Tumorigenicity Evaluation of Umbilical Cord Blood-derived Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) have been identified in multiple types of tissue and exhibit characteristic self-renewal and multi-lineage differentiation abilities. However, the possibility of oncogenic transformation after transplantation is concerning. In this study, we investigated the tumorigenic potential of umbilical cord blood-derived MSCs (hUCB-MSCs) relative to MRC-5 and HeLa cells (negative and positive controls, respectively) both *in vitro* and *in vivo*. To evaluate tumorigenicity *in vitro*, anchorage-independent growth was assessed using the soft agar colony formation assay. hUCB-MSCs and MRC-5 cells formed few colonies, while HeLa cells formed a greater number of larger colonies, indicating that hUCB-MSCs and MRC-5 cells do not have anchorage-independent proliferation potential. To detect tumorigenicity *in vivo*, hUCB-MSCs were implanted as a single subcutaneous injection into BALB/c-nu mice. No tumor formation was observed in mice transplanted with hUCB-MSCs or MRC-5 cells based on macro- and microscopic examinations; however, all mice transplanted with HeLa cells developed tumors that stained positive for a human gene according to immunohistochemical analysis. In conclusion, hUCB-MSCs do not exhibit tumorigenic potential based on *in vitro* and *in vivo* assays under our experimental conditions, providing further evidence of their safety for clinical applications.

Key words: Tumorigenicity, Mesenchymal stem cells, Immunohistochemistry, Soft agar assay

INTRODUCTION

Mesenchymal stem cells (MSCs) have been identified in multiple types of tissue including bone marrow, umbilical cord, and adipose (1). They came to prominence as a potential source of stem cells for cellular and genetic therapies due to their inherent abilities of self-renewal, proliferation, and functional multi-lineage differentiation (i.e., bone, cartilage, neurons, adipocytes, and cardiomyocytes). Clinical use of MSCs is emerging, providing valuable insight into regenerative medicine and tissue engineering (2-6).

However, therapeutic applications of MSCs are limited, owing to concerns that they could be prone to malignant transformation. It was reported that *ex vivo* expansion of mouse bone marrow MSCs induced transformation with subsequent generation of sarcomas after implantation in baboons (7). Furthermore, one study documented that bone marrow-derived cells, presumably MSCs, progressed from metaplasia and dysplasia to gastric epithelial cancer in mice with chronic mucosal injury (8).

Therefore, the tumorigenic potential of MSCs is a major concern in clinical applications, and regulatory guidelines recommend evaluating their tumorigenicity using immuno-compromised animals to assess stem cell safety (9). To use MSCs safely in stem cell therapy in humans, it is important to determine whether human MSCs progress to malignant transformation. The purpose of this study was to investigate the potential malignant transformation of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) using *in vitro* and *in vivo* tumorigenicity assays.

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MATERIALS AND METHODS

Cell lines and experimental animals. hUCB-MSCs were provided by Kang Stem Biotech (Seoul, Korea) and maintained as previously reported (10). Cells at passage 6 were used for all experiments (11). To compare the *in vitro* and *in vivo* tumorigenic potential of hUCB-MSCs, HeLa (human adenocarcinoma) and MRC-5 (human lung fibroblast) cell lines were used as positive and negative controls, respectively (American Type Culture Collection, VA, USA). HeLa and MRC-5 cells were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC, VA, USA) supplemented with 10% fetal bovine serum (FBS, ATCC, VA, USA) in an incubator at 37°C with 5% CO_2.

To examine tumorigenicity *in vivo*, 4-week-old BALB/c-nu/nu female mice were purchased from Japan SLC Co. (Shizuoka, Japan). Mice were housed two per filter-top polycarbonate cage with aspen bedding at 23 ± 3°C with a relative humidity of 50 ± 10%, air ventilation of 10~20 times/hr, and light intensity of 150~300 Lux under a 12-hr light/dark cycle throughout the experimental period. The temperature and relative humidity of the room were maintained automatically, and ventilation frequency and light intensity were monitored periodically. Sterile pellet food (Labdiet #5053, PMI Nutrition International, St. Louis, USA) and water were available *ad libitum*. This animal study was reviewed and assessed by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (KIT).

In vitro tumorigenicity assay. To evaluate tumorigenicity *in vitro*, anchorage-independent growth was assessed with the soft agar colony formation assay using the 96-well plate. Briefly, the base agar layer was prepared from a 0.6% soft agar solution containing Dulbecco's Modified Eagle's Medium (DMEM) containing FBS in a 96-well plate. Then, 1×10^4 cells were suspended in DMEM containing FBS and 0.4% agar solution and plated onto the base layer. Plates were incubated at 37°C with 5% CO_2 for 7 days, after which colony formation was observed under a microscope.

In vivo tumorigenicity assay. Mice were divided randomly into four groups of six. Vehicle control (EMEM), hUCB-MSCs, MRC-5 cells, and HeLa cells (1×10^7 cells/head) were inoculated subcutaneously once into the lateral trunk region. After inoculation, mice were observed for 13 weeks twice daily for clinical signs and twice weekly for the presence of tumors. Tumor dimension was assessed using a vernier caliper, and tumor size was estimated using the following equation: $\frac{1}{2} \times$ minor axis$^2 \times$ major axis. Body weight was measured once weekly throughout the study period.

Thirteen weeks after inoculation, animals were fasted overnight and necropsied under deep isoflurane inhalation anesthesia. Blood samples were collected from the posterior vena cava of animals for hematologic analysis. Complete gross examinations were conducted on all animals. Organs including the liver, brain, spleen, heart, kidney, adrenal gland, and lung were collected and weighed. In addition, relative organ weights were calculated as the ratio of organ weight-to-fasted body weight before necropsy.

Routine hematological examinations were conducted using blood with EDTA-2K, an anticoagulant, on a hematological auto-analyzer (ADVIA120 Hematology System, Bayer, USA) to assess: white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), differential leukocyte count (neutrophil (NEU), lymphocyte (LYM), monocyte (MON), eosinophil (EOS), basophil and large unstained cell (BAS) and reticulocyte count (RET).

The organs collected for histopathology including the kidney, liver, spleen, heart, brain, lung, adrenal gland, mandibular lymph node, stomach, mesenteric lymph node, pancreas, thoracic cavity, skin, and injection site were fixed in 10% neutral-buffered formalin. Fixed organs from all animals were embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and examined under a microscope.

Human gene expression at the injection site. To determine whether inoculated hUCB-MSCs persisted at the injection site and whether tumor formation in HeLa cell-treated mice originated from inoculated cells, immunohistochemistry (IHC) was performed on injection site tissues or observed tumor tissues of animals from each group using an automated staining system (DISCOVERY XT, Ventana, Tucson, USA). Heat-induced epitope retrieval was applied using citrate-based buffer (pH 6.0). Sections were incubated with an anti-human mitochondria mouse monoclonal antibody (1 : 1000, ab92824, Abcam, Cambridge, UK) for 2 hr. For detection, diaminobenzidine chromogen was used and counterstained with hematoxylin.

Statistical analysis. The data were examined for variance homogeneity using Bartlett's test. When Bartlett's test indicated no significant deviations from variance homogeneity, a one-way analysis of variance (ANOVA) was performed at $\alpha = 0.05$. When significance was noted, Dunnett's test for multiple comparisons was conducted to determine which pairs were significantly different. When pairs of group homogeneity were observed, the non-parametric Kruskal-Wallis comparison test was conducted. When a significant difference was observed in the Kruskal-Wallis test, Dunn's Rank Sum test was conducted to determine the specific pairs of group comparison. Statistical analyses were performed using the PATH/TOX System (Version 4.2.2,
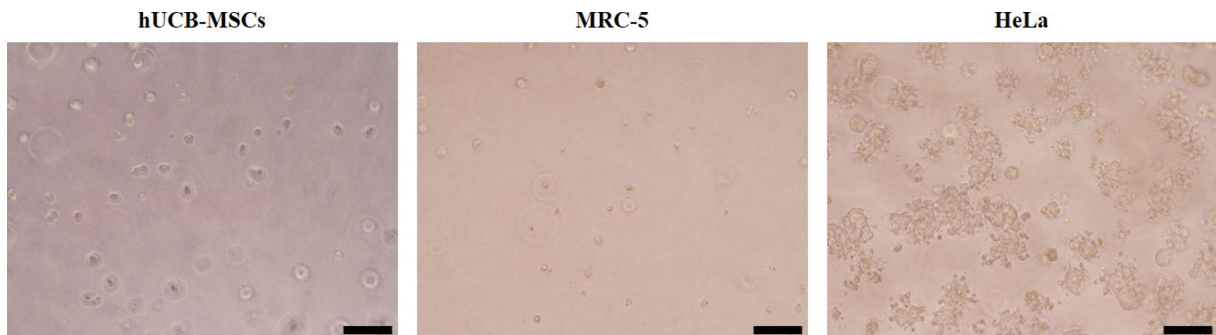
Fig. 1. Representative images of colonies in the soft agar assay for hUCB-MSCs, MRC-5 cells, and HeLa cells. HeLa cells, but not MRC-5 cells or hUCB-MSCs, showed anchorage-independent cell growth in soft agar. Bar = 40 μm.
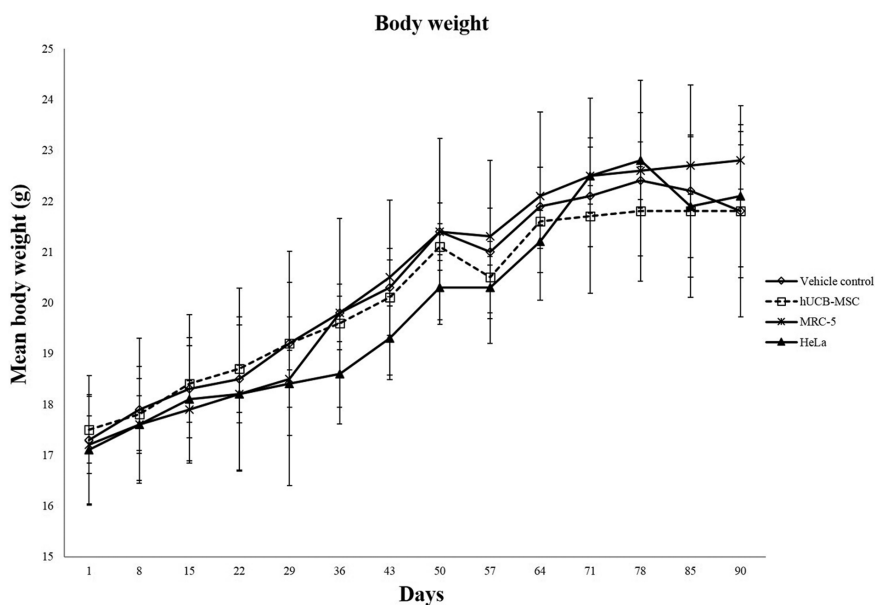

Fig. 2. Body weight changes following subcutaneous transplantation of hUCB-MSCs, MRC-5 cells, and HeLa cells. No significant body weight changes were observed in BALB/c Slc-nu/nu mice after transplantation of hUCB-MSCs, MRC-5, or HeLa cells. Values are expressed as the mean ± SD and significant difference compared to the control ($p < 0.05$).

Xybion Co., Cedar Knolls, USA). Significance was set as $p < 0.05$.

RESULTS

In vitro tumorigenicity assay. To compare colony formation of hUCB-MSCs with HeLa and MRC-5 cells, we performed the soft agar colony assay using 1×10^4 cells/well in a 96-well plate. hUCB-MSCs showed a notably lower ability to form colonies than HeLa cells (positive control). The vehicle control and MRC-5 groups did not form colonies (Fig. 1).

In vivo tumorigenicity assay. No significant body weight changes were observed in any of the groups during the experimental period (Fig. 2). Upon clinical observation,
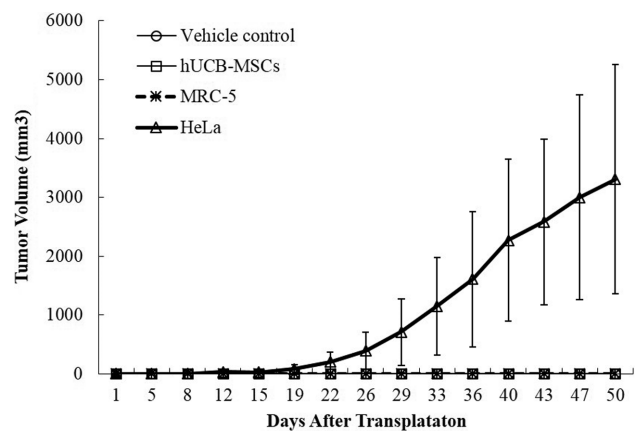

Fig. 3. Tumor volume changes in mice transplanted with vehicle control, hUCB-MSCs, MRC-5 cells, and HeLa cells. Tumor volume was measured twice weekly.

Table 1. Hematology after transplanting hUCB-MSCs, MRC-5 cells, and HeLa cells in BALB/c-nu mice

Parameter	Group			
	Control	hUCB-MSC	MRC-5	HeLa
WBC ($\times 10^9$/L)	3.45 ± 2.443	4.56 ± 1.722	3.36 ± 1.260	61.80 ± 166.819
RBC ($\times 10^{12}$/L)	10.62 ± 0.584	9.90 ± 0.855*	9.37 ± 2.030	9.91 ± 1.237
HGB (g/dL)	1.59 ± 0.98	14.0 ± 1.46**	14.1 ± 3.10	13.7 ± 1.78**
HCT (%)	51.5 ± 2.72	49.0 ± 4.55	46.5 ± 10.10	46.0 ± 5.33**
MCV (fL)	48.6 ± 1.68	49.5 ± 0.79	49.5 ± 1.08	46.6 ± 3.41
MCH (pg)	14.9 ± 0.46	14.2 ± 0.93	15.1 ± 0.36	13.9 ± 1.27*
MCHC (g/dL)	30.7 ± 0.68	28.6 ± 2.23**	30.4 ± 0.65	29.8 ± 0.71**
PLT ($\times 10^9$/L)	1256 ± 205.0	1214 ± 519.4	1170 ± 208.4	1519 ± 426.1*
RET (%)	512.6 ± 384.77	477.7 ± 205.50	347.4 ± 99.68	561.8 ± 179.57
NEU (%)	1.57 ± 0.835	2.29 ± 0.709	1.77 ± 0.560	56.81 ± 154.338*
LYM (%)	1.73 ± 1.641	2.01 ± 1.016	1.42 ± 0.786	1.26 ± 1.843
MON (%)	0.04 ± 0.030	0.05 ± 0.028	0.04 ± 0.018	0.81 ± 2.413
EOS (%)	0.06 ± 0.035	0.10 ± 0.072	0.09 ± 0.105	2.28 ± 7.024
BAS (%)	0.01 ± 0.008	0.02 ± 0.015	0.01 ± 0.013	1.19 ± 3.664

Values are expressed as the mean $\pm$ SD.
*, Significant difference compared to the control ($p < 0.05$).
**, Significant difference compared to the control ($p < 0.01$).

Table 2. Macroscopic findings with abnormal lesions after transplanting hUCB-MSCs, MRC-5 cells, and HeLa cells in BALB/c-nu mice

Parameter	Group			
	Control	hUCB-MSC	MRC-5	HeLa
No. of animals	6	6	6	6
Mass				
Mass in injection site	0	0	0	6
Mass in thoracic cavity	0	0	0	1
Abnormal lesion				
Splenomegaly	0	0	0	3

mice treated with hUCB-MSCs or MRC-5 cells had no clinical signs, but HeLa cell-treated mice exhibited swollen skin (6/6), a palpable mass (6/6), and hard skin (1/6) with an increased tumor volume during the experimental period (Fig. 3). Upon hematologic evaluation, there were no meaningful differences between control and hUCB-MSC-treated

Table 3. Microscopic findings after transplanting hUCB-MSCs, MRC-5 cells, and HeLa cells in BALB/c-nu mice

Parameter	Group			
	Control	hUCB-MSC	MRC-5	HeLa
No. of animals	6	6	6	6
Kidneys				
Inflammatory cell infiltration	0	0	3	0
Liver				
Inflammatory cell infiltration	1	0	1	0
Extramedullary hemopoiesis	0	0	0	1
Spleen				
Extramedullary hemopoiesis	0	0	0	5
Heart				
Cardiomyopathy	1	1	0	0
Inflammatory cell infiltration	0	0	1	0
Lung				
Alveolitis	0	1	0	0
Hemorrhage	0	0	0	2

Table 3. Continued

| Parameter	Group			
	Control	hUCB-MSC	MRC-5	HeLa
Adrenal glands				
Subcapsular cell hyperplasia	5	4	4	4
Corticomedullary vacuolation	5	4	4	2
Mandibular lymph node				
Extramedullary hemopoiesis	3	3	2	3
Stomach				
Erosion/ulcer	0	1	1	2
Mesenteric lymph node				
Extramedullary hemopoiesis	0	0	0	2
Inflammtory cell infiltration, granulocytes	0	1	0	0
Giant cell infiltration	0	1	0	0
Injection site				
Tumor mass/HeLa cells, subcutaneous	0	0	0	6
Thoracic cavity				
Tumor mass	0	0	0	1
Skin				
Keratin cyst	0	0	0	1
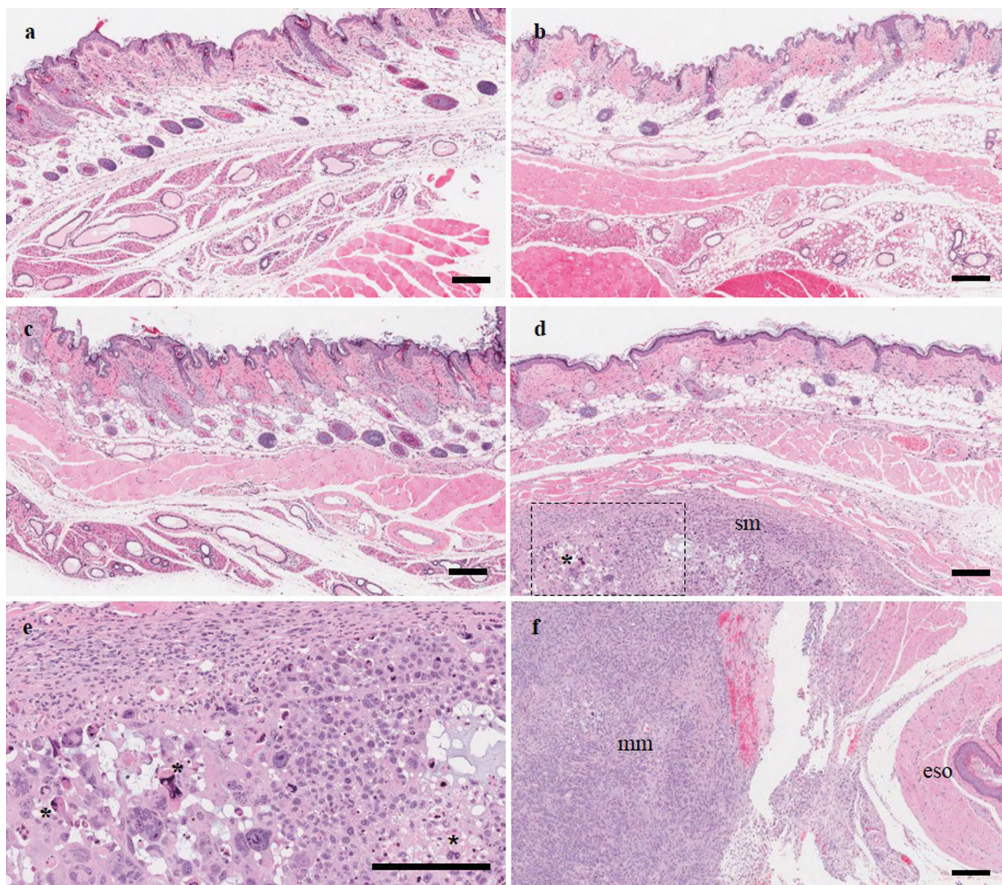
Fig. 4. Histopathology of injection sites and formation of subcutaneous and metastatic cell masses. No cell mass formation was observed in the (a) control, (b) hUCB-MSCs, or (c) MRC-5 cell injection sites. However, the (d) HeLa cell injection site showed a subcutaneous mass (sm). Mitotic figures (asterisks) were observed in the dotted square. These were confirmed in the magnified picture (e), which also showed rounded or pleomorphic cells with hyperchromatic nuclei. (f) A metastatic cell mass (mm) in the thoracic cavity had the same cellular components as the subcutaneous mass. Esophagus (eso). Bar = 200 μm.

groups (Table 1). There were no treatment-related findings according to macroscopic examination in the vehicle control, MRC-5-, or hUCB-MSC-treated mice (Table 2). Microscopic examination revealed no tumors in the vehicle control, MRC-5, or hUCB-MSC-treated groups. However, all mice inoculated with HeLa cells developed tumors at the injection site, with one case presenting metastasis to the thoracic cavity (Table 2, Table 3). The tumor lesions presented mitotic figures, necrosis, and rounded or pleomorphic cells with hyperchromatic nuclei (Fig. 4). In addition, extramedullary hemopoiesis with splenomegaly was observed in HeLa cell-treated mice (Table 3).

Human gene detection at the injection site. Tumor formation of inoculated human cells can be detected by immunostaining the injection site tissue sections of animals with a human-specific antibody (12). No human mitochondrial antigen expression was observed at the injection site in the vehicle control, hUCB-MSCs, or MRC-5 cell-treated groups, while the HeLa cell-treated group showed high human mitochondrial antigen expression in tumors at the injection site (Fig. 5).

DISCUSSION

Mesenchymal stem cells are a promising tool for cell therapies because they are easily sourced from various tissues and have properties of tissue revascularization and repair. Previous non-clinical and clinical studies have reported on the therapeutic value of MSCs (13-16). However, some studies have reported adverse effects of MSCs, including tumor growth and mutation into tumor cells (17-20). Moreover, one patient with the neurodegenerative hereditary disorder ataxia telangiectasia treated with human fetal neural stem cells by intracerebellar and intrathecal injection developed a multifocal brain tumor of non-host origin. This suggested that the tumor was derived from the transplanted neural stem cells (21). These reports indicate the importance of
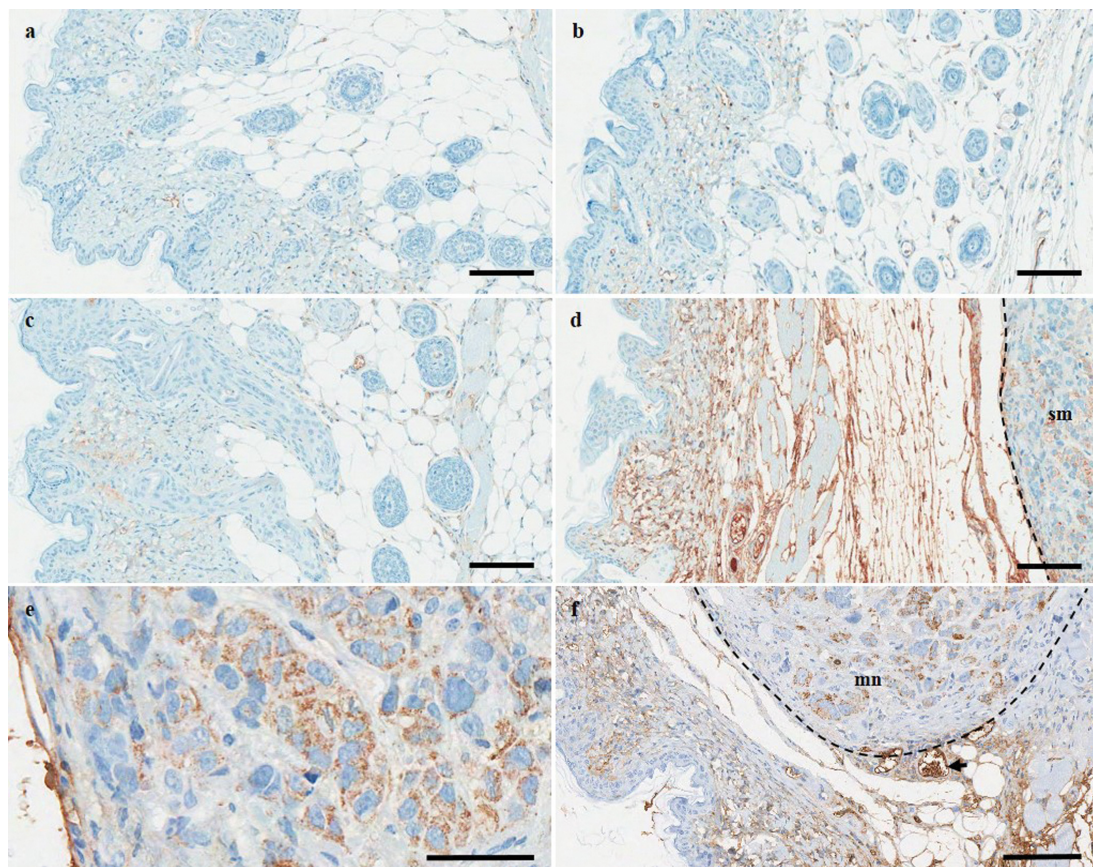

Fig. 5. Immunohistochemical analysis of a human mitochondrial marker at the injection site. Immunohistochemical analysis of a human mitochondrial antigen revealed no expression in the (a) control, (b) hUCB-MSCs, or (c) MRC-5 cell groups. The HeLa cell group showed a tumor mass (sm, right side of the dotted line) in the (d) subcutaneous and metastatic tumor nodules (mn, upper side of the dotted line) in the (f) dermis. (e) The human mitochondrial antigen was expressed well in the cytoplasm of tumor cells. HeLa cell-treated mice showed strong background staining because the tumor mass exhibits dramatic (d) angiogenesis and hemopoiesis. Bar = 50 μm.

investigating the tumorigenicity of MSCs for their use in clinical applications.

In this study, we evaluated the tumorigenicity of hUCB-MSCs *in vitro* with the soft agar colony formation assay, which is used to measure cell anchorage-independent proliferation potential. Anchorage-independent growth is a hallmark of transformation and the most accurate *in vitro* indication of tumorigenicity (22). In this study, hUCB-MSCs formed few colonies, while HeLa cells formed a greater number of larger colonies, indicating that hUCB-MSCs do not possess anchorage-independent proliferation potential. The less tumorigenic effect of hUCB-MSCs *in vitro* than HeLa cells corresponds with recent results of other MSCs (23,24).

With increasing passages of stem cell lines, there is increased potential for chromosomal aberrations, a hallmark of tumor formation (9). The hUCB-MSCs in this study were used at passage 6 and had no evidence of tumorigenicity, suggesting that hUCB-MSCs may be chromosomally stable at least up to passage 6 under our culture conditions, as they were not prone to malignant transformation. In agreement with our results, Ra *et al*. (25) reported that chromosomal abnormalities were not observed in human adipose tissue-derived MSCs (hAdMSCs) from passages 4~12.

In our 13-week tumorigenicity study in which hUCB-MSCs were transplanted into female BALB/c-nu/nu mice, there were no tumor formation-related findings in body weight, clinical signs, gross findings, organ weight, hematology, or macro- and microscopic examination. However, all HeLa cell-treated mice developed tumors. These findings correlate with the clinical observations and macro- and microscopic examinations. Our results obtained from *in vivo* and *in vitro* tumorigenicity studies consistently demonstrated that hUCB-MSCs did not induce tumor formation. These data correspond with a previous study, which reported no tumorigenicity of hAdMSCs in immunocompromised animals (25).

According to immunohistochemical analysis, no human mitochondrial antigen expression was observed in the vehicle control and hUCB-MSC-treated groups, indicating a lack of human cell persistence. However, the tumors observed in the HeLa cell-treated mice likely originated from the inoculated HeLa cells based on the detection of human mitochondrial antigen expression.

In this study, tumorigenicity was studied after 13 weeks. However, when allogeneic bone marrow-derived MSCs were injected intravenously into baboons, high amounts of MSC DNA were detected in the kidney, lung, thymus, liver, gastrointestinal, and skin tissues until 19 months post-treatment. Moreover, hAdMSCs survived in mice with severe combined immunodeficiency for 17 months after subcutaneous injection and differentiated into fibroblasts of the subdermic connective tissue and mature adipocytes of fat tissue at the injection site (26). These reports suggest that

the duration of this study might have been insufficient. Therefore, a long-term study is required to validate these findings.

In conclusion, our data show that hUCB-MSCs have no *in vitro* or *in vivo* tumorigenic potential under our study conditions, providing further evidence of the safety of MSCs for clinical applications. However, further studies are required to address the long-term tumorigenicity of human MSCs in animals.

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