

# Safety of Intravenous Infusion of Human Adipose Tissue-Derived Mesenchymal Stem Cells in Animals and Humans

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Adipose tissue-derived mesenchymal stem cells (AdMSCs) represent an attractive and ethical cell source for stem cell therapy. With the recent demonstration of MSC homing properties, intravenous applications of MSCs to cell-damaged diseases have increased. In the present study, the toxicity and tumorigenicity of human AdMSCs (hAdMSCs) were investigated for clinical application. Culture-expanded hAdMSCs showed the typical appearance, immunophenotype, and differentiation capacity of MSCs, and were genetically stable at least 12 passages in culture. Cells suspended in physiological saline maintained their MSC properties in a cold storage condition for at least 3 days. To test the toxicity of hAdMSCs, different doses of hAdMSCs were injected intravenously into immunodeficient mice, and the mice were observed for 13 weeks. Even at the highest cell dose ( $2.5 \times 10^8$  cells/kg body weight), the SCID mice were viable and had no side effects. A tumorigenicity test was performed in Balb/c-nu nude mice for 26 weeks. Even at the highest cell dose ( $2 \times 10^8$  MSCs/kg), no evidence of tumor development was found. In a human clinical trial, 8 male patients who had suffered a spinal cord injury >12 months previous were intravenously administered autologous hAdMSCs ( $4 \times 10^8$  cells) one time. None of the patients developed any serious adverse events related to hAdMSC transplantation during the 3-month follow-up. In conclusion, the systemic transplantation of hAdMSCs appears to be safe and does not induce tumor development.

## Introduction

MESENCHYMAL STEM CELLS (MSCs) have an inherent ability for self-renewal, proliferation, and differentiation toward mature tissues, depending on the surrounding microenvironment. Such characteristics intrinsic to stem cells make MSCs very attractive for use in cell therapy and regenerative medicine. MSCs are found in various tissues, including bone marrow (BM) [1], umbilical cord blood [2], placenta [3], and fat [4]; these tissues contain a rare population of adult stem cells that have the potential to undergo multilineage differentiation into osteoblasts, adipocytes, and chondroblasts *in vitro* [5].

The MSCs comprise only a minor fraction of BM and other tissues, with bone marrow MSCs (BMMSCs) constituting a mere 0.0001%–0.01% of all BM-nucleated cells [6]. In contrast, adipose tissues contain 100,000 MSCs in each gram of fat [7]. Further, the differential capacity of adipose tissue-

derived MSCs (AdMSCs) is less affected by donor age [8]. Adipose tissue is an accessible, abundant, and reliable site for the isolation of adult stem cells suitable for tissue engineering and regenerative medicine applications. In this regard, the treatment efficacy of AdMSCs for various diseases has been reported using animal models.

Another interesting characteristic of MSCs is their ability to mobilize to areas of tissue injury. MSCs intravenously delivered to rats after myocardial infarction localize in the infarct region and improve ventricular function, whereas MSCs delivered to noninfarcted rats localize to the BM [9]. Localized abdomen irradiation significantly enhances MSC homing specifically to radiation-injured tissues of mice [10]. The potential for the minimally invasive delivery of MSCs via systemic infusion is of particular interest because of the easy, minimally invasive application of stem cells.

Before culture-expanded stem cells can be used in the human clinic, good manufacturing practices (GMP) must be

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developed for the production of clinical-grade human stem cells. The stem cells should be evaluated for aerobic and anaerobic bacteria, endotoxin, and mycoplasma. The GMP must define the distinct biological characteristics of the cells; verify their stability throughout the storage, thawing, and shipping processes; and ensure the quality assurance and control of the components used [11]. The *in vivo* safety of the stem cells, including toxicity and tumorigenicity, also should be confirmed.

To apply human AdMSCs (hAdMSCs) in the human clinic for stem cell therapy, we determined the characteristics, stability, toxicity, and tumorigenicity of hAdMSCs in animals and transplanted them to patients in this study. The characteristics and stability of culture-expanded hAdMSCs produced in a GMP facility were determined. Cell toxicity and cell tumorigenicity was investigated using animal models. Finally, a human clinical trial was conducted for spinal cord injury (SCI). The patients were administered high dose ( $4 \times 10^8$  cells per patient) of autologous AdMSCs intravenously and monitored the safety as well as the efficacy.

## Materials and Methods

### Isolation and culture of hAdMSCs

Human adipose tissues were obtained by simple liposuction from the abdominal subcutaneous fats with an informed consent. Subcutaneous adipose tissues were digested with collagenase I (1 mg/mL) under gentle agitation for 60 min at 37°C. The digested tissues were filtered through a 100- $\mu$ m nylon sieve to remove cellular debris and were centrifuged at 470 g for 5 min to obtain a pellet. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen)-based media containing 0.2 mM ascorbic acid and 10% fetal bovine serum (FBS) obtained from bovine spongiform encephalopathy free herd. The cell suspension was re-centrifuged at 470 g for 5 min. The supernatant was discarded and the cell pellet was collected. The cell fraction was cultured overnight at 37°C/5% CO<sub>2</sub> in DMEM-based media containing 0.2 mM ascorbic acid and 10% FBS. After 24 h, the cell adhesion was checked under an inverted microscope, and nonadherent cells were removed by washing with phosphate-buffered saline (PBS). The cell medium was changed to Keratinocyte-SFM (Invitrogen)-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL rEGF, and 5% FBS. The cells were maintained for 4–5 days until confluent (passage 0). When the cells reached 90% confluency, they were subculture-expanded in Keratinocyte-SFM-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL rEGF, and 5% FBS until passage 3. FBS contaminant from cultured MSCs were completely removed by several washing with PBS and was verified through the test of albumin level below the measurement limit using a bovine albumin ELISA quantitation kit (Bethyl Laboratories). The Korea Food and Drug Administration permitted the FBS-eliminated MSCs for clinical study.

Aliquots of the hAdMSCs are then tested for cell viability and fungal, bacterial, endotoxin, and mycoplasma contamination as demanded by the Code of Federal Regulations, Title 21 (21CFR) before further use. The details of specific standards are found in the 21CFR, Sections 610 [12]. The procedure for hAdMSCs preparation was performed un-

der GMP conditions in the Stem Cell Research Center of RNL BIO.

### Flow cytometry analysis

Trypsinized hAdMSCs ( $2 \times 10^5$  cells) were suspended in 100  $\mu$ L of PBS containing 5% bovine serum albumin. Cells were stained with FITC-conjugated CD31, CD45, HLA-ABC, and HLA-DR (1:100; BD Biosciences) antibodies, and PE-conjugated CD29, CD34, CD73, CD90 (BD Biosciences), and CD105 (R&D Systems) antibodies. The immunophenotype of hAdMSCs was analyzed using a FACS Calibur flow cytometer (BD Biosciences) using CELL Quest software.

### *In vitro* differentiation

**Adipogenic induction.** hAdMSCs were plated at  $1 \times 10^5$  cells/mL in Keratinocyte-SFM-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL rEGF, and 5% FBS. The medium was replaced with adipocyte induction medium (NH Adipodiff medium; Miltenyi Biotec) or control medium when cells reached 50% confluency. Cells were maintained in culture for 21 days, with 90% of the media being replaced every 3 days. Adipogenic differentiation was monitored by Oil red O staining.

**Osteogenic induction.** hAdMSCs were plated at  $1 \times 10^5$  cells/mL in Keratinocyte-SFM-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL rEGF, and 5% FBS. The medium was replaced with osteoblast induction medium (NH Osteodiff medium; Miltenyi Biotec) or control medium. The cells were maintained in culture for 14 days, with 90% of the media being replaced every 3 days. Osteogenic differentiation was detected by Alizarin red S staining.

**Chondrogenic induction.** hAdMSCs were plated at  $2.5 \times 10^5$  cells/mL in Keratinocyte-SFM-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL rEGF, and 5% FBS. Cells were centrifuged at 500 g for 5 min, resuspended in chondrogenic induction medium (NH chondrogenic medium; Miltenyi Biotec), and centrifuged again at 500 g for 5 min to form pellets. The pellets were maintained in culture using polypropylene tubes for 14 days, with 50% of the media being replaced every 3–4 days. Chondrogenic differentiation was assayed by toluidine blue O staining.

**Myogenic induction.** hAdMSCs were plated at  $1 \times 10^5$  cells/mL in Keratinocyte-SFM-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL rEGF, and 5% FBS. When cell growth reached 50%, the medium was replaced with induction medium (SkGM medium containing 0.1% hEGF, dexamethason, GA 1000, 1% insulin, bovine serum albumin and Fetuin; LONZA) and maintained for 14 days. The cells were fixed with 4% paraformaldehyde and incubated with a human myosin antibody (1:500 dilution; Chemicon). After 3 washes in PBS, cells were incubated with a combination of AlexaFluor 488-conjugated donkey anti-mouse secondary antibodies and stained with DAPI for nucleic acid detection.

**Neurogenic induction.** hAdMSCs were plated at  $1 \times 10^5$  cells/mL in DMEM containing 10% FBS, 20 ng/mL rEGF, and 20 ng/mL FGF for 3 days. The medium was replaced with neuronal induction medium (DMEM containing 10% FBS, 2% DMSO, 200  $\mu$ M BHA, 1  $\mu$ M hydrocortisone, 5  $\mu$ g/mL insulin, 0.5 mM IBMX and 1 mM cAMP) or control media.

The cells were maintained for 8 to 10 days at 37°C, fixed with 4% paraformaldehyde, and incubated with human antibodies against MAP2 (1:200 dilution; Chemicon), neuron specific enolase (1:10 dilution; Abcam), beta III tubulin (TUJ1) (1:1,000 dilution; Abcam), or GFAP (1:250 dilution; Chemicon). After 3 washes in PBS, cells were incubated with a combination of AlexaFluor 488- or 555-conjugated donkey anti-mouse or anti-rabbit secondary antibodies. After secondary antibody staining, cells were stained with DAPI for nucleic acid detection.

### *Karyotype analysis*

Karyotyping was performed by the Cytogenomic Services Facility of Samkwang Medical Laboratories (Seoul, Korea). To analyze the karyotype of hAdMSCs, cells from 3 different donors were cultured and harvested at passage 4, 7, 10, or 12. Cell division was blocked in metaphase by adding 0.05 µg/mL colcemid (Invitrogen) for 1 to 2 h. The chromosomes were then observed by GTG-banding.

### *Single nucleotide polymorphism analysis*

Single-nucleotide polymorphisms (SNPs) of hAdMSCs at different passages (0, 3, 6, or 9) were analyzed. Approximately 750 ng of genomic DNA was used for genotyping using an Illumina HumanHap300 BeadChip (Illumina) instrument at SNP Genetics Inc. Samples were processed according to the Illumina Infinium-II assay manual. Briefly, each sample was whole-genome amplified, fragmented, precipitated, and re-suspended in the appropriate hybridization buffer. Denatured samples were hybridized on a prepared HumanHap300 BeadChip for a minimum of 16 h at 48°C. After hybridization, the Beadchips were processed for the single-base extension reaction, stained, and imaged on an Illumina Bead Array Reader. The normalized bead intensity data obtained from each sample were loaded into the Beadstudio 3.0 software (Illumina), which converted fluorescent intensities into SNP genotypes. The SNP clusters for genotype calling were examined for all SNPs using Beadstudio 3.0 software. Genomic profiles were created using the Illumina Genome Viewer and Chromosome Browser of the Illumina Beadstudio 3.0 software, which plots SNP genotyping data to view, identify, and annotate chromosomal aberrations.

### *Stability evaluation*

hAdMSCs from 3 different donors at passage 3 were placed in 1-mL syringes at  $1.5 \times 10^7$  cells/500 µL saline and stored under a cold condition for up to 72 h. The number and survival rate of hAdMSCs and the expression of surface markers were measured 3 times each at 0, 12, 24, 48, 56, 64, or 72 h.

### *Preclinical toxicity test*

The toxicity study was conducted under Good Laboratory Practice (GLP) conditions and appropriate veterinary supervision at Seoul National University Hospital (SNUH) Clinical Research Institute (Seoul, Korea) with approval of Institutional Animal Care and Use Committee (SNUH-IACUC No. 0801801). Six-week-old SCID mice (91 males and 91 females) were purchased from Harlan and were kept for 1

week. For the toxicity test, 80 healthy males (19.9–24.3 g body weight, B.W.) and 80 healthy females (15.7–19.1 g B.W.) were selected and divided into saline control and low-dose ( $5 \times 10^6$  hAdMSCs/kg B.W.), medium-dose ( $3.5 \times 10^7$  hAdMSCs/kg B.W.), and high-dose MSC groups ( $2.5 \times 10^8$  hAdMSCs/kg B.W.), each with 20 males and 20 females. The cells were injected once into the tail vein. All groups were observed for 13 weeks. High-dose MSCs were administered particularly slowly and carefully at a rate of 10 µL/5 s to avoid embolism. The cells were suspended in 10 mL saline/kg B.W.

The animals were observed twice a day for clinical symptoms (vital signs, appearance, presence and extent of any abnormal response, etc.). The body weight and food and drink intake of the animals were measured before the injection and every week until study termination. Five male or female mice from each group were randomly selected for urine and ophthalmic tests at 13 weeks postinjection.

At the study completion, animals that survived were anesthetized using isoflurane, their blood was collected from the inferior vena cava, and an autopsy was performed. Organ weight and tissue pathological examinations were performed on 10 mice from each group. The remaining mice were observed by the naked eye at the time of autopsy.

Statistical analyses were performed by analysis of variance followed by the Dunnett *t*-test or Duncan/Tukey test using SPSS software (version 12.0K for Windows). Statistical differences were considered significant for  $P < 0.05$ .

### *Tumorigenicity test*

The tumorigenicity study was also conducted under GLP conditions with approval of IACUC (SNUH-IACUC No. 0603303) at SNUH Clinical Research Institute. Six-week-old BALB/c-nu nude mice (58 male and 58 female) were purchased from Harlan and kept for 1 week. Fifty healthy males and 50 healthy females were selected for the tumorigenicity test. Test groups were divided into low- ( $2 \times 10^6$  hAdMSCs/kg B.W.), medium- ( $2 \times 10^7$  hAdMSCs/kg B.W.), and high-dose MSC groups ( $2 \times 10^8$  hAdMSCs/kg B.W.). To compare tumor formation, negative (MRC-5 cells; human fetal lung cell line) and positive (A375 cells; human malignant melanoma cell line) control groups were also prepared. Each group had 10 males and 10 females.

Cells were injected subcutaneously once and the animals were observed for 26 weeks. All animals were observed twice a day for their clinical symptoms. The presence of tumors was assessed twice a week between the first injection and study completion. The tumor dimensions were measured using a caliper, and the tumor size was calculated using the equation ( $\pi/6 \times \text{length} \times \text{width} \times \text{height}$ ). According to the recommendation of the SNUH-IACUC, animals were euthanized when the diameter of the tumor exceeded 250 mm.

### *Human clinical trial for SCI*

The phase I study of autologous adipose stem cells for SCI was approved by the Korea Food and Drug Administration with Investigational New Drug Application number of BPD-455 (April 29, 2009) and the Institutional Review Board (No. 2009002-RNL-ASTROSTEM) at SAM Anyang Hospital (Anyang, Korea). The clinical trial (clinicaltrials.gov Identifier: NCT01274975) started in July 1, 2009, and completed in

February 15, 2010. The candidates were given the information regarding the expected efficacy and safety of the trial resulted from preclinical tests, in which the SCI model animals had significantly improved motor functions with human adipose stem cells and showed no adverse effect. Procedures were performed after obtaining written informed consent from each participant.

Eight male patients were enrolled who had suffered traumatic SCI at least 12 months before this study. The following ASIA impairment scale [13] is used in grading the degree of impairment: A=Complete. No sensory or motor function is preserved in the sacral segments S4-S5. B=Incomplete. Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5. C=Incomplete. Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade <3. D=Incomplete. Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade greater than or equal to 3. E=Normal. Sensory and motor function is normal.

The inclusion criteria were grade A or B on the ASIA Impairment Scale and an age of 19–60 years. Exclusion criteria included ventilator-assisted breathing, history of malignancies within the past 5 years, concomitant infectious diseases such as HIV or HBV infection, anemia, myocardial infarction, chronic renal failure, fever (above 38°C), mental confusion or dysphasia, use of immunosuppressant therapy, or a serious pre-existing medical disease.

Before liposuction operation, patients were assessed for hematology, blood chemistry, and urinalysis, and were screened for HIV, HBV/HCV, and VDRL. A chest X-ray, pulmonary function test, spinal cord independence measure (SCIM), visual analog scale, spinal magnetic resonance imaging, electrophysiological examination of motor and somatosensory evoked potentials (MEP and SEP, respectively), and a neurological examination using ASIA were obtained for each patient.

The hAdMSCs were harvested under sterile conditions and delivered directly at cold temperature without cryopreservation procedure. The quality control test including cell viability, fungal, bacterial, endotoxin, and mycoplasma contamination carried out before the time of delivery. Four 100-mL normal saline containing 100 million cells/each were prepared and a total of  $4 \times 10^8$  autologous hAdMSCs per patient was introduced into the cephalic vein over 3 to 4 h. Patients were interviewed and monitored extensively on days 1, 4, and 7 and weeks 4 and 12 after transplantation. Patients were followed up for a total of 12 weeks post-transplantation.

## Results

### Morphology and phenotype of culture-expanded hAdMSC

AdMSCs were spindle-shaped with a fibroblast-like morphology and were attached to the plate during cell culture. These characteristics were well preserved during repeated subculture. For immunophenotypic characterization of hAdMSCs, culture-expanded cells at passage 3 were prepared from 5 donors, and surface protein expression was examined by flow cytometry. The results of FACS analysis

TABLE 1. IMMUNOPHENOTYPE OF CULTURE-EXPANDED HUMAN MESENCHYMAL STEM CELLS AT PASSAGE 3

Surface markers	The positive percentage (%) (n=5)
CD29	99.81 ± 0.18
CD31	0.50 ± 0.34
CD34	1.95 ± 0.87
CD44	99.77 ± 0.18
CD45	0.60 ± 0.39
CD73	98.12 ± 1.64
CD90	99.99 ± 0.01
CD105	99.95 ± 0.03
HLA-DR	0.68 ± 0.52
HLA-ABC	98.91 ± 0.93

Data are expressed as mean ± standard deviation.

with 5 different donor samples are listed in Table 1. The hAdMSCs were positive for CD29, CD44, CD73, CD90, CD105, and HLA-ABC, but were negative for CD31, CD34, CD45, and HLA-DR.

To investigate immunophenotypic changes during subsequent subculture, hAdMSCs harvested at different passages (1, 2, 4, 7, or 10) from 3 donors were analyzed for the expression of CD73, CD90, CD31, CD34, or CD45. Every passage of hAdMSCs showed a homogenous population of cells with high expression levels of CD73 and CD90 and very low expression levels of CD31, CD34, and CD45 (Table 2). CD73 was expressed at a high level ( $\geq 98\%$ ) up to passage 7, and decreased to 80% in passage 10. The expression of CD90 continued to be  $\geq 97\%$  up to passage 10. Negative markers such as CD31, CD34, and CD45 remained very low throughout all passages. Cell viability evaluated by trypan blue exclusion was  $>95\%$  before cell transplantation (data not shown). To minimize the risk of contamination, all cell culture steps took place in a GMP-grade clean room facility and the clinical and/or preclinical lot was produced under GMP conditions. No evidence of bacterial, fungal, or myco-

TABLE 2. IMMUNOPHENOTYPE OF CULTURE-EXPANDED HUMAN MESENCHYMAL STEM CELLS AT DIFFERENT PASSAGES (1–10)

Donor (sex)	Passages	Surface markers [positive percentage (%)]				
		CD73	CD90	CD31	CD34	CD45
#60018 (Male)	P1	99.89	99.98	1.88	0.51	0.53
	P2	98.25	99.80	3.48	3.76	3.36
	P4	99.62	99.98	1.20	1.39	1.19
	P7	99.89	99.93	1.64	0.71	1.40
	P10	99.89	99.71	2.02	1.55	1.73
#60023 (Male)	P1	90.24	99.96	0.52	1.89	0.40
	P2	86.54	99.88	1.00	0.78	0.50
	P4	99.75	99.99	1.24	1.16	1.03
	P7	99.40	99.81	3.26	0.81	2.00
	P10	81.52	98.73	0.77	0.27	0.22
#60028 (Female)	P1	96.47	99.96	0.50	1.42	0.31
	P2	98.26	99.88	4.54	3.29	3.04
	P4	99.26	99.99	1.29	1.14	0.80
	P7	98.87	99.91	4.04	1.09	3.28
	P10	83.62	97.39	0.82	0.20	0.25

plasmal contamination was observed in cells tested before shipping and cell viability evaluated by trypan blue exclusion was >95% before cell transplantation (data not shown).

#### *Differentiation capability of culture-expanded hAdMSCs*

The ability of hAdMSCs to differentiate into various cell types was investigated. Culture-expanded cells at passage 3 were capable of *in vitro* differentiation into adipocytes as assessed by Oil red O staining (Fig. 1A), osteoblasts as assessed by Alizarin red S staining (Fig. 1B), chondroblasts as assessed by toluidine blue O staining (Fig. 1C), myoblasts as assessed by myosin immunostaining (Fig. 1D), and neuronal cells as assessed by MAP2, neuron specific enolase, TUJ1, and GFAP immunostaining (Fig. 1E–H).

#### *Stability of culture-expanded hAdMSCs*

The hAdMSCs from 3 different donors were culture-expanded to prove their genetic stability during proliferation by karyotyping, which is often used to analyze the genetic stability of stem cells [14–16]. Chromosomal analysis was performed on hAdMSCs at passages 4, 7, 10, and 12. No chromosomal abnormality was observed in any sample up to passage 12. A representative karyotype image is shown in Fig. 2.

Karyotyping would be sufficient to identify the numerical and structural chromosomal aberrations if a large DNA area is affected. If a small DNA area (single gene) is mutated; however, karyotype making is not sufficient and additional molecular genetic method, such as SNP genotyping, shall be applied. Karyotyping and SNP genotyping regarded as a necessary control for the genetic integrity of embryonic stem cells by the International Stem Cell Initiative [17]. The SNP

assay results did not show substantial genotypic differences regardless of passage number within the same origin (Fig. 3). The call rates for hAdMSCs at passages 3, 6, 9, and 12 were 99.78%, 99.90%, 99.91%, and 99.93%, respectively, and the reproducibility of each sample was 100%.

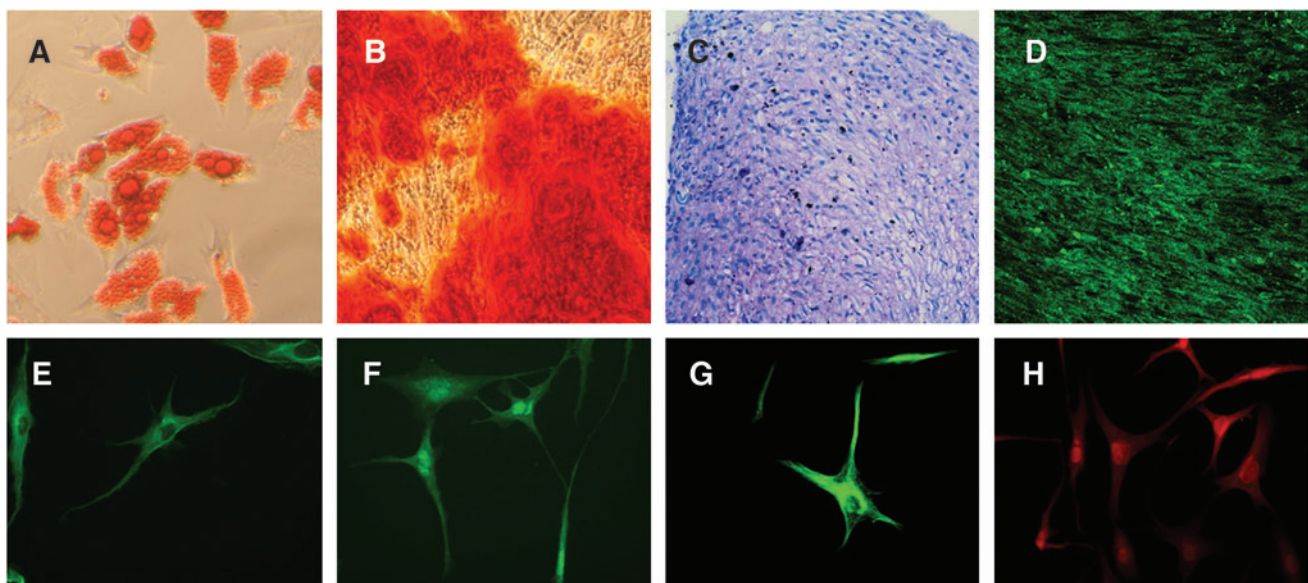
To evaluate the hAdMSC stability in saline solution depending on the storage time, the cell number, survival rate, and purity were measured 3 times at each time point up to 72 h. No significant decrease in the survival rate or total cell number was observed at any time point (Table 3). Cultured hAdMSCs had a survival rate of  $\geq 80\%$  for at least 72 h. A high level of purity was maintained throughout all time points, as demonstrated by the consistent expression of positive or negative surface antigen for MSC up to 72 h.

#### *Preclinical toxicity test*

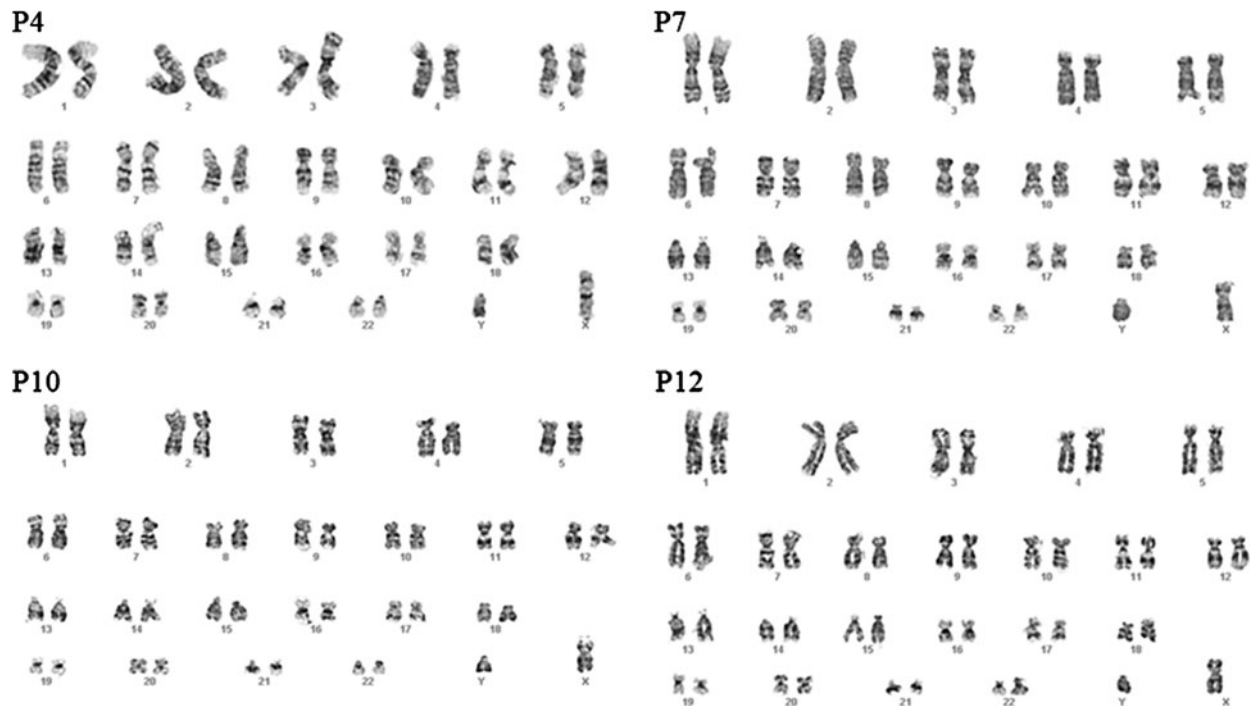
A preclinical toxicity test of the systemic transplantation of hAdMSCs in mice was conducted at a GLP facility for 13 weeks. Among the 120 mice in stem cell-treated groups, one male and 2 female died instantly after the intravenous injection of high-dose AdMSCs ( $2.5 \times 10^8$  cells/kg B.W.). In necropsies, however, specific pathological changes were not observed in these mice organs including lungs.

During the observation period, one female and one male in the low-dose group ( $5 \times 10^6$  hAdMSCs/kg B.W.) and one male in the medium-dose group ( $3.5 \times 10^7$  hAdMSCs/kg B.W.) as well as one male in the saline control group died. The cause of death for these mice was lymphoblastic lymphoma, which naturally occurs in SCID mice, and thus independent from the testing material. No abnormal clinical signs or behavioral symptoms related to the testing material were observed in other mice.

Body weight and food and water consumption were recorded weekly. No differences were seen in the change in



**FIG. 1.** Multilineage differentiation of culture-expanded hAdMSCs. (A) Oil red O staining of induced adipocytes from hAdMSCs. (B) Differentiation of hAdMSCs into osteoblasts as evaluated by Alizarin red S staining. (C) Chondrogenic potential of hAdMSCs as shown by toluidine blue O staining. (D) Differentiated myocytes were detected by immunostaining with myosin antibody (E). The hAdMSCs showed neurogenic differentiation by immunostaining with MAP2, (F) neuron specific enolase, (G) TUJ1, and (H) GFAP antibodies. hAdMSC, human adipose tissue-derived mesenchymal stem cells.

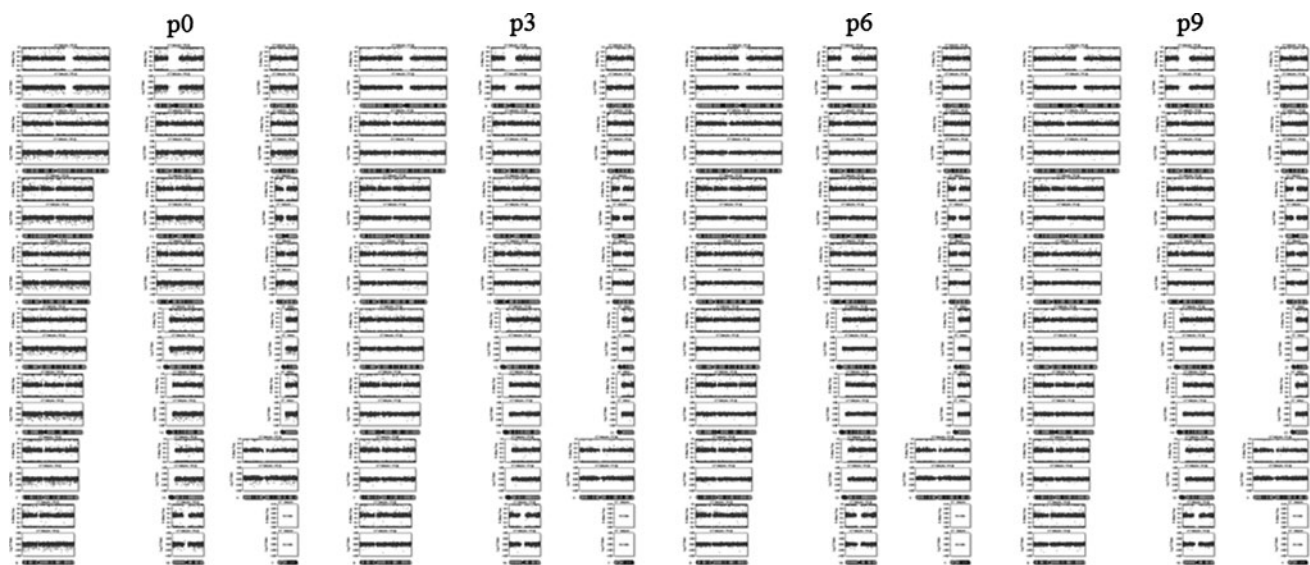


**FIG. 2.** Representative karyotype of culture-expanded hAdMSCs at different passages (4, 7, 10, and 12). The ideogram shows that the hAdMSCs retained their normal karyotype during repeated in vitro proliferations.

body weight or food consumption among the stem cell-treated groups and control groups in both sexes (Supplementary Fig. S1A–D; Supplementary Data are available online at [www.liebertonline.com/scd](http://www.liebertonline.com/scd)). Water intake was increased in the medium-dose group at 3 weeks (3.86 vs. 4.23 g) ( $P < 0.05$ ) and 4 weeks (3.86 vs. 4.42 g) ( $P < 0.01$ ) and in all treated groups at 7 weeks (3.64 vs. 4.02 vs. 4.00 vs. 3.98 g) ( $P < 0.05$ ) postinjection compared to the control in male mice

(Supplementary Fig. S1E). No difference in water consumption was observed in the female groups (Supplementary Fig. S1F). The finding that water consumption was sporadically elevated in the male groups was not related to dose levels or duration time.

Some variations were observed among the groups in the hematology, blood biochemistry, urine, and ophthalmic test results. However, no significant change related to stem cell



**FIG. 3.** Single-nucleotide polymorphism genotyping of culture-expanded hAdMSCs at different passages (0, 3, 6, and 9). The genotype of hAdMSCs was maintained over the subcultures.

TABLE 3. STABILITY OF HUMAN ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS IN SALINE SOLUTION DURING COLD STORAGE

Donor	Time (h)	Viability (%)	Total cell count	Surface markers [positive percentage (%)]				
				CD73	CD90	CD31	CD34	CD45
#60019	0	94.23±2.68	1.39±0.03	95.2±0.9	95.6±0.4	0.90±0.37	0.58±0.20	0.52±0.08
	12	92.57±1.99	1.40±0.02	89.7±5.8	90.0±4.5	0.26±0.08	0.24±0.04	0.21±0.07
	24	88.07±2.68	1.38±0.03	96.6±0.3	96.2±1.0	0.23±0.07	0.20±0.02	0.20±0.01
	48	91.97±2.00	1.38±0.02	92.9±1.8	91.7±2.9	1.41±0.39	0.69±0.25	0.64±0.25
	56	89.40±4.36	1.40±0.02	94.9±2.0	96.3±0.6	0.63±0.06	0.45±0.01	0.40±0.05
	64	90.20±1.10	1.40±0.02	94.3±4.1	92.8±3.3	0.93±0.14	0.84±0.17	0.80±0.18
	72	90.37±1.45	1.38±0.02	96.4±0.4	96.5±0.4	1.00±0.19	0.80±0.14	0.73±0.16
#60029	0	95.90±1.28	1.40±0.02	92.5±2.4	92.0±3.3	0.47±0.07	0.44±0.06	0.37±0.05
	12	92.83±1.74	1.39±0.02	94.7±0.7	96.1±1.0	0.37±0.31	0.29±0.22	0.25±0.19
	24	90.07±1.62	1.38±0.03	94.8±2.6	96.6±0.1	0.42±0.07	0.26±0.09	0.26±0.05
	48	88.70±1.42	1.40±0.02	94.3±1.0	96.6±0.3	0.25±0.04	0.24±0.02	0.26±0.08
	56	84.87±3.01	1.38±0.03	94.8±1.0	96.5±0.1	0.27±0.08	0.25±0.01	0.26±0.11
	64	83.87±0.87	1.38±0.03	84.9±2.3	95.8±0.0	0.41±0.03	0.34±0.05	0.29±0.03
	72	84.97±5.66	1.37±0.01	94.9±1.3	96.5±0.7	0.49±0.25	0.32±0.05	0.26±0.03
#70629	0	94.10±1.18	1.38±0.02	94.2±0.6	94.2±0.7	1.47±0.40	1.03±0.10	0.88±0.02
	12	92.07±1.62	1.37±0.01	94.4±0.1	94.7±0.3	0.69±0.23	0.54±0.08	0.57±0.13
	24	87.37±0.70	1.37±0.03	92.3±0.6	93.1±0.6	1.46±0.23	1.49±0.22	1.28±0.24
	48	85.33±0.80	1.38±0.02	93.4±0.7	94.1±0.6	1.26±0.16	1.28±0.36	0.95±0.19
	56	86.13±4.48	1.35±0.03	89.4±7.2	95.4±0.6	1.16±0.07	1.19±0.24	0.23±0.06
	64	81.83±1.20	1.35±0.02	85.5±2.4	95.4±0.3	0.29±0.07	0.23±0.04	0.22±0.07
	72	81.00±2.36	1.38±0.03	91.2±1.3	95.8±0.8	0.31±0.20	0.23±0.12	0.19±0.09

Measurements were repeated 3 times for each time point. Data are expressed as mean±standard deviation.

transplantation was observed. At autopsy, no significant difference was observed in the absolute and/or relative organ weights among the groups, except the adrenal glands in male and female mice of the low-dose group. In male mice, the absolute weight (0.0016 vs. 0.0012 g) ( $P<0.01$ ) and relative weight (0.0059 vs. 0.0041 g) ( $P<0.01$ ) of the right adrenal gland were decreased significantly in the low-dose group compared to the control group. In female mice, the relative weight (0.0162 vs. 0.0129 g) ( $P<0.05$ ) of the left adrenal gland was decreased significantly in the low-dose group compared to the control group.

Histopathological analysis revealed no abnormal findings. Various histopathologic lesions, including lymphoma, leaky thymus, and mineral deposition in the heart (chalky linear streaks), were observed in both male- and female-treated mice and control mice at autopsy. These lesions are common in SCID mice, and therefore were thought to be independent from the injection of the testing material.

Taken together, these findings indicated that the no-observed-effect level for the developmental toxicity of hAdMSCs is  $>2.5\times 10^8$  cells/kg B.W.

#### Tumorigenicity test in animals

The preclinical tumorigenicity test of hAdMSCs in mice was conducted at a GLP facility for 26 weeks. During the experiment, one male mouse in the low-dose group ( $5\times 10^6$  hAdMSCs/kg B.W.) died at 10 weeks post-transplantation. However, histological examination indicated that the death was not related to the infused cells. In the positive control (A375 cells) group, 2 female mice were dead on days 49 and 62 due to excessive tumor growth. No clinical or behavioral abnormality was observed, except for scars on the backs and

buttocks of numerous male mice, probably due to their attacks on each other. The male group did not show any differences in body weight throughout the entire period of the experiment.

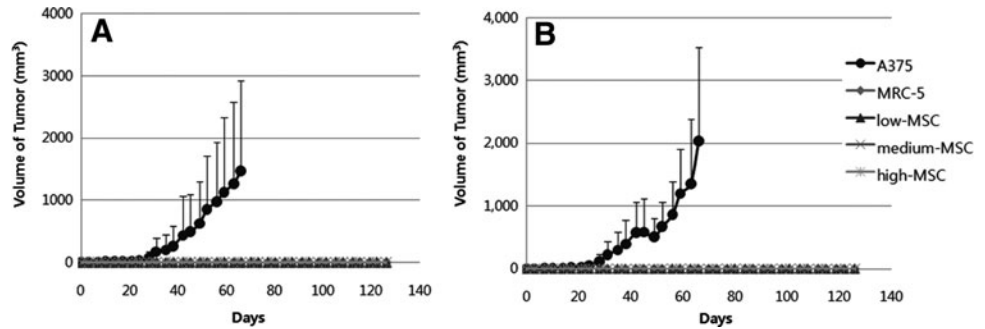
Among the female mice, the low-dose group showed an increasing pattern of body weight at 49, 56, 77, 168, and 182 days after injection compared to the negative control group. However, the increase of the body weight did not correlate with the injection dose and was thought to be due to intermittent changes. Comparing the absolute and/or relative organ weights, there were some changes in the kidney, adrenal gland, lung, and brain compared to the negative control group mice, but these changes occurred sporadically and were not correlated to the number of transplanted cells. Mice in the positive control group were excluded from the statistical analysis of organ weights due to their early death during the study.

Mice were inspected for tumor development twice a week for 26 weeks after injection of the testing material. All mice in the positive control group developed tumors, but no mice in any other group developed a tumor (Fig. 4). For humane treatment with regard to the excessive tumor growth, all mice in the positive control group were euthanized at 67 days post-transplantation. The results indicated that hAdMSCs did not induce tumors up to  $2\times 10^8$  cells/kg B.W.

#### Clinical phase I trial for SCI

Male SCI patients aged 23–54 years were included in the study. The time period since SCI ranged 1.07–7.88 years. Among the 8 patients enrolled in this study, 7 patients had quadriplegia and 1 patient had paraplegia with no evidence of ongoing SCI recovery. Causes of SCI in the 8 patients

**FIG. 4.** Tumor volume changes in (A) male and (B) female BALB/c-nu mice inoculated subcutaneously with 3 different doses of hAdMSCs, MRC-5 cells (negative control), or A375 cells (positive control) in the tumorigenicity test. The tumor volume was measured twice a week for 26 weeks. However, A375 group mice were sacrificed on day 67 because of animal welfare concerns regarding excessive tumor growth. Error bars represent standard deviation.

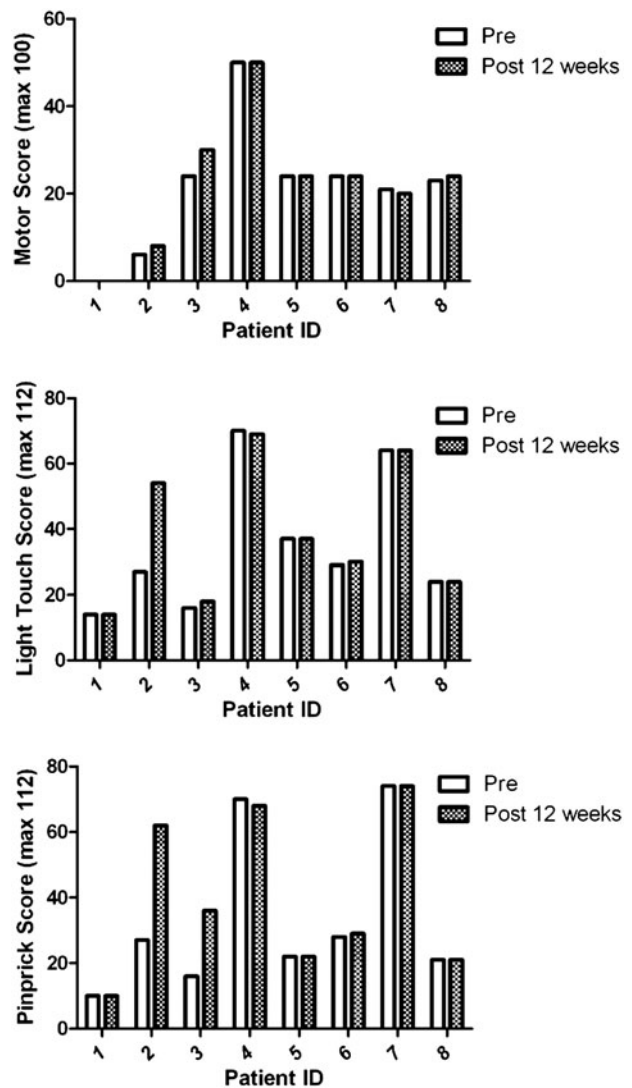


included 4 traffic accidents, 2 falls, 1 fall from a horse, and 1 diving accident. The safety of infused hAdMSCs was evaluated by adverse events, lab findings, electrocardiogram, physical examination, and vital signs.

Regardless of the correlation with cell transplantation, 19 adverse events were observed in the 8 recipients, including chest pain, chest tightness, mild fever, furuncle on the upper thigh, musculoskeletal pain, painful neck and shoulder, increased sputum, upper respiratory infection, urinary incontinence, urinary tract infection, aggravation of spasticity, neuropathic pain, exacerbation of pain, headache, low thyroid stimulating hormone, and somnolence. No patient experienced serious complications. All adverse events resolved or stabilized during follow-up, except for one patient who showed an abnormal thyroid function. This patient still showed low thyroid stimulating hormone levels in the follow-up evaluation, but the finding was not considered clinically important. There were no significant differences between the pre- and 12-weeks postinjection lab findings, electrocardiogram, physical examination, or vital signs. The areas of spinal damage estimated by magnetic resonance imaging decreased from  $134.50 \pm 95.69 \text{ mm}^2$  to  $122.93 \pm 99.45 \text{ mm}^2$  at 12 weeks, but the difference was not significant ( $P=0.8047$ ).

The amplitude and latency of MEP and SEP were measured before and 12 weeks after injection (Fig. 5). Arm MEP was conducted in one patient with paraplegia. An additional 6 patients were tested with arm SEP and 3 patients were tested with leg SEP. No statistical differences were seen in the latency or amplitude of arm SEP. However, the latency of the left side in leg SEP increased from  $41.93 \pm 4.39$  to  $48.27 \pm 3.93$  ( $P < 0.05$ ). The latency of the right side also increased but not significantly ( $P=0.0516$ ). In one patient (001-S006), the amplitude and latency of the arm SEP developed after treatment, whereas there had been no response before treatment.

In one patient (001-S003), the ASIA impairment grade changed from ASIA A to ASIA C. This patient improved by 6 points for motor, 20 points for sensory pin prick, and 2 points for sensory light touch (Fig. 5). In other patients, ASIA impairments did not change at 12 weeks. The ASIA sensory scoring of one patient (001-S002) improved by 27 and 35 points for sensory pin prick and sensory light touch, respectively (Fig. 5). No significant differences were seen in the pulmonary function test, SCIM, and visual analog scale



**FIG. 5.** Motor and sensory scores in patients with spinal cord injury at pre- and 12 weeks post-therapy. Motor (upper), sensory light touch (middle), and sensory pinprick (lower) scores were assessed according to the ASIA impairment scale.



findings. However, one patient (001-S003), who had been unable to eat without assistance or adaptive devices and needed total assistance for dressing before treatment, could hold a cup and could dress with partial assistance after treatment. This finding increased the SCIM score of the patient by 3 points in the self-care category. Another patient (001-S008) with constipation had a sphincter management score that increased by 5 points after treatment. He had regular stools with aid.

## Discussion

Use of MSCs from adipose tissues, BM, or cord blood is a new concept technology that breaks the limits of current drug and medical technologies. In regenerative medicine, stem cells can replace injured cells and tissues. They can also be used to address various incurable diseases that cannot be adequately treated with medication or surgery, including critical limb ischemia, arthritis, and SCI.

To achieve therapeutic clinical results with MSCs, large quantities of the cells must be generated by *ex vivo* expansion. However, long-term culture can alter the quality of MSCs, including their proliferative capacity [18], differentiation potential [19], and trophic activity [20]. Such alterations, including genetic abnormalities, abnormal nucleus type, and changes in cellular characteristics, have also been reported in embryonic stem cells [21,22]. With regard to the safety of culture-expanded stem cells, researchers must verify that the passaged MSCs are genetically stable. They must also obtain consistent data on the morphological, immunophenotypic, and differentiation characteristics, as well as data concerning their toxicity and tumorigenicity.

To address these issues, we evaluated the safety of AdMSCs obtained from human adipose tissues in animal models and in a phase I human clinical trial. The hAdMSCs were attached to the bottom of a culture dish, where they grew rapidly and acquired a spindle shape. More than one billion cells were easily obtained at passage 3 or 4 from <10 g of fat tissue. Subsequent cultivation up to passage 10 did not alter the cell morphology or surface marker expression. The immunophenotype of hAdMSCs showed the expression of MSC antigens (CD29, CD44, CD73, CD90, CD105, and HLA-ABC) and the absence of hematopoietic and endothelial antigens (CD31, CD34, CD45, and HLA-DR). The hAdMSCs were capable of differentiating into multiple lineages, including adipogenic, osteogenic, chondrogenic, myogenic, and neurogenic.

There is some reporting about the risk of transformation MSCs during the culture process. In the study of Rubio et al., spontaneous transformation of adipose-derived human MSCs was found to occur with prolonged culture [23] and seemed to involve a mesenchymal-epithelial transition [24]. However, all the data concerning the transformation were later found to be related to contamination by an epithelial cancer cell line during the experimental procedures [25,26]. Another report of concerning of human BM MSC transformation by Røsland et al. [27] was also retracted due to similar reason [28].

A normal karyotype of the MSCs in this study was maintained at least 12 subcultures. We did not evaluate the karyotype of the MSCs >12 passage because the final GMP-grade MSCs were considered to be used to human trial at

passage 3 and the 4 times long subculture period is enough to verify the genetic stability of the hAdMSC. Moreover, SNP arrays did not show any genome abnormalities in the culture-expanded hAdMSCs. Our findings are consistent with results of other groups on genetically stable MSCs during culture [29,30] and comments on a review article [31]. These findings indicate that the cell preparations fulfill the requirements for MSCs suggested by the ICST [5], and that the cell culture procedure can be used to reliably obtain a large number of culture-expanded hAdMSCs.

Although the culture-expanded MSCs displayed stem cell properties and retained their genetic stability, stem cells must be prepared under simple conditions (ie, physiological saline rather than culture medium) if they are to be used clinically. If the MSCs lose their characteristics in an environment other than culture medium, then the utility of the MSCs is severely limited and the quality of the results is questionable. We tested the stability of MSCs suspended in a saline solution under cold conditions for 3 days. This observation time was determined considering that 3 days is a reasonable time period between shipping and using stem cells in the clinic. Cells in saline remained stably viable and maintained their original immunophenotype for at least 72 h. Thus, the demand for MSCs in an area that is 2 to 3 days away from an MSC source can be met, allowing MSCs to be used abroad. However, the physical vibration during shipment may negatively impact viability of a cell product and that further analysis would be to evaluate this matter.

In this study, the toxicity of hAdMSCs was evaluated in immunodeficient mice with various dose levels. Establishing a single cell dose limit will be very useful for applying MSC therapy clinically. A recent study of stem cells in a rat model of middle cerebral artery occlusion reported the greatest therapeutic benefit when a single high cell dose injection was used, rather than multiple infusions of smaller cell doses over several time points [32]. On the other hand, the risk of intravascular transplantation of cultured MSCs was also reported [33]. Furlani et al. described the death of small laboratory animals was due to their relatively large cell size inducing pulmonary sequestration [33].

In the present study, we injected the cultured hAdMSCs into tail vein at 3 different concentrations ( $5 \times 10^6$  or  $3.5 \times 10^7$  or  $2.5 \times 10^8$  cells/kg). Although caution was taken to avoid accidental bolus injection during administration of the highest dose ( $\sim 4\text{--}5 \times 10^6$  cells per mouse), 3 animals in the high-dose group died soon after cell injections because of accidental rapid injections by hand. Slow injections of high dose cells into the mice substituted for dead mice did not induce any adverse events, confirming that the deaths were not related to stem cell toxicity. Except sudden death of 3 mice due to mal-manipulation before starting the toxicity test, no abnormal findings related to the cells were observed. We concluded that the no-observed-effect level for hAdMSCs is  $>2.5 \times 10^8$  cells. Similar results were reported for the systemic administration of allogenic BMMSCs; the IV infusion of  $5 \times 10^6$  MSCs/kg B.W. of BMMSCs did not change the overall health or immune status of recipient baboons [34].

In a separate experiment, *in vivo* distribution of intravenous hAdMSCs was investigated in SCI model rats using fluorescence labeled hAdMSCs. Relative organ distributions of hAdMSCs in brain, spinal cord, spleen, thymus, kidney,

liver, lung, and heart were analyzed using a fluorescence microscopy and human specific *Alu* PCR. We found that the cells largely remained spleen (40%), thymus (21%), and surprisingly, spinal cord (13%) (manuscript in preparation).

Although the safety of intravenous cultured MSCs was confirmed in patients [35] and many human clinical studies of MSCs have been implied to treat diseases such as osteogenesis imperfect [36], metachromatic leukodystrophy [37], acute myocardial infarction [38] and graft-versus-host disease [39], there were some reports presenting that MSCs can induce sarcoma [40] or facilitate the growth of tumor [41]. Therefore, the tumorigenicity of hAdMSCs was investigated in an animal model. In this tumorigenicity test, hAdMSCs were injected into immunodeficient mice and observed for 26 weeks. Even when high doses of cells ( $2 \times 10^8$  hAdMSCs/kg B.W.) were injected into mice, tumors were not found in any of the animals. In contrast, all mice that received A375 cells as a positive control developed tumors. Our results indicated that hAdMSCs in the present study do not induce tumor formation. In line with our result, Vilalta et al. reported that the implanted hAdMSCs tended to maintain a steady state, and no detectable chromosomal abnormalities nor tumors formed during the 8 months of residence in the host's tissues [42]. Therefore, tumor formation is considered as dependent on source of MSCs (species, tissues), quality of MSCs, and affected tumor type. Notably, the development of sarcoma in the study of Tolar et al. was due to the cytogenetically abnormal cultured MSCs [40]. In addition, Izadpanah et al. demonstrated that the long-term culture of MSCs resulted in their transformation to malignant cells [43]. This is one reason why the culture of MSCs is processed under a clinical grade GMP conditions and the expanded cells is verified for genetic stability and preclinical tumorigenicity before human trials.

Finally, the safety of hAdMSCs was investigated in a human clinical trial. The number of hAdMSCs were determined based on the results of preclinical efficacy test, in which the SCI model rats had improved behavioral impairment in a dose-dependent manner, ranging from 0.1 to 2.5 million cells/300 g B.W. (20–500 million cells/60 kg B.W. in human) by intravenous infusion of hAdMSCs (manuscript in preparation). Because of behavioral restrictions of SCI patients, the body weight of patients was not measured and all patients were given the same amount of  $4 \times 10^8$  autologous hAdMSCs in human trial. No serious adverse event associated with the intravenous administration of  $4 \times 10^8$  hAdMSCs was observed during the completion of the phase I study. These results confirm the animal model results of hAdMSC toxicity in humans. Some adverse events were found in patients but these were symptoms common to SCI patients. The adverse events improved spontaneously or were alleviated with medication. One idiopathic case of asymptomatic hyperthyroidism that did not require medical treatment remained sustained in follow-up.

The efficacy of hAdMSC administration in SCI patients was also investigated. According to a previous longitudinal study [44], most traumatic SCI patients (94.4%) who had a neurologically complete injury at 1 year remained complete at 5 years postinjury, with 3.5% improving to ASIA grade B and up to 1.05% each improving to ASIA grades C or D. In the current clinical trial, one patient out of 4 (25%) with ASIA grade A showed improvement to ASIA grade C. Motor score improvement was shown in 3 patients. In ASIA grade B, a

patient with sensory pin prick should have a better prognosis for regaining functional ambulation than patients with sensory light touch [45,46]. Because the pin prick scores were mostly improved compared to the light touch score in this trial, favorable prognosis seemed to be expected.

Although there are some restorations of function, the efficacy of hAdMSCs from the present results in SCI patients cannot be determined due to the following limitations in the study design. First, although commonly observed in phase I trials, the control group was omitted. Second, the number of patients was too small to have adequate statistical power to detect the frequency of adverse events or the recovery rate. Third, the observation time was relatively short, prohibiting us from verifying the possibility of recovery.

Based on the toxicity and tumorigenicity test results, we conclude that the transplantation of up to  $2 \times 10^8$  cells/kg B.W. autologous hAdMSCs may be safe when given by slow intravenous infusion.

### Author Disclosure Statement

No competing financial interests exist.

### References

- Friedenstein AJ, RK Chailakhjan and KS Lalykina. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 3:393–403.
- Erices A, P Conget and JJ Minguell. (2000). Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 109:235–242.
- Zhang Y, C Li, X Jiang, H Li, P Tang and N Mao. (2004). Comparison of mesenchymal stem cells from human placenta and bone marrow. *Chin Med J* 117:882–887.
- Zuk PA, M Zhu, H Mizuno, J Huang, JW Futrell, AJ Katz, P Benhaim, HP Lorenz and MH Hedrick. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 7:211–228.
- Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, D Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8:315–317.
- Pittenger MF, AM Mackay, SC Beck, RK Jaiswal, R Douglas, JD Mosca, MA Moorman, DW Simonetti, S Craig and DR Marshak. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science (New York, N.Y.)* 284:143–147.
- Sen A, YR Lea-Currie, D Sujkowska, DM Franklin, WO Wilkison, YD Halvorsen and JM Gimble. (2001). Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. *J Cell Biochem* 81:312–319.
- Jo JY, SK Kang, IS Choi and JC Ra. (2009). Comparison of neural cell differentiation of human adipose mesenchymal stem cells derived from young and old ages. *Dev Reprod* 13:227–237.
- Saito T, J Kuang, B Bittira, A Al-Khaldi and RCJ Chiu. (2002). Xenotransplant cardiac chimera: immune tolerance of adult stem cells. *Ann Thorac Surg* 74:19–24; discussion 24.
- Mouisseddine M, S François, A Semont, A Sache, B Allenet, N Mathieu, J Frick, D Thierry and A Chapel. (2007). Human mesenchymal stem cells home specifically to radiation-

- injured tissues in a non-obese diabetes/severe combined immunodeficiency mouse model. *Br J Radiol* 80 Spec No 1:S49–S55.
11. Gimble JM, F Guilak and BA Bunnell. (2010). Clinical and preclinical translation of cell-based therapies using adipose tissue-derived cells. *Stem Cell Res Ther* 1:19.
  12. CFR - Code of Federal Regulations Title 21, 2010. [www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm](http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm).
  13. Maynard FM, MB Bracken, G Creasey, JF Ditunno, WH Donovan, TB Ducker, SL Garber, RJ Marino, SL Stover, CH Tator, RL Waters, JE Wilberger and W Young. (1997). International standards for neurological and functional classification of spinal cord injury. *American Spinal Injury Association. Spinal Cord* 35:266–274.
  14. Brimble SN, X Zeng, DA Weiler, Y Luo, Y Liu, IG Lyons, WJ Freed, AJ Robins, MS Rao and TC Schulz. (2004). Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. *Stem Cells Dev* 13:585–597.
  15. Lange C, F Cakiroglu, A Spiess, H Cappallo-Obermann, J Dierlamm and AR Zander. (2007). Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. *J Cell Physiol* 213:18–26.
  16. Meza-Zepeda LA, A Noer, JA Dahl, F Micci, O Myklebost and P Collas. (2008). High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. *J Cell Mol Med* 12:553–563.
  17. Loring JF and MS Rao. (2006). Establishing standards for the characterization of human embryonic stem cell lines. *Stem Cells (Dayton, Ohio)* 24:145–150.
  18. Wislet-Gendebien S, P Leprince, G Moonen and B Rogister. (2003). Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. *J Cell Sci* 116:3295–3302.
  19. Bonab MM, K Alimoghaddam, F Talebian, SH Ghaffari, A Ghavamzadeh and B Nikbin. (2006). Aging of mesenchymal stem cell in vitro. *BMC Cell Biol* 7:14.
  20. Briquet A, S Dubois, S Bekaert, M Dolhet, Y Beguin and A Gothot. (2010). Prolonged ex vivo culture of human bone marrow mesenchymal stem cells influences their supportive activity toward NOD/SCID-repopulating cells and committed progenitor cells of B lymphoid and myeloid lineages. *Haematologica* 95:47–56.
  21. Imreh MP, K Gertow, J Cedervall, C Unger, K Holmberg, K Szöke, L Csöregi, G Fried, S Dilber, E Blennow and L Ahrlund-Richter. (2006). In vitro culture conditions favoring selection of chromosomal abnormalities in human ES cells. *J Cell Biochem* 99:508–516.
  22. Hanson C and G Caisander. (2005). Human embryonic stem cells and chromosome stability. *APMIS* 113:751–755.
  23. Rubio D, J Garcia-Castro, MC Martín, R de la Fuente, JC Cigudosa, AC Lloyd and A Bernad. (2005). Spontaneous human adult stem cell transformation. *Cancer Res* 65:3035–3039.
  24. Rubio D, S Garcia, T De la Cueva, MF Paz, AC Lloyd, A Bernad and J Garcia-Castro. (2008). Human mesenchymal stem cell transformation is associated with a mesenchymal-epithelial transition. *Exp Cell Res* 314:691–698.
  25. de la Fuente R, A Bernad, J Garcia-Castro, MC Martín and JC Cigudosa. (2010). Retraction: spontaneous human adult stem cell transformation. *Cancer Res* 70:6682.
  26. Garcia S, A Bernad, MC Martín, JC Cigudosa, J Garcia-Castro and R de la Fuente. (2010). Pitfalls in spontaneous in vitro transformation of human mesenchymal stem cells. *Exp Cell Res* 316:1648–1650.
  27. Røsland GV, A Svendsen, A Torsvik, E Sobala, E McCormack, H Immervoll, J Mysliwicz, J Tonn, R Goldbrunner, PE Lønning, R Bjerkvig and C Schichor. (2009). Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 69:5331–5339.
  28. Torsvik A, GV Røsland, A Svendsen, A Molven, H Immervoll, E McCormack, PE Lønning, M Primon, E Sobala, J Tonn, R Goldbrunner, C Schichor, J Mysliwicz, TT Lah, H Motaln, S Knappskog and R Bjerkvig. (2010). Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track—letter. *Cancer Res* 70:6393–6396.
  29. Bernardo ME, N Zaffaroni, F Novara, AM Cometa, MA Avanzini, A Moretta, D Montagna, R Maccario, R Villa, MG Daidone, O Zuffardi and F Locatelli. (2007). Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 67:9142–9149.
  30. Kim J, JW Kang, JH Park, Y Choi, KS Choi, KD Park, DH Baek, SK Seong, H Min and HS Kim. (2009). Biological characterization of long-term cultured human mesenchymal stem cells. *Arch Pharm Res* 32:117–126.
  31. Philippe B, S Luc, P Valérie, R Jérôme, B Alessandra and C Louis. (2010). Culture and use of mesenchymal stromal cells in phase I and II clinical trials. *Stem Cells Int* 2010:503593.
  32. Omori Y, O Honmou, K Harada, J Suzuki, K Houkin and JD Kocsis. (2008). Optimization of a therapeutic protocol for intravenous injection of human mesenchymal stem cells after cerebral ischemia in adult rats. *Brain Res* 1236:30–38.
  33. Furlani D, M Ugurlucan, L Ong, K Bieback, E Pittermann, I Westien, W Wang, C Yerebakan, W Li, R Gaebel, R Li, B Vollmar, G Steinhoff and N Ma. (2009). Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. *Microvasc Res* 77:370–376.
  34. Beggs KJ, A Lyubimov, JN Borneman, A Bartholomew, A Moseley, R Dodds, MP Archambault, AK Smith and KR McIntosh. (2006). Immunologic consequences of multiple, high-dose administration of allogeneic mesenchymal stem cells to baboons. *Cell Transplant* 15:711–721.
  35. Lazarus HM, SE Haynesworth, SL Gerson, NS Rosenthal and AI Caplan. (1995). Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Bone Marrow Transplant* 16:557–564.
  36. Horwitz EM, PL Gordon, WKK Koo, JC Marx, MD Neel, RY McNall, L Muul and T Hofmann. (2002). Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc Natl Acad Sci USA* 99:8932–8937.
  37. Koç ON, J Day, M Nieder, SL Gerson, HM Lazarus and W Krivit. (2002). Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). *Bone Marrow Transplant* 30:215–222.
  38. Chen S, W Fang, F Ye, Y Liu, J Qian, S Shan, J Zhang, RZ Chunhua, L Liao, S Lin and J Sun. (2004). Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* 94:92–95.

39. Le Blanc K, F Frassoni, L Ball, F Locatelli, H Roelofs, I Lewis, E Lanino, B Sundberg, ME Bernardo, M Remberger, G Dini, RM Egeler, A Bacigalupo, W Fibbe and O Ringdén. (2008). Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 371:1579–1586.
40. Tolar J, AJ Nauta, MJ Osborn, A Panoskaltis Mortari, RT McElmurry, S Bell, L Xia, N Zhou, M Riddle, TM Schroeder, JJ Westendorf, RSMcIvor, PCW Hogendoorn, K Szuhai, L Oseth, B Hirsch, SR Yant, MA Kay, A Peister, DJ Prockop, WE Fibbe and BR Blazar. (2007). Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells* (Dayton, Ohio) 25:371–379.
41. Zimmerlin L, AD Donnenberg, JP Rubin, RJ Landreneau, P Basse and VS Donnenberg. (2011). Regenerative therapy and cancer: in vitro and in vivo studies of the interaction between adipose-derived stem cells and breast cancer cells from clinical isolates. *Tissue Engineering Part A* 17:93–106.
42. Vilalta M, IR Dégano, J Bagó, D Gould, M Santos, M García-Arranz, R Ayats, C Fuster, Y Chernajovsky, D García-Olmo, N Rubio and J Blanco. (2008). Biodistribution, long-term survival, and safety of human adipose tissue-derived mesenchymal stem cells transplanted in nude mice by high sensitivity non-invasive bioluminescence imaging. *Stem Cells Dev* 17:993–1003.
43. Izadpanah R, D Kaushal, C Kriedt, F Tsien, B Patel, J Dufour and BA Bunnell. (2008). Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. *Cancer Res* 68:4229–4238.
44. Kirshblum S, S Millis, W McKinley and D Tulsy. (2004). Late neurologic recovery after traumatic spinal cord injury. *Arch Phys Med Rehabil* 85:1811–1817.
45. Crozier KS, V Graziani, JF Ditunno and GJ Herbison. (1991). Spinal cord injury: prognosis for ambulation based on sensory examination in patients who are initially motor complete. *Arch Phys Med Rehabil* 72:119–121.
46. Waters RL, RH Adkins, JS Yakura and I Sie. (1994). Motor and sensory recovery following incomplete tetraplegia. *Arch Phys Med Rehabil* 75:306–311.

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