage of its unique sheet form, it may be applicable elsewhere, such as in the regeneration of skin, blood vessels, ligaments, and periosteum, in addition to bone tissue engineering.

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Gene Therapy (2004) 11, 1475–1481

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RESEARCH ARTICLE

Brain transplantation of genetically modified bone marrow stromal cells corrects CNS pathology and cognitive function in MPS VII mice

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Current therapies for lysosomal storage diseases (LSDs), enzyme replacement therapy and bone marrow transplantation are effective for visceral organ pathology of LSD, but their effectiveness for brain involvement in LSDs is still a subject of controversy. As an alternative approach, we transplanted genetically modified bone marrow stromal (BMS) cells to lateral ventricle of newborn mucopolysaccharidosis VII (MPS VII) mice. MPS VII is one of LSDs and caused by deficiency of beta-glucuronidase (GUSB), resulting in accumulation of glycosaminoglycans (GAGs) in brain. At 2 weeks after transplantation, the GUSB enzyme-positive cells were identified in olfactory bulb, striatum and cerebral cortex, and the enzymatic activities in various brain areas increased. The GAGs contents in brain were reduced to near normal level at 4 weeks after transplantation. Although GUSB activity declined to homozygous level after 8 weeks, the reduction of GAGs persisted for 16 weeks. Microscopic examination indicated that the lysosomal distention was not found in treated animal brain. Cognitive function in MPS VII animals as evaluated by Morris Water Maze test in treated mice showed a marked improvement over nontreated animals. Brain transplantation of genetically modified BMS cells appears to be a promising approach to treat diffuse CNS involvement of LSDs.
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Keywords: *bone marrow stromal cells; MPS VII; intraventricular transplantation; beta-glucuronidase; Morris Water Maze*

Introduction

Mucopolysaccharidosis type VII (MPS VII, Sly syndrome) is one of lysosomal storage diseases (LSDs) caused by deficiency of beta-glucuronidase (GUSB), resulting in progressive accumulation of undegraded glycosaminoglycans (GAGs) in various tissues including the brain.[1,2] A murine model of MPS VII is available and its clinical, biochemical and pathological features closely reflect those of human MPS VII.[3–5] Using this mouse model, various therapeutic approaches including enzyme replacement therapy,[6–8] bone marrow transplantation (BMT),[9–11] gene therapy[12–14] and cell therapy[15–17] have previously been attempted with varying degree of success. In case of enzyme replacement therapy, patients must continue to receive enzyme replacement in their whole life and the cost of recombinant enzyme is another big burden to patients. BMT results in high mortality and morbidity rate, especially at early infancy. In gene therapy, although AAV vectors appear to have a promise, it is still difficult to produce large amount of recombinant virus. The safety issue of lentivirus vector is another major concern for use of this virus vector to human. In cell therapy, although transplantation of neural stem cells or amniotic cells shows a great hope to treat CNS involvement in MPS VII, ethical and immunologic problems must be cleared.

Recent studies have demonstrated that the bone marrow stromal (BMS) cells have ability to differentiate into osteocytes, chondrocytes, adipocytes, muscle and also into neural cells,[18–22] and that the brain transplantation of BMS cells induces good clinical outcome in neurological diseases such as brain infarction[23] and LSD.[24] Patient's BMS cells could be harvested by a simple bone marrow aspiration and grow rapidly in culture, and autologous transplantation can overcome ethical and immunologic problems associated with transplantation of neural stem cells or amniotic cells. In the present study, we demonstrate that the intraventricular transplantation of BMS cells overexpressing GUSB in neonatal MPS VII mice corrects the CNS pathology and function in these mutant mice.

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Results

Transduction of KUSA/A1 by MND/HBG

KUSA/A1, mouse bone marrow stromal cell, was transduced with human GUSB gene by retrovirus vector. The GUSB activity in transduced cell (KUSA/HBG) was increased from 5.9×10^1 to 1.9×10^6 nmol/h/mg. Histochemical staining of GUSB-bearing KUSA/HBG cells exhibited positive staining for GUSB, while parental

KUSA/A1 cells were negative for the staining (data not shown). These results are consistent with the data of enzymatic activity.

Distribution of GUSB activity in the brain after transplantation

At 2 and 8 weeks after transplantation, GUSB activities in the recipient brains were determined. Serial coronal sections of mouse brain as shown in Figure 1 were collected and GUSB activity was assayed quantitatively in each section. GUSB activity in all brain regions of treated MPS VII mice 2 weeks after transplantation was higher than in those of age-matched untreated MPS VII mice (Figure 1). GUSB activity of treated MPS VII mouse brain was approximately 50 times higher than that of untreated MPSVII mice in each section. This value corresponds to approximately 20% of heterozygote mouse level. At 8 weeks after transplantation, GUSB activity in treated mouse brain declined to homozygote mouse level.

Biochemical response of the brain in treated MPS VII mice

It has been known that the enzyme activity of other lysosomal enzymes such as α-galactosidase A (α-Gal) and hexosaminidase (Hex) are elevated in MPS VII mouse brain, and that the reduction of these enzymes by treatment is well correlated with therapeutic effect.[8–10] Thus, to monitor the therapeutic effect of cell transplantation, we assayed enzyme activities of α-Gal and Hex in the brain of mice at 2 weeks after transplantation. Activities of both enzymes in the treated mouse brain decreased to those of heterozygous mouse level (Figure 2a, b).

Histochemical detection of GUSB-positive cells in treated mouse brain

At 2 weeks after transplantation, we examined the survival and distribution of transplanted cells by GUSB histochemical analysis. In transplanted mice GUSB positive cells (red) were found not only in meninges and subventricular regions (Figure 3a) but also in

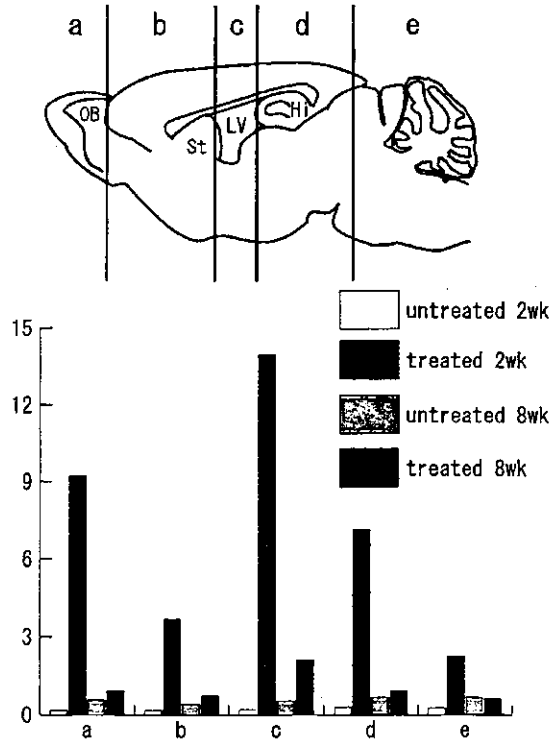

Figure 1 GUSB activity in various regions of the brain after transplantation. Average of GUSB activity in various regions of the brain of mice at 2 (n = 2) and 8 (n = 3) weeks after transplantation and age-matched untreated mutant mice. Mouse brains were divided into serial five coronal sections. The regions were defined by anatomical landmark: a, olfactory bulb; b, from end of olfactory bulb to beginning of striatum; c, striatum to the rostal edge of the hippocampus; d, hippocampus to colliculus and midbrain; e, cerebellum and brain stem. GUSB activity was expressed as nmol/h/mg protein.

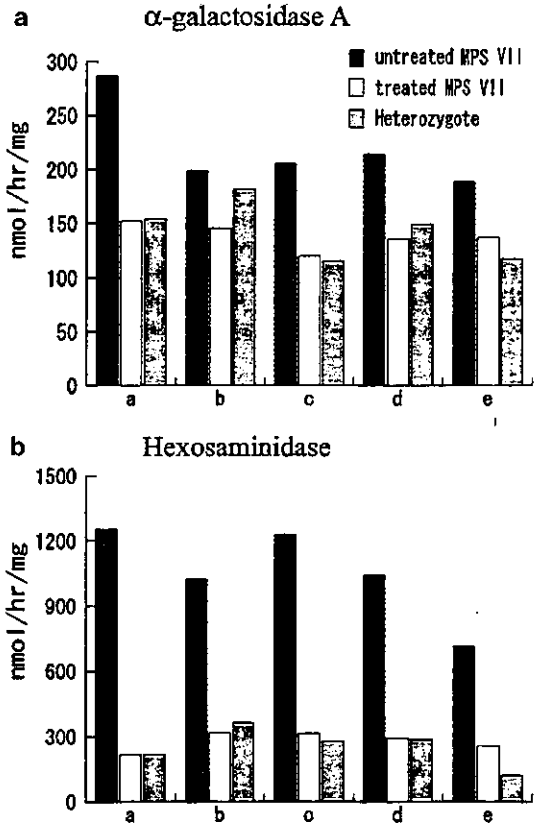

Figure 2 α-Galactosidase A (α-Gal) and hexosaminidase (Hex) activity in various brain regions of treated MPS VII mice. At 2 weeks after transplantation, average activities of α-Gal (a) and Hex (b) in the same tissues (n = 2) as described in Figure 1. These enzymatic activities in the brain of treated MPS VII decreased to heterozygous level. These activities are expressed as nmol/h/mg protein.

Figure 3 Histochemical detection of GUSB-positive cells in treated mouse brain. Cryosection of the brain in treated MPS VII mice at 2 weeks after transplantation. The GUSB positive cells were detected in various regions, (a) subventricle, (b) cerebral cortex, and (c) olfactory bulb, of transplanted MPS VII mouse brain. (bar = 200 μm).

cerebral cortex (Figure 3b) and olfactory bulb (Figure 3c), while GUSB-positive cells were not found in control untreated brain.

Correction of lysosomal distention in the brain of treated MPS VII mice

To evaluate lysosomal distention of MPS VII mouse brain, histopathological analysis was performed in variety of brain regions, such as cerebral cortex, olfactory bulb, and striatum at 4 weeks after transplantation (Figure 4). Profound lysosomal distention in neural cell was observed in age-matched untreated MPS VII mice (indicated by arrow, Figure 4a–c), while this was not observed in olfactory bulb (Figure 4d), striatum (Figure 4e) or cortex (Figure 4f) in treated MPS VII mouse brain. There was no lysosomal distension in the brain of the heterozygous mouse (data not shown).

Reduction in levels of GAGs in the brain of treated MPS VII mice

Levels of GAGs in the brain were determined in treated MPS VII mice at 4, 8, 16 weeks after transplantation (Table 1). In this study, we assayed amount of total chondroitin sulfate (T-CS) and hyaluronic acid (HA), which are known to accumulate in the MPS VII brain. At 4 weeks after transplantation, both of these two GAGs in treated MPS VII mice were reduced significantly as compared to age-matched untreated MPS VII mice (T-CS; $P = 0.0012$, HA; $P = 0.0002$ respectively). Although the enzymatic activity diminished to homozygous level 8 weeks after transplantation, a significant reduction (T-CS; $P = 0.0071$, HA; $P = 0.0001$) of GAGs in the brain of treated MPS VII mice persisted up to 16 weeks after treatment.

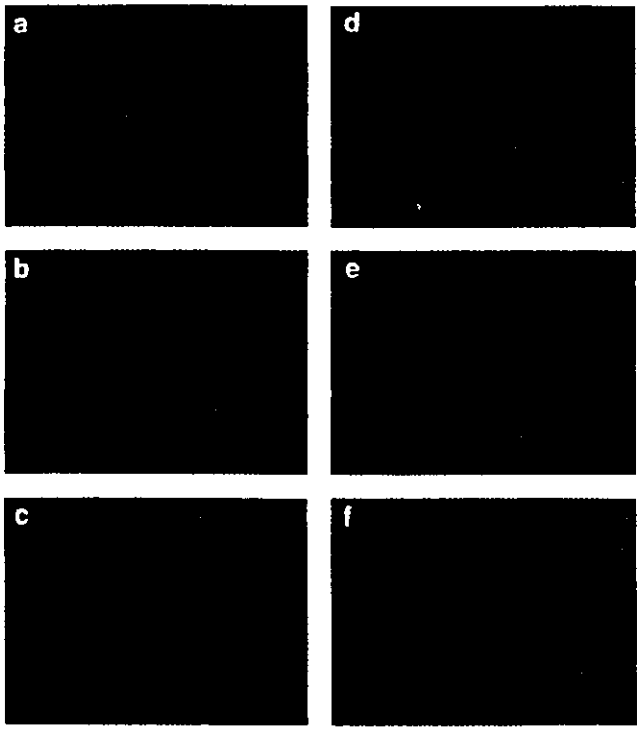

Figure 4 Correction of lysosomal distention in the brain of treated MPS VII mice. Pathological analysis of the MPS VII mouse brain. (a–c) Untreated MPS VII mouse brain. (d–f) Treated MPS VII mouse brain. (a and d) olfactory bulb, (b and e) striatum and (c and f) cerebral cortex of the mouse brain. Although there were many cells with lysosomal distention in the multiple brain regions of untreated MPS VII mice (a–c), all regions of treated MPS VII mouse brain 4 weeks after transplantation (d–f) have no lysosomal storage vacuoles. (bar = 40 μm).

Table 1 Reduction in levels of glycosaminoglycans (GAGs) in the brain of treated MPS VII mice

	Age (n)	*T-CS (μg/g wet-tissue)*	*HA (μg/g wet-tissue)*
Untreated	4 wk ($n = 3$)	316.600 (±14.860)	87.967 (±2.835)
MPS VII	8 wk ($n = 3$)	531.733 (±39.434)	82.667 (±4.447)
	16 wk ($n = 3$)	523.067 (±32.452)	87.400 (±1.877)
Treated	4 wk ($n = 4$)	131.975 (±21.276)*	34.275 (±3.95)**
MPS VII	8 wk ($n = 3$)	255.167 (±15.048)*	34.900 (±2.757)**
	16 wk ($n = 3$)	312.467 (±25.894)*	45.500 (±2.100)**
Normal	4 wk ($n = 3$)	85.800 (±3.980)	24.467 (±1.110)
	8 wk ($n = 3$)	182.400 (±8.228)	36.733 (±5.584)
	16 wk ($n = 3$)	145.267 (±19.368)	44.967 (±5.109)

*$P < 0.01$, **$P < 0.001$.
Versus untreated MPS VII mice.
Total chondroitin sulfate (T-CS) and hyaluronic acid (HA) of GAGs were measured biochemically 4, 8 and 16 weeks after transplantation by HPLC methods. *Indicates significant reduction ($P < 0.01$) *versus* age-matched untreated MPS VII mice. **Indicates significant reduction ($P < 0.001$) *versus* age-matched untreated MPS VII mice.

Improvement of cognitive function with the Morris Water Maze (MWM) test

We performed the visible test before nonvisible test and there was no significant difference of normal mice and untreated MPS VII mice at 6 weeks old (data not shown). These data indicate that there is no significant difference of motor function between wild-type mouse and mutant mouse. At 6 weeks after transplantation, we performed the MWM to evaluate improvement of cognitive function by the treatment. Mice were adapted to the pool at the day before the acquisition phase. The acquisition phase: All of mice were trained from day 1 to day 5 to memorize the platform place. The probe test (Figure 5): The probe test was performed on day 6, immediately after the acquisition phase. The platform was removed and the amount of time spent in each quadrant was monitored. It is well known that normal mice spend longer time in target quadrant where the platform was placed at the acquisition phase than the mean of other three nontarget quadrants. Average of the time spent in the target quadrant by treated mice was 22.4 (s), whereas average of the time spent by untreated mice was 18.2 (s). Treated MPS VII mice spent significantly more time in the target quadrant than in the mean of other three nontarget quadrants ($P = 0.0028$), whereas in untreated MPS VII mice the difference of time spent in the target quadrant and the mean of the other three nontarget quadrants was not statistically significant ($P = 0.3201$).

Discussion

Current available therapies for LSDs are BMT and enzyme replacement therapy. However, both approaches have inherent problems. BMT has high mortality and morbidity rate and enzyme replacement therapy has economic problems and life-long infusion of enzyme

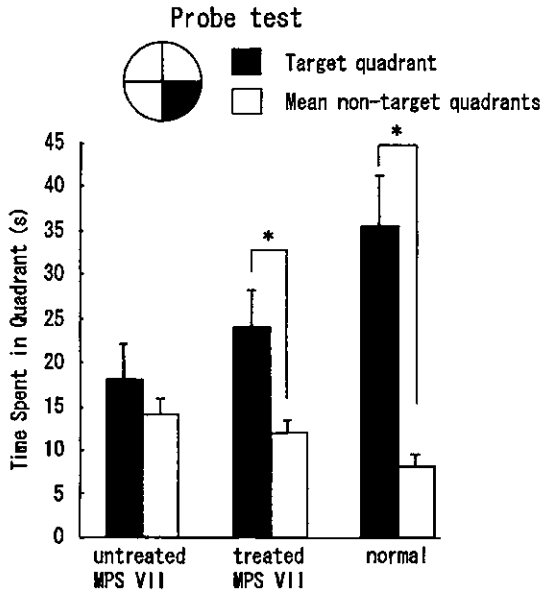

Figure 5 Improvement of cognitive function with the Morris Water Maze Test. The probe test was performed on day 6 immediately after the acquisition phase. After the platform was removed, the amount of time spent in each quadrant was monitored. Filled bars indicate the time spent in the target quadrant, and open bars indicate the average time spent in each of the three nontarget quadrants. Treated MPS VII mice and normal mice spent significantly more time in the target quadrant than in the other three nontarget quadrants ($P<0.001$), whereas in untreated MPS VII mice the difference of time spent in target quadrant and the other three nontarget quadrants was not statistically significant ($P=0.320$). Untreated MPS VII mice ($n=5$), treated MPS VII mice ($n=6$), normal mice ($n=5$).

replacement is required. Moreover, it is still controversial whether both approaches have a therapeutic effect for CNS involvement in the LSDs. To circumvent these problems, various approaches have been attempted to develop a new treatment for CNS involvement of LSDs, such as gene therapy and cell therapy. However, both approaches still have limitations. In gene therapy, although AAV vectors appear to have a promise, it is still difficult to produce large amount of recombinant virus. The safety issue of lentivirus vector is another major concern for use of this virus vector to human. In cell therapy, although transplantation of neural stem cells or amniotic cells shows a great hope to treat CNS involvement in MPS VII, ethical and immunologic problems must be cleared.

In the present study, we tested BMS cells as a vehicle to deliver the missing enzyme to wide region of the brain of LSD mouse model. The BMS cells have several advantages as compared to other stem/progenitor cells. First, the BMS cells are readily accessible from bone marrow and grow well *in vitro*. Second, the autologous transplantation overcomes the ethical and immunologic concerns. BMS cells have capability to differentiate into various neural cell lineages. We generated GUSB overexpressing BMS cell (KUSA/HBG) and KUSA/HBG was transplanted into lateral ventricle of newborn MPS VII mice. At 2 weeks after transplantation, expression of GUSB was observed in all of brain lesions. GUSB histochemical staining revealed that transplanted cells migrated to various brain regions not only ventricles also brain parenchyma, including cerebral cortex and olfactory bulb. Even though the number of transplanted cells in brain parenchyma was small, the pathological improvement is significant. Lysosomal storage in most of cells, such as endothelial cells, glial cells and neurons from various brain regions, was reduced. The amount of T-CS and HA, which was accumulated GAGs in MPS VII brain, was also reduced markedly.

To assess the functional improvement of the treated animals, we performed the MWM testThe MWM is known as test to evaluate cognitive functions. At 6 weeks after the transplantation, treated MPS VII mice spent significantly more time in the target quadrant than in the other three nontarget quadrants and the time spent in the target quadrant by treated MPS VII increased compared with by untreated MPS VII mice. Earlier report also demonstrated that the enzyme replacement therapy in neonatal period,[7] gene therapy using lentivirus vector[14] successfully restored brain function of MPS II mice. The expression of GUSB in the brain of treated animals 8 weeks after transplantation was markedly reduced. This relatively early reduction of GUSB activity in transplanted cells might be due to a mismatched allograft transplantation. KUSA/A1 cells are derived from C3H mouse, while MPS VII mice are C57BL/6 background. In addition, because KUSA/HBG expresses huge amount of human enzyme and the mouse immune system might recognize transplanted cell as a foreign cells. Thus, there is a possibility that transplanted cells were rejected by immunological mechanisms. Another possibility is that the transplanted cells underwent apoptosis from other unspecified mechanisms. We previously transplanted human neural stem cells into lateral ventricle of newborn MPS VII mice.[16] The human neural cells transduced with GUSB gene successfully corrected CNS pathology and restored enzyme levels, but grafted cells later underwent cell death by apoptosis mechanisms. The experiment is currently under way to understand the mechanism(s) for early death of KUSA/HBG cells in mouse brain. In the present study, although GUSB activity of treated mice 8 weeks after transplantation declined to those of untreated mice, interestingly, the restored GAGs contents in treated mouse brain were maintained up to 16 weeks after transplantation. Moreover the MWM showed that the brain function of treated mice still improved.

These results suggest that once MPS VII mice receive certain amount of GUSB cells in early stage, the brain function was maintained even after the considerable reduction of enzymatic activity. GAGs synthesis and GAGs turn over in adult MPS VII brain may be small so that GAGs do not re-accumulate. Large amount of GUSB may not be necessary for adult MPS VII brain after newborn brain treatment with GUSB.

Another question is what kinds of cell types transplanted cells differentiate into following brain transplantation. Previous reports have shown that the marrow stromal cells transplanted into the brain migrate to the parenchyma and differentiate into neural cells.[21,22] Number of KUSA/HBG cells in brain parenchyma was too small to perform double immunostaining, thus we performed another set of experiments. Briefly, KUSA/A1 cells were transduced by a LacZ expressing retrovirus vector and transplanted into striatum of adult C57BL/6 mice. After transplantation, frozen sections were made and double stained with anti-LacZ antibody and cell

type specific antibody, such as anti-NeuN for neurons, and anti-GFAP for astrocytes. Although a small number of LacZ positive cells expressed NeuN, majority of LacZ positive cells did not express any cell type specific makers (data not shown). These observations suggest that even if these cells have a migrating capacity in mouse brain, most of cells lost differentiation capability *in vivo*. We do not know the reason why our results differ from those of previous reports. However, for our purpose, transplanted cells do not necessarily differentiate into certain neural cells, such as neuron and glial cells. In another words, in order to treat the brain pathology of MPS VII mice, enzyme competent cell should migrate to various brain lesions and cross correct enzyme deficient neighboring cells.[25] In this regard, even if transplanted BMS cells did not differentiate into neural cells, BMS cell transplantation can correct the brain involvement of MPS VII. There are still some obstacles to be overcome to use BMS cell as a vehicle for treatment of brain involvement of LSDs. We transplanted BMS cells during neonatal period. However, it is quite rare that LSDs patients were diagnosed in this period. The development of newborn screening system for early detection of LSDs patients may overcome this problem. We do not know if BMS cell transplantation should have beneficial effect for the CNS involvement in MPS VII in later stage. Since a previous study has demonstrated that the BMS cell brain transplantation in Niemann–Pick disease mice at 3 weeks of age was effective for CNS involvement,[24] this may be the case for MPS VII as well. The transplantation of BMS cell into the brain in MPS mice at 3–4 weeks of age is underway.

In conclusion, we have demonstrated that the intraventricular transplantation of BMS cells overexpressing GUSB in neonatal MPS VII mice corrects the biochemical defects, CNS pathology and cognitive behavior in these mutant mice.

Materials and methods

Animals

Breeding pairs of (+/mps) were purchased form the Jackson Laboratory and bred in our institutional animal facility. Mutant mice (−/−) were identified by genetic analysis of DNA from mouse tail.[26] Enzymatic activity of tail was also measured as described below to confirm the diagnosis. Animals were maintained on a 12-h light/dark cycle and given a standard rodent chow.

BMS cell

Mouse BMS cell line, KUSA/A1, was established by limiting dilution of Dexter long-term cultures from C3H/He female mouse. The detailed methods and characters of this cell line were described previously.[20] This cell line is able to differentiate into osteoblasts and neural cells, including neurons, astrocytes and oligodendrocytes under specific conditions. This cell line was cultivated in DMEM/10% FCS at 37°C and 5% CO_2.

In vitro *retroviral transduction*

Full-length human GUSB cDNA (provided by Dr WS Sly of Saint Louis University) was cloned into *Eco*RI site of retrovirus vector plasmid pMNDXSN (provided by Dr DB Kohn of University Southern California).[27] The recombinant retrovirus expressing human GUSB was generated by transfection of this plasmid to PA317 packaging lines. The condition medium of packaging cells were collected and filtered through 0.45 μm filter. The resultant condition medium was added to ~50% confluent KUSA/A1 cells and incubated at 37°C for 2 h in the presence of polybrene (8 μg/ml). After incubation, the condition medium was removed and fresh medium (DMEM/10% FCS) was added. About 24 h later, the cells were selected with G418 (400 μg/ml) and resistant cells were pooled (KUSA/HBG). The GUSB activity in KUSA/HBG was assayed as described below to confirm successful transduction. Cytochemical analysis of GUSB was also performed.

Intraventricular transplantation of KUSA/HBG

KUSA/HBG was washed and harvested by 0.1% trypsin. The cells were resuspended in PBS and injected into the both lateral ventricles of mice within 48 h after birth using a 30G needle attached to a Hamilton syringe.[28] At this age, mutant mice do not exhibit any clinical and pathological symptoms. Thus, we expect maximized therapeutic effect. Injected volume of cell suspension was approximately 5 μl and the number of transplanted cells per mouse was from 1×10^5 to 1×10^6.

Histochemical/cytochemical analysis of GUSB

Transplanted mice were killed at 2 weeks after transplantation. Brain was removed and immediately embedded with OCT compound (Miles) and frozen in a liquid nitrogen bath. The samples were cut 10 μm sections by cryostat. The sections were fixed with chloral-formal-acetone fixative. The histochemical analysis of GUSB activity was performed using naphthol-AS-BI β-D-glucuronide as a substrate. The detail method of this staining was described elsewhere.[26] After hisitochemical staining of GUSB, sections were counterstained with 1% methyl green. The culture cells were fixed and stained with same method as stated above.

Lysosomal enzymes activities

Cells were washed briefly with PBS twice and harvested by 0.1% trypsin. After centrifugation, cell pellets were stocked at −80°C until assay. Cell pellets were resuspended in water and sonicated. The cell lysate was centrifuged at 14 000 *g* for 10 min at 4°C. The treated mice, age-matched mutant (−/−) and heterozygote (+/−) mice were killed at 2 and 8 weeks after transplantation. Harvested mouse brains were also stocked at −80°C until assay. To assess the distribution of transplanted cells, brain was cut into five serial coronal sections (Figure 1). To make tissue lysate, tissue was homogenized in water using a glass homogenizer. The homogenates were also centrifuged at 14 000 *g* for 10 min at 4°C and the resultant supernatant was used as enzyme source. The enzymatic activities of GUSB, α-Gal and Hex were measured fluorometrically using the artificial substrate, 4-methylumbelliferyl (4MU) β-D-glucuronide, 4MU α-D-galactopyranoside and 4MU *N*-acetyl-β-D-glucosaminide dihydrate (Sigma) respectively.[26,29,30] Protein concentration was determined by the BCA kit (Pierce) following the manufacture's instruction. Activity was expressed as nanomoles of 4-methylumbelliferone released per mg protein per hour.

Histopathological analysis of lysosomal distension

At 4 weeks after transplantation, treated mice, age-matched mutant and heterozygote mice were killed. Small blocks ($3 \times 3 \times 3\ mm^3$) of various brain regions (olfactory bulb, cerebral cortex, and striatum) were immersed in 2% glutaraldehyde in PBS and fixed for 2–3 weeks. After fixation, the samples were embedded in Epon-araldite. Ultrathin sections (1.0 μm) were cut and were stained with toluidine blue to evaluate lysosomal distention.[5,26]

Analysis of GAG contents

Brain contents of T-CS and HA, which is substrate of GUSB, were measured by enzyme digestion and HPLC method.[31] The amount of T-CS and HA were expressed as μg/g wet tissue.

The MWM test

The MWM test was carried out at 6 weeks after transplantation. The acquisition phase and the probe test of the MWM test were performed as previously described.[32,33] The MWM test was slightly modified for this test. Briefly, the pool of a diameter 100 cm was filled 30 cm deep with water at 20°C. A transparent circular plexiglass platform 10 cm in a diameter was placed 1 cm below the surface of the water and 20 cm from the wall of the pool. The pool was designed as divided into four quadrants. The mice were released to the pool facing the wall at three points, four trials per a day were made for each mouse and the order of the releasing points was selected at random but all mice were followed the same order. If the mouse found the platform within 60 s, it was allowed to stay on the platform for 15 s. If the mouse could not find the platform within 60 s, it was guided to the platform by experimenter and it was allowed to stay on the platform for 15 s. All of the mouse traces were recorded by the over-head video camera. The acquisition phase of the test was performed for the 5 consecutive days (days 1–5). On day 6, the platform was removed and mice were released from the opposite side of the original platform place and the amount of time spent in each quadrant was monitored (the probe test).

Statistical analysis

A two-tailed Student's *t*-test was used for comparing the significance. The data are presented as mean ± s.e. *P*-value less than 0.05 was considered as significant.

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Expression of a Novel Human Gene, *Human Wings Apart-Like (hWAPL)*, Is Associated with Cervical Carcinogenesis and Tumor Progression

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ABSTRACT

In *Drosophila melanogaster*, the *wings apart-like* (*wapl*) gene encodes a protein that regulates heterochromatin structure. Here, we characterize a novel human homologue of *wapl* (termed *human WAPL*; *hWAPL*). The *hWAPL* mRNA was predominantly expressed in uterine cervical cancer, with weak expression in all other normal and tumor tissues examined. hWAPL expression in benign epithelia was confined to the basal cell layers, whereas in dysplasias it increasingly appeared in more superficial cell layers and showed a significant correlation with severity of dysplasia. Diffuse hWAPL expression was found in all invasive squamous cell carcinomas examined. In addition, NIH3T3 cells overexpressing hWAPL developed into tumors on injection into nude mice. Furthermore, repression of *hWAPL* expression by RNA interference induced cell death in SiHa cells. These results demonstrate that hWAPL is associated with cell growth, and the *hWAPL* expression may play a significant role in cervical carcinogenesis and tumor progression.

INTRODUCTION

The *wings apart-like* (*wapl*) gene of *Drosophila melanogaster* encodes a protein that regulates heterochromatin structure (1). Mutations of *wapl* prevent the normal close apposition of sister chromatids in heterochromatin regions but do not appear to affect either heterochromatin condensation or chromosomal segregation (1). This evidence suggests that *wapl* is required to hold sister chromatids together in mitotic heterochromatin. *wapl* has also been implicated in both heterochromatin pairing during female meiosis and the modulation of position effect variegation (1). In addition, a *P* element screen of *Drosophila* identified *wapl* as a modifier of chromosome inheritance (2).

Among all varieties of cancer, uterine cervical cancer is unique because of its association with high-risk human papillomavirus (HPV) infection, with strains like HPV-16 and HPV-18. High-risk HPVs encode two oncoproteins, E6 and E7, which subvert crucial cellular regulatory mechanisms that reactivate and maintain DNA synthesis in the host cell. E6 accelerates proteosomal degradation of the p53 tumor suppressor, and E7 inactivates the retinoblastoma protein, interfering with the action of both $p16^{INK4a}$ (3) and the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (4, 5). Both the E6 and E7 high-risk HPV oncoproteins independently induce genomic instability in normal human cells (6, 7). Only a small portion of precursor lesions infected with HPV, however, develops into invasive carcinomas (8). Therefore, additional genetic and microenvironmental factors subsequent to HPV infection are thought to play an important role in the initiation and progression of cervical neoplasia (8–10).

In this study, we describe the isolation and characterization of a novel human *wapl*-related gene termed *human WAPL* (*hWAPL*). We have also demonstrated that *hWAPL* has the characteristics of an oncogene and is associated with uterine cervical cancer.

MATERIALS AND METHODS

cDNA Cloning and Construction of the hWAPL Expression Vector. To isolate the complete *hWAPL* cDNA sequence, we used a human testis Marathon-Ready cDNA kit (Clontech, Palo Alto, CA).

To create an expression vector encoding *hWAPL*, a *Hin*dIII-*Eco*RI cDNA fragment containing the complete coding region of hWAPL was amplified by PCR using the primers 5′-TTAAGCTTTGAAACTGGTGTCAAAATGACATCCAGATT-3′ and 5′-TTGAATTCAAGCAATGTTCCAAATATTCAATCACTCTAGAG-3′ and inserted into the hemagglutinin (HA)-tagged mammalian expression vector, pHM6 (HA-hWAPL; Roche Diagnostics, Mannheim, Germany).

Northern Blot and Quantitative Real-Time PCR Analysis. RNA isolation (11) and Northern blot analysis (11, 12) were performed as described. The 674-bp *Dpn*II fragment of *hWAPL* cDNA was used as a probe and labeled with ^{32}P using the Rediprime II random prime labeling system (Amersham Biosciences, Piscataway, NJ). A human β-actin cDNA control probe (Clontech) was used as a control.

First-strand cDNA synthesis was performed as described (13). Real-time PCR analysis was performed using the Smart Cycler System (Cepheid, Sunnyvale, CA) with SYBR Green I (Cambrex, Washington, DC). Real-time PCR used the *hWAPL*-specific primers 5′-GAATTCATAGGCACAGCGCTGAACTGTGTG-3′ and 5′-TTGAATTCCTAGCAATGTTCCAAATATTCA-3′ and β-actin-specific primers 5′-GGGAAATCGTGCGTGACATTAAG-3′ and 5′-TGTGTTGGCGTACAGGTCTTTG-3′. Reaction mixtures were denatured at 95°C for 30 s and then were subjected to 40 PCR cycles at 95°C for 3 s, 68°C for 30 s, and 87°C for 6 s. *hWAPL* mRNA levels were normalized to β-actin signals.

Immunohistochemistry and Immunoblot Analysis. To generate mouse monoclonal antibodies against hWAPL, we immunized mice against a 6 × histidine-tagged hWAPL COOH terminus (amino acids 814-1037) fusion protein. Spleen cells of an immunized mouse were fused with P3UI mouse myeloma cells as described previously (14). Of the 128 hybrids generated, one clone (clone R929) showed exclusive reactivity with hWAPL by ELISA. We used the supernatant of this clone as anti-hWAPL antibody.

Immunohistochemical assays were performed on formalin-fixed, paraffin-embedded sections using Ventana HX System Benchmark (Ventana Medical Systems Inc., Tucson, AZ). Immunohistochemical stains for hWAPL were interpreted semiquantitatively by assessing the intensity and extent of staining on the entire tissue sections present on the slides as described (9).

Immunoblot analyses were performed as described previously (15). The anti-HA (Roche Diagnostics; 3F10) and monoclonal anti-α-tubulin clone B-5-1-2 (Sigma Chemical Co., St. Louis, MO; T-5168) antibodies were purchased.

Animals and Treatment. BALB/cAJc1-nu female mice (4 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan).

The tumorigeneicity of the stable NIH3T3 transformants overexpressing hWAPL *in vivo* was examined as described previously (16).

Cell Culture and small interfering RNA (siRNA) Transfection. SiHa and NIH3T3 cells were grown in DMEM (Sigma) containing 10% fetal bovine serum at 37°C in a 5% CO_2 environment. For the transfection of siRNA, we

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generated siRNAs using a Silencer siRNA Construction Kit (Ambion, Austin, TX). siRNA transfection was performed in DMEM without serum using Oligofectamine Reagent (Invitrogen Japan, Tokyo, Japan) and Opti-MEM I (Invitrogen Japan).

For cell quantitation, we harvested the cells from the wells of a 12-well plate and resuspended them in 100 μl of PBS. Trypan blue solution (100 μl, 0.4%; Sigma) was added to each sample, and viable cell numbers were quantitated using an erythrometer. The results shown are representative of three independent cell count analyses.

RESULTS

Molecular Cloning of *hWAPL*. To isolate *wapl*-related genes from human cells, we searched DNA databases and identified a cDNA fragment, KIAA0261 (17), and three expressed sequence tag clones, BE410177, BF79516, and BE257022, containing the KIAA0261 sequence. We also performed 5′ rapid amplification of cDNA ends. From these DNA sequences, we cloned and confirmed the full-length coding region sequence of the cDNA containing KIAA0261. We named this gene *hWAPL* (GenBank accession no. AB065003) to reflect its homology to *wapl*. The *hWAPL* gene product shows high sequence similarity in the WAPL-conserved region (amino acids 627-1169, 34% identical and 56% similar) and low similarity throughout the other regions to the *wapl* gene product. Several additional stretches of amino acids are also present in wapl protein (Fig. 1*A*).

High-Level Expression of hWAPL in Human Cervical Cancer. As *wapl* is involved in sister chromatid cohesion, hWAPL may modify chromosomal inheritance. Deregulation of the expression of genes involved in chromosomal inheritance directly induces a variety of disorders associated with aneuploidy, including birth defects and cancer. Northern blot analysis detected *hWAPL* mRNA expression in several invasive cervical cancer samples, examined in tandem with additional human cancers and normal tissues (Fig. 1*B*). We confirmed the *hWAPL* expression in cervical cancers by quantitative real-time PCR analysis of tumor and normal tissue samples. The levels of *hWAPL* mRNA expression in cervical cancers were significantly higher than the levels observed in either normal cervical controls or endometrial, ovarian, breast, lung, stomach, renal, and colon cancers (Fig. 1*C*).

To investigate the connection between hWAPL expression and oncogenesis in cervical malignancies, we examined the expression of hWAPL by immunohistochemistry in a series of clinical samples of the various grades of cervical dysplasia [cervical intraepithelial neoplasia (CIN) I–III] and invasive squamous cell carcinoma. We found nuclear immunostaining for hWAPL in all samples (Fig. 2*A*). hWAPL expression in benign squamous epithelia was confined to the basal and parabasal cell layers. In contrast, hWAPL expression in squamous dysplasia and invasive carcinoma increasingly appeared in the more superficial cell layers and was significantly increased compared with the adjacent benign epithelia ($P = 0.0002$ for CIN I, $P = 0.0003$ for CIN II, $P = 0.0001$ for CIN III, and $P = 0.0001$ for invasive squamous cell carcinoma; Wilcoxon's signed rank test). CIN I and II cases showed hWAPL expression in the basal 50 and 70% of the epithelial thickness, respectively, whereas CIN III and invasive squamous cell carcinoma showed hWAPL expression in the full thickness of the dysplastic epithelia (Fig. 2*A*). Furthermore, the mean hWAPL staining score increased remarkably with increasing grade of dysplasia (Fig. 2*B*). These data strongly suggest that the unscheduled high-level expression of hWAPL may play a significant role in cervical carcinogenesis and tumor progression.

hWAPL Has Oncogenic Characteristics. Because we observed high-level expression of *hWAPL* in tumors, we sought to determine whether hWAPL overexpression promotes tumor development. We transfected NIH3T3 cells with an HA-tagged hWAPL expression vector (HA-hWAPL 3T3) or HA expression vector (HA-3T3). Then, we compared the ability of HA-hWAPL 3T3 with HA-3T3 cells to grow as tumors in nude mice. We injected 10^6 cells into three s.c. sites of each nude mouse. HA-hWAPL 3T3 cells produced tumors in all nude mice within 10 days after injection of cells (100%, $n = 18$; Fig. 3*A*). HA-3T3 failed to produce tumors in any mice (0%, $n = 18$). We confirmed high hWAPL expression levels in the resultant tumors by Western blot analysis (Fig. 3*B*). These results suggest that *hWAPL* has the characteristics of an oncogene.

Repression of hWAPL Expression Induces Cell Death. We examined hWAPL function by suppressing hWAPL expression. Initial attempts to generate a *WAPL*-deficient mouse demonstrated that the loss of *WAPL* was embryonic lethal (data not shown). Therefore, we designed two 21-nucleotide, double-stranded siRNAs, siRNA(I) and siRNA(II), to repress *hWAPL* expression (Refs. 18 and 19; Figs. 1*A*

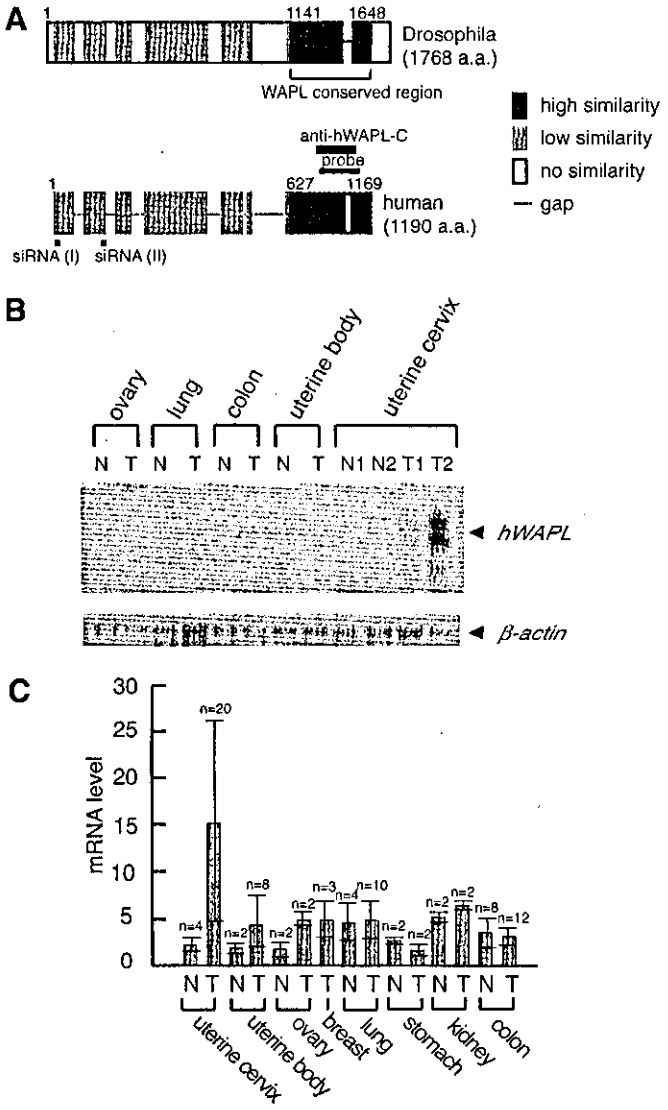

Fig. 1. Structures of wings apart-like (*WAPL*) proteins and *human WAPL* (*hWAPL*) expression in normal and tumor human tissues. *A*, schematic structure of the *hWAPL* and *Drosophila wapl* gene products. The site corresponding to the probe sequence used for Northern blot analysis is indicated by "probe." The antibody recognition site is indicated by "hWAPL-C." The small interfering RNA (*siRNA*) targeting sites are indicated by "siRNA(I)" and "siRNA(II)." *B*, Northern blot analysis of *hWAPL* in several normal (*N*) and tumor (*T*) human tissues. *C*, quantitative real-time PCR analysis demonstrating *hWAPL* mRNA levels in various normal (*N*) and tumor (*T*) human tissues. *Columns*, the means of examined samples. The minimum mRNA expression level was arbitrarily set to 1 in the graphical presentation; all other mRNA signals were normalized to this value. *Bars*, SD.

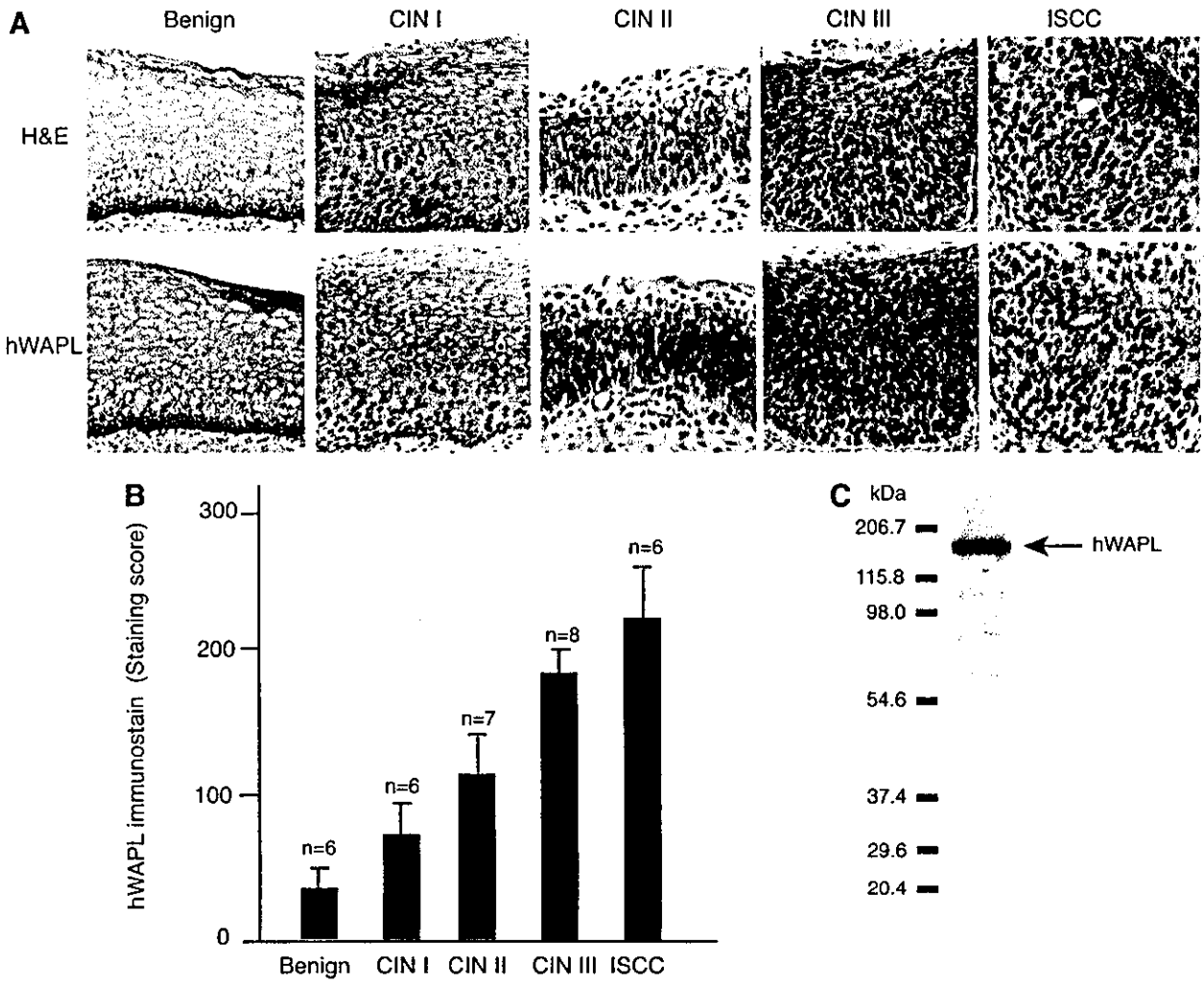

Fig. 2. Immunohistochemical analysis of human wings apart-like (*hWAPL*) expression in uterine cervical epithelia of normal, dysplasia, and carcinoma. *A*, immunohistochemical staining of hWAPL expression in benign squamous epithelium, various grades of squamous dysplasia [cervical intraepithelial neoplasia (*CIN*) grades I, II, and III], and invasive squamous cell carcinoma (*ISCC*). hWAPL was stained with hematoxylin counterstain; H&E. *B*, graphical representation of the increase of the hWAPL expression with increasing severity of dysplasia in cervical squamous epithelia. The mean hWAPL staining scores were calculated as described (9). *Bars*, SD. *C*, Western blot analysis with the total extract from a uterine cervical cancer-derived cell line, SiHa, to confirm the specificity of the anti-hWAPL monoclonal antibody hWAPL-C.

and 4*A*). We examined various human cancer-derived cell lines and found that cervical cancer-derived cell lines containing both HPV-positive and -negative cells exhibited higher levels of hWAPL expression compared with the other cell lines (data not shown). Then, we examined the effects of suppressing *hWAPL* in a cervical cancer-derived cell line, SiHa. siRNA transfection at a concentration of either 1 nM siRNA(I) or siRNA(II) reduced *hWAPL* mRNA levels (Fig. 4*B*). siRNA(I) was more effective at reducing *hWAPL* mRNA than siRNA(II). Thus, we used siRNA(I) in the subsequent experiments. hWAPL protein levels were also significantly reduced after siRNA(I) transfection (Fig. 4*C*). Interestingly, siRNA(I) repressed the growth of the cells and subsequently induced cell death (Fig. 4, *D* and *E*). siRNA(II) repressed cell growth in a similar manner as siRNA(I) (Fig. 4*D*), suggesting that the effects of these siRNAs on proliferation and viability are likely caused by the repression of *hWAPL* expression. Similar results were obtained in another cervical cancer-derived cell line, CaSki, with 10 nM siRNA(I) (data not shown). On the contrary, we did not observe any effects of siRNA(I) on cells expressing relatively low levels of *hWAPL*, such as Saos-2 and HCT116 (data not shown).

To investigate the fate of cells transfected with siRNA(I), we analyzed siRNA-transfected cells by flow cytometry (Fig. 5). In siRNA(I)-transfected cells, the population of cells exhibiting S phase DNA content increased (Fig. 5; 48 and 72 h). In addition, there was an increase in the number of apoptotic cells exhibiting subG$_1$ DNA content (Fig. 5; 72 h). Many cells showing S phase DNA content may also be apoptotic cells at G$_2$-M phase. Taken together, these results suggest that a malfunction in the hWAPL pathway activates an S phase checkpoint or another apoptotic pathway and consequently leads to cell death.

DISCUSSION

In this study, we report the isolation and characterization of a novel human gene termed *hWAPL*. We were unable to identify additional genes similar to *wapl* within the human genome sequence database. Thus, although the high-sequence conservation between hWAPL and wapl is limited to a third of the protein sequence encoded by *wapl* (Fig. 1*A*), we consider hWAPL to be the human homologue of wapl. We did not find any protein sequence motifs in hWAPL, except for the WAPL-conserved region (Fig. 1*A*). We therefore expect that hWAPL has similar functions to the wapl protein. Two hybridization signals for *hWAPL* were visible by Northern blot analysis (Fig. 1*B*). Western blot analysis, however, detected only a single band for

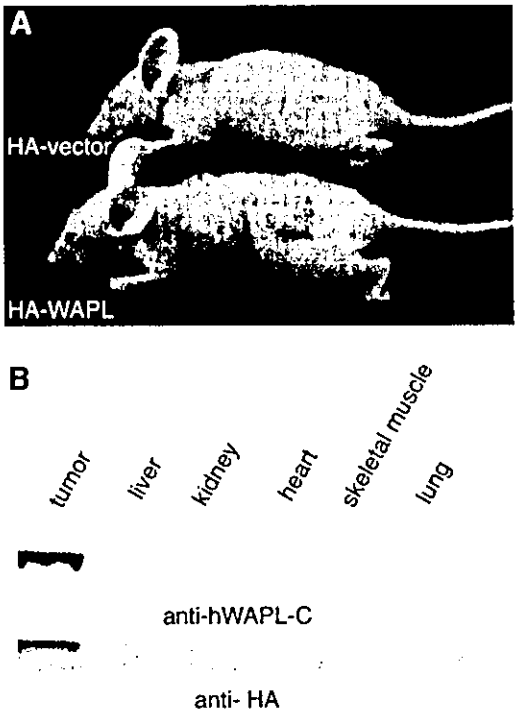

Fig. 3. Human wings apart-like (*hWAPL*) overexpression promotes tumor development. *A*, tumorigenecity of HA-hWAPL 3T3 in nude mice. The lower mouse in the *panel* is shown 10 days after the injection of HA-hWAPL-3T3 at three s.c. sites. The upper mouse was injected with the control HA-3T3 cells. *B*, Western blot analysis of hWAPL protein in tumor and other control tissues from HA-hWAPL-3T3-injected nude mice. *Top panel*, anti-hWAPL antibody; *bottom panel*, anti-HA antibody.

hWAPL (Fig. 2*C*). In addition, we did not obtain additional nucleotide sequences similar to the open reading frame of *hWAPL* by PCR analysis with various PCR primers (data not shown). Thus, we consider that the two hybridization signals may reflect the difference of the length of the untranslated regions of the *hWAPL* mRNA.

High-level expression of *hWAPL* was observed in cervical cancers (Fig. 1, *B* and *C*). Furthermore, hWAPL-overexpressing 3T3 cells developed into tumors on injection into nude mice (Fig. 3). These results suggest that *hWAPL* has oncogenic characteristics. Cervical cancer is a serious health problem, with ~500,000 women developing the disease each year worldwide. In many developing countries, it is the most common cause of cancer death and years of life lost because of cancer (20). Although the fundamental role of high-risk HPV infection in the pathogenesis of cervical carcinoma is well established, other factors are thought to play a role in cervical carcinogenesis (8, 21). Because all of uterine cervical samples examined were HPV positive (data not shown), it is still to be confirmed whether hWAPL expression is inducible by HPV infection. However, HPV-positive normal cervical tissue samples exhibited low hWAPL expression (Fig. 1, *B* and *C* and data not shown), and an HPV-negative, uterine cervical cancer-derived cell line, C33A, showed high hWAPL expression (data not shown). Thus, hWAPL expression is likely to be more closely related with cervical carcinogenesis than HPV infection. Recently, Acs *et al.* (9) found significant correlation among expression of Epo receptor, $p16^{INK4a}$, and *bcl*-2 in benign and dysplastic squamous epithelia. In our results, hWAPL showed similar expression pattern to Epo receptor and $p16^{INK4a}$ in benign and dysplastic cervical squamous epithelia and invasive squamous cell carcinomas (Fig. 2, *A* and *B*). Although we did not find any evidence for hWAPL being involved in hypoxia-inducible Epo signaling, hWAPL may cooperate with the Epo signaling in the progression of cervical neoplasia. These observations indicate that hWAPL overexpression can be used as a useful diagnostic tool in the detection of cervical dysplasia like $p16^{INK4a}$ (22) and Epo receptor (9). In addition, our results provide the necessity to investigate the potential of hWAPL as a cancer therapeutic target.

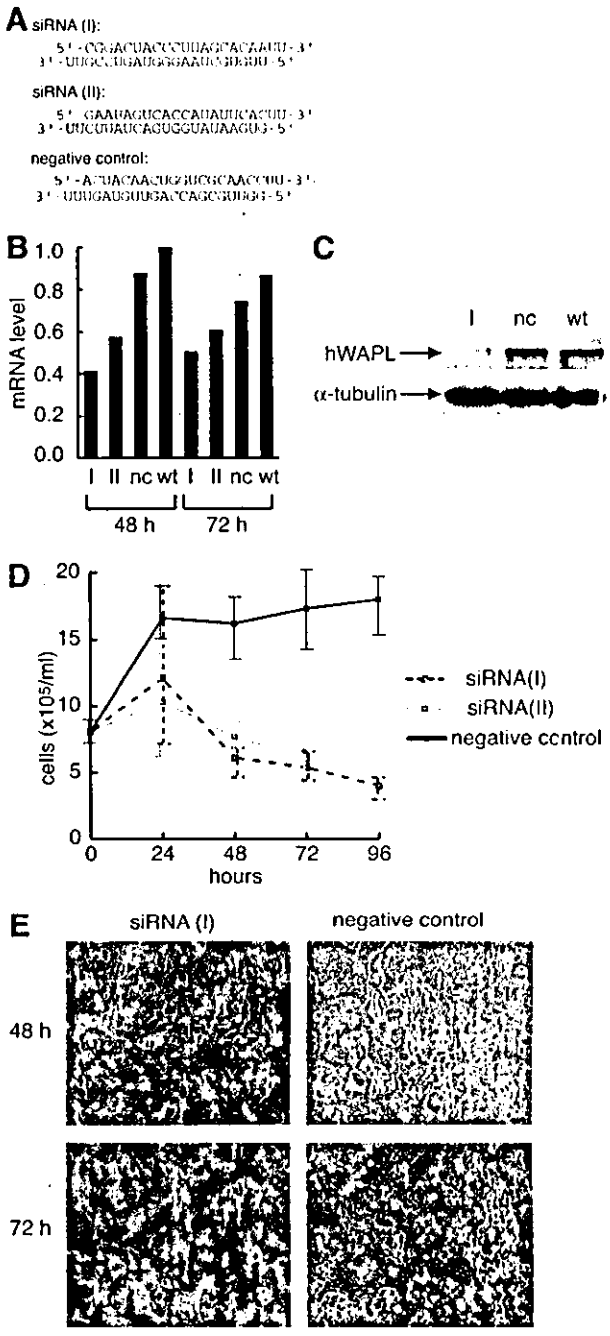

Fig. 4. Repression of human wings apart-like (*hWAPL*) expression by small interfering RNA (*siRNA*) treatment induces cell death. *A*, sequences and structures of siRNAs. The negative control siRNA possesses the same nucleotide composition as siRNA(I) but lacks homology to any known human genes. *B*, reduction of the *hWAPL* transcript by siRNA in SiHa cells. After siRNA transfection, SiHa cells were harvested at either 48 or 72 h. Total RNA was extracted from the cells and subjected to real-time PCR analysis. *I*, siRNA(I); *II*, siRNA(II); *nc*, negative control siRNA; *wt*, untransfected wild type. Data were normalized to a maximum mRNA level that was arbitrarily set to 1 in the graphical presentation. *C*, reduction of hWAPL protein levels by siRNA. Western blot analysis of total cell extracts from untreated SiHa or SiHa cells 72 h after transfection with siRNA(I) or negative control siRNA. α-tubulin is shown as a loading control. *D*, active siRNA specific for *hWAPL* induces cell death. SiHa cells transfected with siRNA(I), siRNA(II), or negative control siRNA were harvested at 24, 48, 72, and 96 h after transfection. Cell numbers were counted using an erythrometer. *Bars*, SE. *E*, representative phase-contrast images of SiHa cells transfected with siRNA(I) and negative control siRNA are shown.

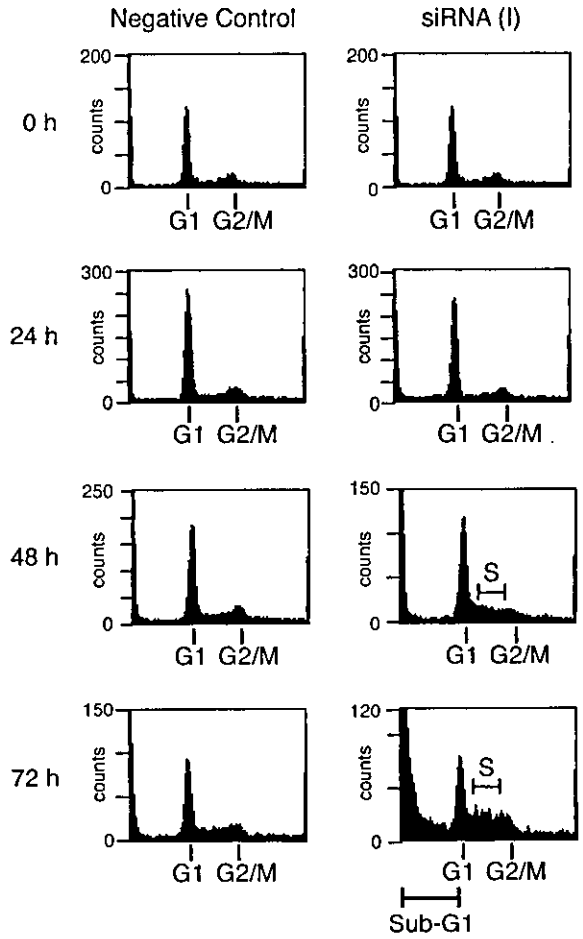

Fig. 5. Flow cytometric analysis of SiHa cells after small interfering RNA (*siRNA*) transfection. SiHa cells were transfected with either siRNA(I) or negative control siRNA, then harvested at 24, 48, and 72 h after transfection. Cells were stained with propidium iodide and subjected to flow cytometric analysis to examine DNA content. A total of 50,000 cells was counted for the sample siRNA(I) 72 h, and 20,000 cells were counted for the other samples.

Loss of WAPL was embryonic lethal in mouse (data not shown), and repression of hWAPL expression in SiHa cells led to cell death (Fig. 4). Flow cytometry analysis demonstrated that malfunction of hWAPL may cause apoptosis and/or arrest of cells at S phase (Fig. 5). In addition, *Drosophila wapl* is associated with regulation of chromatin organization (1). Thus, we expect that hWAPL is also associated with regulation of chromatin structure, and deregulation of hWAPL expression may induce chromosomal instability. Although additional investigations are necessary to elucidate the actual function of hWAPL in normal and malignant cells, our results have demonstrated that the novel oncogene, *hWAPL*, is one of the essential genes for development and cell growth and may play a significant role for cervical carcionogenesis and tumor progression.

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RESEARCH ARTICLE

Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation?

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Abstract

Background Cell transplantation has recently been challenged to improve cardiac function of severe heart failure. Human mesenchymal stem cells (hMSCs) are multipotent cells that can be isolated from adult marrow stroma, but because of their limited life span, it is difficult to study them further. To overcome this problem, we attempted to prolong the life span of hMSCs and investigate whether the hMSCs modified with cell-cycle-associated genes can differentiate into cardiomyocytes *in vitro*.

Methods We attempted to prolong the life span of hMSCs by infecting retrovirus encoding bmi-1, human papillomavirus E6 and E7, and/or human telomerase reverse transcriptase genes. To determine whether the hMSCs with an extended life span could differentiate into cardiomyocytes, 5-azacytidine-treated hMSCs were co-cultured with fetal cardiomyocytes *in vitro*.

Result The established hMSCs proliferated over 150 population doublings. On day 3 of co-cultivation, the hMSCs became elongated, like myotubes, began spontaneously beating, and acquired automaticity. Their rhythm clearly differed from that of the surrounding fetal mouse cardiomyocytes. The number of beating cardiomyocytes increased until 3 weeks. hMSCs clearly exhibited differentiated cardiomyocyte phenotypes *in vitro* as revealed by immunocytochemistry, RT-PCR, and action potential recording.

Conclusions The life span of hMSCs was prolonged without interfering with cardiomyogenic differentiation. hMSCs with an extended life span can be used to produce a good experimental model of cardiac cell transplantation and may serve as a highly useful cell source for cardiomyocytic transplantation.

Keywords Bmi-1; marrow stroma; cardiomyocytes; immortalization; papillomavirus; senescence

Introduction

Cell transplantation has recently been attempted to improve cardiac function in severe heart failure. Many types of cells, such as embryonic stem cells [1,2], fetal cardiomyocytes [3–5], myoblasts [6,7], bone marrow hematopoietic cells [8,9], and mesenchymal stem cells (MSCs) [10–12], have been transplanted to functionally restore damaged or diseased tissue in animal models, and mononuclear cells [13–16] or myoblasts [17] have been injected into ischemic hearts clinically.

MSCs can be a useful source of cells for transplantation for several reasons: they have the ability to proliferate and differentiate into mesodermal tissues, including heart, they entail no ethical or immunological problems, and bone marrow aspiration is an established routine procedure. When placed in appropriate *in vitro* and *in vivo* environments, MSCs can give rise to all major mesenchymal tissues, such as bone, cartilage, muscle, and adipose tissue [18]. Murine MSCs can also differentiate into cardiomyocytes and start to beat synchronously *in vitro* [19], and direct injection of murine MSCs into the heart has been shown to be feasible in murine models of ischemic heart disease and normal mouse heart. Thus far, only endothelial cells have been shown to exhibit '*in vitro* cardiomyogenesis' in humans [20].

Large numbers of cells must be injected into damaged sites in ischemic heart disease to restore cardiac function in humans, and cells need to be injected into the entire heart in cardiomyopathy. Until now, however, there have been no reports of a sufficient number of differentiated human cardiomyocytes ever having been obtained to restore the function of a failing heart. One of the reasons for this is that the life span of human cells *in vitro* is limited. Human cells reach senescence or stop cell growth after a limited number of cell replications [21], and the average number of hMSC population doublings (PDs) has been found to be 38 [22], implying that it would be difficult to obtain enough cells to restore the function of a failing human heart.

To resolve these problems and to establish a model of cell therapy of the failing heart, we attempted to prolong the life span of hMSCs by using the system to infect retrovirus encoding bmi-1, human telomerase reverse transcriptase (TERT), and human papillomavirus E6 and E7 genes. Both Rb/p16INK4a inactivation with E7 and telomerase activation with E6 are required to extend the life span of human epithelial cells [23]. bmi-1, a c-myc cooperating oncogene in murine lymphomas, reduces expression of p16INK4a, stimulates cell proliferation [24], and is required for maintenance of self-renewing hematopoietic stem cells [25,26]. This method was highly efficient in extending the life span of hMSCs. In the present study we investigated whether hMSCs with an extended life span have the ability to differentiate into cardiomyocytes *in vitro*.

Materials and methods

Isolation and cell culture of hMSCs

After obtaining signed informed consent, bone marrow cells were harvested from a 91-year-old human female donor with the approval of the Ethics Committee of Keio University School of Medicine (Tokyo). Cells were resuspended in bone marrow stromal cell culture medium (10% fetal bovine serum in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose [DMEM-HG]) with antibiotic/antimycotic supplements (Gibco), and cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. When the cultures reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA, and replated with one half of the harvested cells. After a series of passages, the attached marrow stromal cells were devoid of hematopoietic cells. Several bone marrow stromal cell strains were then generated by the limiting-dilution method, and one of them was designated H4-1. The H4-1 cells were cultured in MSC growth medium (MSCGM) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

Preparation and infection of recombinant retroviruses

The full-length human bmi-1 cDNA was cloned by RT-PCR using RNA extracted from K562 cells. Thermoscript reverse transcriptase (Invitrogen) and KOD polymerase (TOYOBO, Japan) were used for the RT and PCR reactions, respectively. The forward primer, 5′-ACGCGTCGACCGCCATGCATCGAACAACGAGAAT-3′, and reverse primer, 5′-CGGATCCTCAACCAGAAGAAGTTGCTG-3′, were designed to obtain the coding sequence of human bmi-1 flanked by the *Sal*I site (underlined) and the Kozak consensus sequence at the 5′-end and the *Bam*HI site (underlined) at the 3′-end. The *Sal*I-*Bam*HI segment of the PCR product was cloned between the *Xho*I and *Bgl*II sites of pCLXSN to generate pCLXSN-bmi1. The coding sequence of the cDNA was confirmed to be identical to the published sequence (NCBI ACC# NM_005 180.4). Construction of pCLXSH-hTERT has been described previously [27]. The gateway system (Invitrogen) was used to subclone a deletion mutant of HPV16 E6 (16E6SDD151) that lacked transforming activity to 3Y1 cells [28] into pCMSCVpuro. pCMSCVpuro comprises the CMV/LTR fusion promoter, the packaging signal Psi, and the multicloning sequence from pCLXSN (Imgenex Corp., San Diego, CA, USA) followed by the PGK-puro cassette and the 3′ long terminal repeat of murine embryonic stem cell virus from pMSCVpuro (Clontech). The destination vector pCMSCVpuro-DEST was constructed by inserting a modified cassette containing attR sites and ccdB (Invitrogen) between the *Eco*RI and *Bgl*II sites of pCMSCVpuro. 16E6SDD151 was first recombined into pDONR201 by BP reaction, and then into the destination vector by LR reaction according to the manufacturer's instructions (Invitrogen) to generate pCMSCVpuro-16E6SDD151. Production of recombinant retroviruses has been described previously [29,30]. Briefly, the retroviral vector together with the packaging construct, pCL-10A1, was transfected into 293T cells, and the culture fluid was harvested 48–72 h post-transfection. The preparation of the LXSN-16E7 retrovirus and the infection protocols have been described previously [31], except that FLYA13 [32] was used as the packaging cell line instead of PG13. The titers of the recombinant viruses were greater than 5×10^5 drug-resistant colony-forming units per milliliter on HeLa

cells, and 1 ml of the culture fluid was added to the cells in the presence of polybrene (8 μg/ml). Following inoculation with the viruses, hMSCs were grown in the presence of G418 (100 μg/ml), hygromycin B (50 μg/ml), or puromycin (1 μg/ml), and a polyclonal drug-resistant cell line was established and further analyzed. To achieve combinations of retroviral infections, cells were sequentially transduced with LXSN-E7 or LXSN-bmi-1, and LXSH-hTERT, and then MSCVpuro-16E6SDD151, if indicated, and selected with G418, hygromycin B, and puromycin, respectively. The stably transduced cells with an expanded life span were designated UBT-5, UBET-7, UEET-1, UEET-11, and UET-13.

Flow cytometric analysis

Cells were detached and stained for 30 min at 4 °C with primary antibodies and immunofluorescent secondary antibodies. After washing, the cells were analyzed on an EPICS ALTRA analyzer (Beckman Coulter). Antibodies (anti-human CD13, CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD90, CD105, CD117, CD133, CD140a, CD166, Flk-1) were purchased from Beckman Coulter, Immunotech, Cytotech, and Pharmingen Pharmaceutical, Inc.

Introduction of the GFP and β-galactosidase genes

Recombinant adenovirus expressing β-galactosidase and the green fluorescent protein (GFP) was prepared as described [33]. Cells were infected with these viruses at 10 plaque-forming units/cell. hMSCs were examined cytochemically *in vitro* for expression of the β-galactosidase gene and by fluorescent confocal microscopy for expression of the GFP gene. By 7 days post-infection nearly all the cells expressed β-galactosidase and GFP.

Preparation of murine fetal cardiomyocytes

Fetal cardiomyocytes were obtained from the hearts of day 14 mouse fetuses. Hearts were minced with scissors and washed with phosphate-buffered saline (PBS), and the minced hearts were incubated in PBS with 0.05% trypsin and 0.25 mM EDTA for 5 min at 37 °C. After adding DMEM supplemented with 10% fetal bovine serum (FBS), the cardiomyocytes were centrifuged at 1000 rpm for 5 min. The pellet was then resuspended in 10 ml DMEM with 10% FBS and incubated on glass dishes for 1 h to separate the cardiomyocytes from fibroblasts. The floating cardiomyocytes were collected and replated at $1 \times 10^5/cm^2$.

hMSC and murine fetal cardiomyocyte co-culture system

Human MSCs were plated on dishes at $5 \times 10^4/cm^2$, and infected with EGFP-expressing adenovirus on the next day. The supernatant was then removed, and the cells were cultured for 2 days in DMEM supplemented with 10% FBS. The cells were then exposed to 10 μM of 5-azacytidine for 24 h to induce cell differentiation. The 5-azacytidine-treated hMSCs were harvested with 0.25% trypsin and 1 mM EDTA and overlaid onto the fetal cardiomyocytes at $5 \times 10^3/cm^2$. The morphology of the beating hMSCs was evaluated under a fluorescent microscope.

RT-PCR

Total RNA was prepared from co-cultured hMSCs and mouse heart with Isogen (Nippon Gene). Human cardiac RNA was purchased (Clontech). RNA for RT-PCR was converted to cDNA with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotec) according to the manufacturer's recommendations. RT-PCR of the bmi-1, E6, E7, TERT, myosin light chain-2a (MLC-2a), Nkx2.5, and human atrial natriuretic peptide (hANP) genes was performed, and the PCR primers used are listed in Table 1. RT-PCR was performed with PCR primers that can amplify human but not mouse genes. PCR primers of 18S used as a positive control react with both human and murine genes. PCR was performed with TaKaRa Z-Taq (Takara Shuzo Co., Ltd) for 30 cycles, with each cycle consisting of 98 °C for 5 s, 68 °C or 60 °C for 1 s, and 72 °C for 10 s, with an additional 30-s incubation at 72 °C after completion of the final cycle.

Action potential recording and microinjection of dye

An inverted microscope (IX-70, Olympus, Tokyo, Japan) with a fluorescence filter (U-MNIBA2, Olympus) was used for action potential (AP) recording. The microscope was equipped with a recording chamber and a noise-free heating plate (Microwarm Plate, Kitazato Supply, Fujinomiya, Shizuoka, Japan). A 10 mmol/l volume of HEPES was added to the culture medium to stabilize the pH of the perfusate at 7.5–7.6. Standard glass microelectrodes having a DC resistance of 25–35 MΩ when filled with pipette solution were used. Alexa 568 compound was dissolved to a concentration of 0.5 mmol/l in 2 mol/l of KCl solution in order to completely dissolve the Alexa 568 in the pipette solution. The electrodes were positioned with a motor-driven micromanipulator (PCS-5000, Burleigh Instruments, Inc., New York, USA) under optical control. Spontaneously beating GFP-positive cells were selected as targets, and, after the APs of the targeted cells had been recorded, the dye was injected by iontophoresis (−7 nA for 30–60 s). The extent

Table 1. PCR primers used in this study

Gene product	Primer (sense)	Primer (anti-sense)	Annealing temperature (°C)	Product size (bp)
Bmi-1	TCATCCTTCTGCTGATGCTG	GCATCACAGTCATTGCTGCT	60	220
E6	GACCCAGAAAGTTACCACAG	GCAACAAGACATACATCGAC	60	397
E7	ATGACAGCTCAGAGGAGGAG	TCCTAGTGTGCCCATTAACAG	60	178
TERT	CGGAAGAGTGTCTGGAGCAA	GGATGAAGCGGAGTCTGGA	60	144
MLC-2a				
1st	TCGTGATGGCATCATCTGCAAGG	ACAGAGTTTATTGAGGTGCCCC	60	429
2nd	AAGGTGAGTGTCCCAGAGG	ATGGGTGTCAGGGCGAACATC	60	259
NkX2.5				
1st	CTTCAAGCCAGAGGCCTACG	CCGCCTCTGTCTTCTCCAGC	60	233
2nd	CTTCACCGGCCAAGTGTGCGTC	CCGCCTCTGTCTTCTCCAGC	60	152
hANP				
1st	GAACCAGAGGGGAGAGACAGAG	CCCTCAGCTTGCTTTTTAGGAG	60	406
2nd	GTCAGACCAGAGCTAATCCC	ACCTCCATCTCTCTGGGCTG	68	223
18S	GTGGAGCGATTTGTCTGGTT	CGCTGAGCCAGTCAGTGTAG	60	200

of dye transfer was monitored under a fluorescence microscope, and digital images were recorded with a digital photo camera (D100; Nikon, Tokyo, Japan) mounted on the microscope with a fluorescence filter (U-MWIG2; Olympus). The recording pipette was connected to a patch-clamp amplifier (Axopatch 200B; Axon Instruments), and the signal was low-pass filtered at 2 kHz and digitized with an A/D converter with sampling frequency of 10 kHz (Digidata 1322A; Axon Instruments) connected to a computer with Pentium4. Signals were monitored, recorded as electric files, and analyzed offline with pCLAMP 8.2 software (Axon Instruments). The rhythm was considered regular if the maximum beating rate minus the minimum beating rate divided by the max beating rate was <0.4.

Immunohistochemistry

The hMSCs co-cultured with fetal cardiomyocytes *in vitro* were fixed with 4% PFA and stained with anti-β2microglobulin antibody at 1 : 1000, mouse monoclonal antibody against troponin I (Hytest, Euro, Finland) at 1 : 200, anti-desmin antibody at 1 : 100, and anti-β-galactosidase antibody (Chemicon) at 1 : 500. hMSCs expressing GFP were fixed with 4% PFA.

Results

Establishment of hMSCs with an extended life span

H4-1 cells were obtained from primary culture by limiting dilution (Figure 1A). The cells proliferated for a limited number of passages and then underwent senescence, as evidenced by the cells assuming a broad and flattened shape (Figures 1B and 1C). To extend the life span of H4-1 cells, and obtain a large number of cells for cardiac transplantation, four different types of cells were obtained by transferring combinations of bmi-1, E6, E7, and/or TERT genes. Cells transduced with bmi-1 and TERT were designated UBT-5 cells; cells transduced with bmi-1, E6, and TERT were named UBET-7 cells; cells transduced with E7 and TERT were designated UET-13 cells; and cells transduced with E6, E7, and TERT were named UEET-1 and UEET-11 cells (Figures 1D, 1E, and 1F). To simplify nomenclature and avoid confusion, we use the name UEET-1 to refer to cells transduced with E6, E7, and TERT although they have recently been reported as ThMSC1 [29]. The cells were subcloned after each gene transfer, and thus were clonal. The UEET-1 cells were spindle-shaped, and longer than the parental H4-1 cells (Figures 1B, 1D, and 1E). Characteristics of cells with a prolonged life span were investigated. UEET-11 and UET-13 proliferated more than 150 PDs in 400 days, and UBET-7 and UBT-5 proliferated more than 50 PDs in 400 days, while H4-1 stopped dividing at 38 PDs (approximately 200 days). The growth rates of UEET-11 and UET-13 were higher than those of UBT-5 and UBET-7. Chromosome analysis revealed parental H4-1 and UET-13 to exhibit normal karyotypes, while the other cells transduced with E6 and E7 showed chromosome aberrations at low frequencies (data not shown). The transduced cells did not generate tumors, at least for the first 60 days after subcutaneous transplantation into immunodeficient mice.

Surface analysis of hMSCs

Surface markers of the UEET-1, UEET-11, UBT-5, UBET-7, and UET-13 cells were evaluated by flow cytometric analysis. The results showed that all of the MSCs were positive for CD13, CD29 (integrin β1), CD44 (Pgp-1/ly-24), CD55, CD59, CD90 (Thy-1), CD105 (endoglin), CD133, CD140a (PDGFRα or PDGFR2), and CD166 (ALCAM), and negative for CD14 (a marker for macrophage and dendritic cells), CD24, CD31 (PECAM-1), CD34, CD45 (leukocyte common antigen), CD50 (ICAM-3), CD54, CD117 (c-kit), and Flk-1 (Figure 2). Parental H4-1 cells had the same pattern of surface markers as UEET-1, UEET-11, UBT-5, and UBET-7 cells, implying that the surface markers were not influenced by

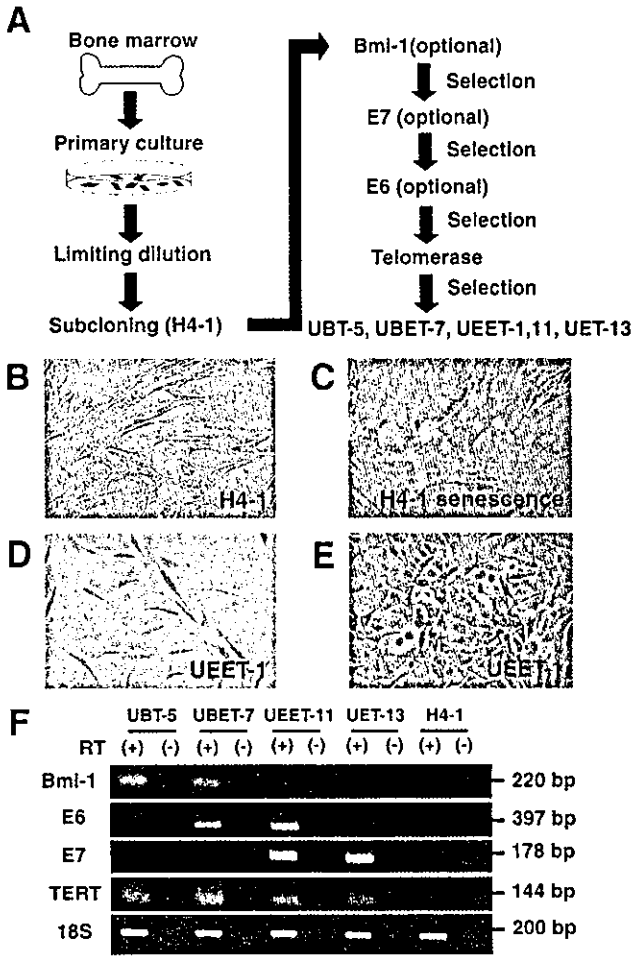

Figure 1. Experimental scheme. (A) Bone marrow stromal cells were obtained from a human donor and subcloned by limiting dilution. One of the cells isolated was designated H4-1 cells, and they were transduced with E6, E7, TERT, or bmi-1 genes to extend their life span. The combinations of genes transferred were: (1) bmi-1 and TERT; (2) bmi-1, E7, and TERT; (3) E7 and TERT; and (4) E6, E7, and TERT. (B) H4-1 cells in the growth phase. (C) H4-1 cells at senescence. The cells showed a broad and flattened shape. (D) H4-1 cells after transfer of E6, E7, and TERT genes were designated UEET-1 cells. (E) UEET-1 cells at confluence. Original magnification, B–E: x100. (F) The gene expression in each cell line was analyzed using RT-PCR

the exogeneously expressed bmi-1, E6, E7, and/or TERT genes.

Cardiomyogenic differentiation of hMSCs and stably transduced hMSCs

To determine whether H4-1 cells could be induced to undergo cardiomyogenic differentiation, the cells were exposed to 10 μM of 5-azacytidine for 24 h as previously reported in murine stromal cells [19]. All of the transduced hMSCs did not exhibit spontaneous beating despite continuous culturing for up to 3 months. Immunocytochemical analysis revealed the presence of desmin, a myocytic marker, in the hMSCs with an extended life span, i.e., UBT-5 cells and UBET-7 cells (Figure 3A). However, all cells tested were negative for the cardiomyocyte marker troponin-I (Figure 3B).

We employed a co-culture system with fetal cardiomyocytes to induce cardiac differentiation (Figure 4), since *in vitro* simulation of the heart by the environment has been shown to be an efficient means of induced differentiation of human endothelial progenitor cells and murine marrow stromal cells [20,34]. After exposing GFP-labeled UBT-5, UBET-7, UEET-11, and UET-13 cells to 10 μM of 5-azacytidine for 24 h, these cells were co-cultured with fetal cardiomyocytes. On day 3 after the start of co-cultivation, a few GFP-positive UBET-7 cells started to contract (Figure 5A). The contraction was stronger when beating cells were clustered than when scattered (Figure 5B). On day 7, the beating of the UBET-7 cells was synchronous with that of adjacent cells and was independent of that of the surrounding murine cardiomyocytes (Figures 5C and 5D). Repetition of these experiments confirmed the results to be reproducible, and the percentages of UBT-5, UBET-7, UEET-11, and UET-13 cells that underwent cardiomyogenic differentiation were almost the same, implying that cardiomyogenic differentiation is independent of the genes transferred. The number of beating cells increased for up to 3–4 weeks, when the fetal cardiomyocytes spontaneously detached from the dishes (Figure 5E). UBET-7 cells not treated with 5-azacytidine were co-cultured with fetal cardiomyocytes to determine whether environmental factors alone can induce cardiac differentiation, but fewer beating cells were observed (Figure 5F). No significant difference was detected in the number of differentiated cells between parental H4-1 and UBET-7 (Figure 5G).

Expression of cardiomyocyte-specific genes and proteins and the action potential of differentiated hMSCs

We analyzed the co-cultured UBET-7 cells in terms of gene expression and by immunocytochemistry and electrical recording. RT-PCR was performed with primers that react with human cardiomyocyte-specific genes but not with murine orthologues. Differentiated UBET-7 cells expressed MLC-2a, hANP, and the cardiomyocyte-specific transcription factor, Nkx2.5/Csx (Figure 6). Sequence analysis revealed that the cDNAs matched the sequences of the human MLC-2a, hANP, and Nkx2.5/Csx genes.

Action potentials were recorded from spontaneously beating cells. Alexa 568 was injected into cells via a recording microelectrode to stain the cells and confirm that the action potential was generated by GFP-positive UBET-7 cells (Figures 7A and 7B). Since the dye did not diffuse into the murine cardiomyocytes, there were no tight cell-to-cell heterologous connections, i.e., gap junctions. In some experiments, Alexa 568 diffused into the GFP-positive satellite UBET-7 cells, suggesting that a homologous cell-to-cell connection had been established at least 1 week after co-cultivation. The measured parameters of the recorded action potential were averaged

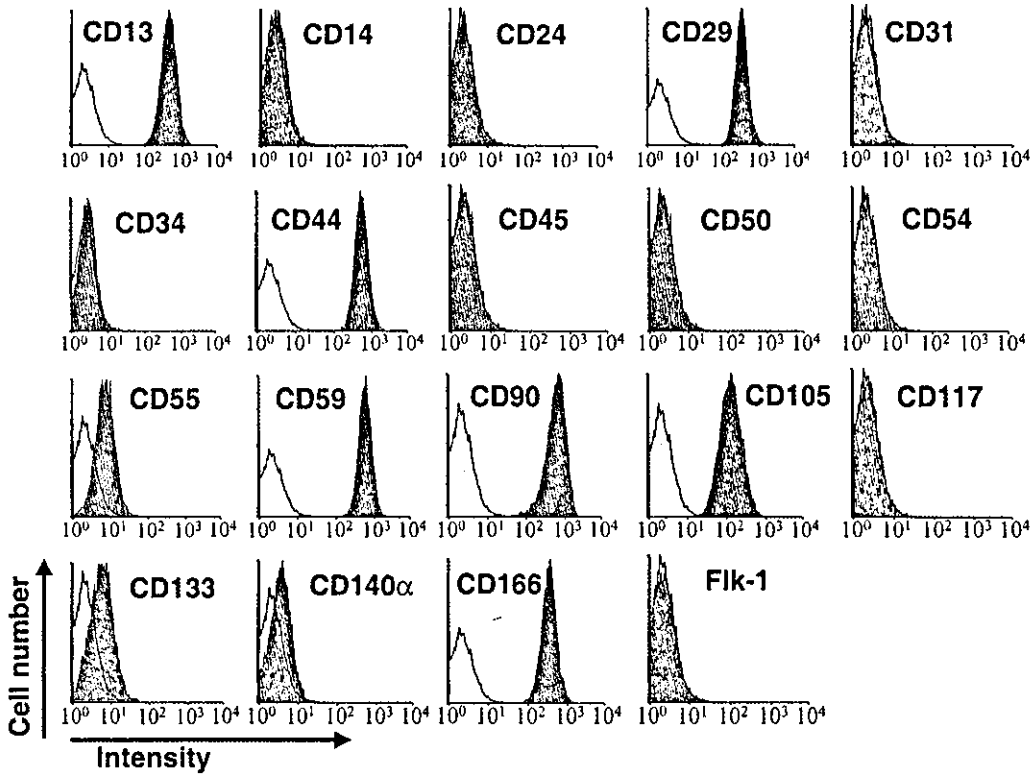

Figure 2. Flow cytometric analysis of UEET-1 cells. UEET-1 cells were labeled with FITC-coupled antibodies against CD13, CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD90, CD105, CD117, CD133, CD140a, CD166, and Flk-1 and analyzed with an EPICS ALTRA analyzer

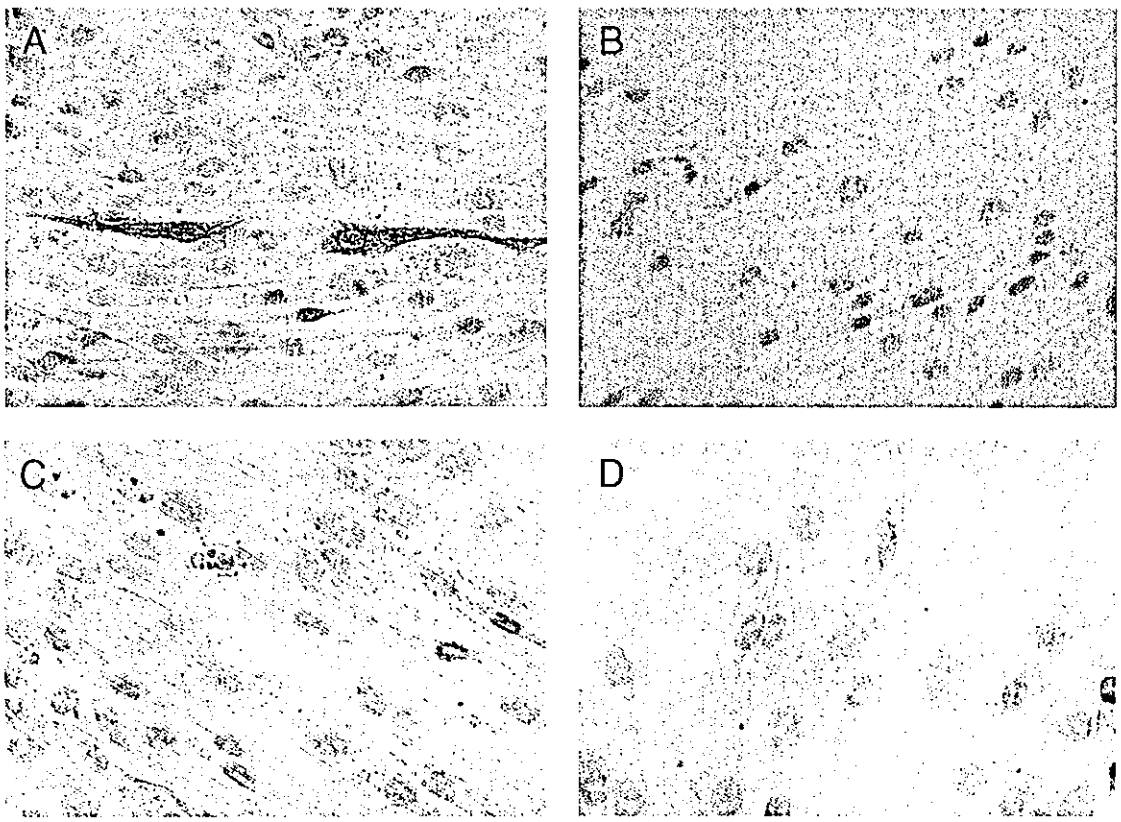

Figure 3. Immunostaining of hMSCs with anti-desmin and anti-troponin-I antibodies after exposure to 5-azacytidine. UBET-7 cells were exposed to 10 μM of 5-azacytidine for 24 h and stained for desmin (A) and cardiac troponin I (B). UBET-7 cells not treated with 5-azacytidine were also stained for desmin (C) and cardiac troponin I (D). Original magnification: ×400