Clearance of senescent cells with ABT-263 improves biological functions of synovial mesenchymal stem cells from osteoarthritis patients

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Clearance of senescent cells with ABT-263 improves biological functions of synovial mesenchymal stem cells from osteoarthritis patients

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Abstract

Background: Osteoarthritis (OA) is an age-related joint disease characterized by progressive cartilage loss. Synovial mesenchymal stem cells (MSCs) are anticipated as a cell source for OA treatment; however, synovial MSC preparations isolated from OA patients contain many senescent cells that inhibit cartilage regeneration through their senescence-associated secretory phenotype (SASP) and poor chondrogenic capacity. The aim of this study was to improve the biological function of OA synovial MSCs by removing senescent cells using the senolytic drug ABT-263.

Methods: We pretreated synovial MSCs derived from 5 OA patients with ABT-263 for 24 h and then evaluated senescence-associated beta galactosidase (SA-β gal) activity, apoptosis, surface antigen expression, colony formation ability, and multipotency.

Results: The ABT-263 pretreatment significantly decreased the percentage of SA-β gal–positive cells and induced early- and late-stage apoptosis. Cleaved caspase-3 was expressed in SA-β gal–positive cells. The pretreated MSCs formed greater numbers of colonies with larger diameters. The expression rate of CD34 was decreased in the pretreated cells. Differentiation assays revealed

that ABT-263 pretreatment enhanced the adipogenic and chondrogenic capabilities of OA synovial MSCs. In chondrogenesis, the pretreated cells produced greater amounts of glycosaminoglycan and showed lower expression of senescence markers (p16 and p21) and SASP factors (MMP-13 and IL-6).

Conclusion: Pretreatment of synovial MSCs from OA patients with ABT-263 can improve the function of the cells by selectively eliminating senescent cells. These findings indicate that ABT-263 could hold promise for the development of effective cell-based OA therapy.

Keywords

Mesenchymal stem cells, senescence, senolytic drug, osteoarthritis, ABT-263, synovium.

Background

Knee osteoarthritis (OA) is the most common joint disease worldwide. It is characterized by a progressive loss of articular cartilage and meniscus degeneration associated with aging and synovitis [1] and has recently been designated an age-related inflammatory disease [2]. OA patients suffer from a

variety of symptoms (e.g., pain, stiffness, swelling, and loss of mobility), so the recommended therapies can range from pain control to surgery [3]. However, none of the available treatments can improve cartilage loss sufficiently to affect patients fundamentally. New treatments are therefore needed to overcome this limited effectiveness, because the number of OA patients is anticipated to increase in today's aging society.

One promising OA treatment that has emerged in the last two decades is the use of mesenchymal stem cells (MSCs). These cells are considered a viable therapeutic alternative for OA due to their pleiotropic abilities, such as self-renewal, multipotency, and secretion of cytokines and growth factors [4, 5]. MSCs can be obtained from various tissues, but synovial MSCs have a higher colony formation ability and chondrogenic potential than MSCs from other tissues, and are therefore promising for treating damaged cartilage [6-9]. We previously demonstrated that synovial MSCs transplanted into rabbit cartilage or meniscus defects could promote regeneration by producing abundant cartilage matrices [10, 11]. Similarly, intra-articular injection of synovial MSCs suppressed the progression of OA in rats [12]. Our recent clinical study in human OA patients showed that transplantation of autologous synovial MSCs improved the clinical

outcomes and the amounts of cartilage that could be assessed by automatic magnetic resonance imaging (MRI) [13].

One issue with the use of MSCs as an OA treatment is that the MSC preparations typically contain senescent cells. Senescence in cells, including MSCs, is triggered by some type of stress that causes the activation of the p16 or p21 pathways. This puts the cells into permanent cell-cycle arrest, resulting in resistance to apoptosis [14]. The senescent cells remain metabolically active and release factors referred to as senescence-associated secretory phenotype (SASP) factors, which include proinflammatory cytokines (e.g., interleukin-6; IL-6) and proteinases (e.g., matrix metalloprotease-13; MMP-13) [14]. SASP plays a crucial role in accelerating the senescence of other neighboring cells and deteriorating their function[14].

In OA knees, chondrocyte senescence can be induced by a number of different stresses [1], and recent reports indicate that senescent cells also accumulate in the OA synovium and in the MSCs isolated from it [15, 16]. This means that senescent cells are also present in cultures of synovial MSCs that are used as OA therapeutics. The cultures containing senescent MSCs lack full OA therapeutic efficacy due to their release of SASP factors and their poor

chondrogenic capacity [16]. Therefore, clearance of senescent cells from MSC cultures intended for therapeutic use is necessary to maximize the efficacy of OA synovial MSCs.

Senescent cells can be selectively removed from cultures by drugs known as senolytics. Several senolytics have been introduced in several previous reports; however, ABT-263 (Navitoclax) is one of the best studied of these senolytic drugs. ABT-263 is an inhibitor of the anti-apoptotic proteins of the BCL-2 protein family, and its suppression of BCL-2 activity can induce senescent cells in various tissues to undergo apoptosis [17]. Grezella et al. demonstrated that ABT-263 exerted a senolytic effect in replicative senescent human bone marrow derived MSCs [18]. However, no studies have yet focused on the use of ABT-263 for the qualitative alteration of MSCs, and little is known about the effects of this drug on senescent OA synovial MSCs.

Our aim in the present study was to use ABT-263 to selectively eliminate senescent cells from synovial MSC samples derived from patients with OA as a way to improve the quality of MSCs used for OA therapy. We pretreated OA synovial MSCs with ABT-263 for a short period and then evaluated the extent of senescent cell clearance and the in vitro biological potencies of the treated MSC

samples.

Methods

Isolation of human synovial MSCs

Human synovial samples were acquired from 5 OA donors (age range 71–80 years; one male and four females) who underwent total knee arthroplasty. The synovium was digested with 3 mg/mL collagenase (Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C for 3 h. Debris was then removed by passing the digest through a 70 μm cell strainer (Greiner Bio-One, Kremsmünster, Austria) and the filtrate was collected. The cells were washed with phosphate buffered saline (PBS), and the nucleated cells were counted and plated in 145 cm^2 dishes at a density of 2,000 cells/cm^2 in a growth medium consisting of α-modified essential medium (α-MEM; Thermo Fisher Scientific, Waltham, MA, USA) and 10% fetal bovine serum (Thermo Fisher Scientific), and 1% antibiotic-antimycotic (Thermo Fisher Scientific). After 2 weeks of culture, the cells were detached with 0.25% trypsin and 1 mM EDTA (Thermo Fisher Scientific) and then cryopreserved in growth medium supplemented with 5% dimethyl sulfoxide (DMSO; Wako, Tokyo, Japan).

Pretreatment with ABT-263

ABT-263 was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Human synovial MSCs at passage 1 were used for all experiments. The cells were treated for 24 h with 0.1% DMSO (control group) or with 20 μM ABT-263 in DMSO (ABT-263 group). After the treatment, the culture medium was changed to the growth medium, and the cells were further expanded for 6 days. Apoptosis assays were performed immediately after the treatment. An experimental scheme is shown in Fig. 1.

Senescence associated-β galactosidase (SA-β-gal) staining

SA-β-gal staining was performed using a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's instructions. Briefly, the cells were fixed with fixative solution and then incubated at 37 °C in staining solution at pH 6.0 for 16 h. Senescent cells were identified as blue-stained cells under brightfield microscopy. Cells positive for SA-β-gal staining were counted manually in four fields at ×10 magnification.

Apoptosis assay

Human synovial MSCs were treated with 0.1% DMSO or 20 μM ABT-263 for a day. After the treatment, the cells were detached with trypsin, suspended in PBS, and the cell suspension was incubated with FITC-annexin V and propidium iodide (PI) using an FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, NJ, USA). The fluorescence intensity was evaluated using a FACSVerse II system (BD Biosciences). The obtained data were analyzed using FlowJo version 8.7.1 (Ashland, OR, USA).

Co-staining of SA-β gal and cleaved caspase-3

Human synovial MSCs were cultured in 8-well chamber slides (Thermo Fisher Scientific). The cells were treated for 6 h with ABT-263 and then stained for SA-β gal. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized, and blocked with 0.3% Triton and 5% normal goat serum. The cells were then incubated overnight at 4 °C with antibody against cleaved caspase-3 (1:400; Cell Signaling Technology). After three washes with PBS, the cells were incubated with anti-rabbit secondary antibodies with horseradish peroxidase (Abcam, Cambridge, UK) for 1 h at room temperature.

159 Diaminobenzidine (DAB) solution (Dako North America, Carpinteria, CA, USA)
160 was applied for 10 min.
161
162 **Colony-forming assays**
163 Cells pretreated with ABT-263 were seeded at 100 cells/dish in 145 cm^2
164 dishes (Thermo Fisher Scientific) and cultured for 2 weeks. The cells were then
165 fixed with 10% neutral buffered formalin (Muto Pure Chemicals, Tokyo, Japan),
166 followed by staining with 0.5% crystal violet (Wako). Colonies were counted
167 manually, and the diameter was assessed by Image J (National Institute of Health,
168 Bethesda, MD, USA). Colonies less than 2 mm in diameter and faintly stained
169 colonies were ignored.
170
171 **Surface antigens**
172 Human synovial MSCs were detached with trypsin and suspended in
173 PBS containing 2% FBS and 5 mM EDTA. The MSCs were then stained for 30
174 min at 4 °C with CD44-APCH7, CD73-V450, CD90-PE-Cy7, CD105-APC, CD34-
175 PE, and CD45-PerCP-Cy5.5 antibodies (BD Biosciences), using Ghost Dye
176 Violet (Tonbo Biosciences, San Diego, CA, USA) to remove dead cells. Isotype

177 controls were prepared as negative controls. The percentage of antigen-positive
178 cells was evaluated using a FACSVerse II system (BD Biosciences).
179
180 **Differentiation assays**
181 For adipogenesis, 100 pretreated human synovial MSCs were plated in
182 10 cm dishes (Thermo Fisher Scientific) and cultured for 14 days to form colonies.
183 The adherent cells were then cultured for an additional 21 days in an adipogenic
184 induction medium consisting of growth medium supplemented with 100 nM
185 dexamethasone (Wako), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 50 mM
186 indomethacin (Sigma-Aldrich), 4.5 mg/mL D-(+)-glucose (Wako), and 10 μg/mL
187 recombinant human insulin (Wako). The cells were then fixed with 10% neutral
188 buffered formalin and stained with oil red O (Sigma-Aldrich), and the oil red O-
189 positive colonies were counted manually. Each dish was then stained with 0.5%
190 crystal violet to determine the percentage of oil red O-positive colonies.
191 Osteogenesis assays were performed by plating 100 pretreated MSCs in
192 10 cm dishes and culturing for 14 days to form colonies. Adherent cells were then
193 cultured in an osteogenic induction medium consisting of growth medium
194 supplemented with 50 μg/mL ascorbic acid 2-phosphate (Wako), 1 nM

dexamethasone, and 10 mM β-glycerophosphate (Sigma-Aldrich). After 21 days, the cells were fixed with 10% neutral buffered formalin and stained with alizarin red (Sigma-Aldrich). The alizarin red-positive colonies were counted manually. Each dish was then stained with 0.5% crystal violet to determine the percentage of alizarin red-positive colonies.

For chondrogenesis, 2.5×10^5 pretreated MSCs were transferred to a 15 mL polypropylene tube (Thermo Fisher Scientific) and centrifuged at 580 × g for 10 min. The pelleted cells were then cultured in chondrogenic induction medium consisting of high glucose Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific), 1 % insulin-transferrin-selenium (ITS; BD Biosciences), 50 μg/mL ascorbate-2-phosphate, 40 μg/mL L-proline (Sigma Aldrich), 100 nM dexamethasone, 100 μg/mL pyruvate (Sigma Aldrich), 1 % antibiotic-antimycotic, 10 ng/mL transforming growth factor-β3 (Miltenyi Biotec, Bergisch Gladbach, Germany), and 500 ng/mL bone morphogenetic protein-2 (Medtronic, Minneapolis, MN, USA). After 3 weeks of cultivation in a chondrogenic induction medium, 5-6 pellets in each group were weighed. Two pellets each were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 μm thick sections. The sections were stained with safranin O (Chroma Gesellschaft

Schmid & Co., Munster, Germany) and fast green (Wako) to evaluate the production of Glycosaminoglycan (GAG). Three or four pellets each were used for biochemical analysis.

Biochemical analysis

GAG and deoxyribonucleic acid (DNA) were quantified by digesting the pellets with 100 μg/mL papain (Sigma-Aldrich) at 65°C for 16 h. DNA content was measured using Hoechst 33258 dye (Dojindo, Tokyo, Japan). Fluorescence intensity was measured with a microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. Calf thymus DNA (Sigma-Aldrich) was used to generate a standard curve. The GAG content was then quantified with a Blyscan Kit (Biocolor, Westbury, NY, USA) according to the manufacturer's instructions. The optical density was measured at 656 nm with a microplate reader, and the total GAG content was calculated. For comparison of the GAG-producing ability, the GAG content was normalized to the DNA content (GAG/DNA). Each experiment was performed in duplicate.

Immunohistochemistry

Paraffin-embedded sections from cartilage pellets were immersed in 10 mM Tris containing 1 mM EDTA (pH 9.0) and heated at 95 °C for 1 h to retrieve antigens. The slides were immersed in methanol containing 0.3% H_2O_2 for 30 min and then washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T buffer). After blocking with 5% normal goat serum, the sections were incubated overnight at 4 °C with antibody against p16, p21, MMP-13, and IL-6 (1:200; all from Abcam). After three washes with TBS-T, the sections were incubated with secondary antibodies conjugated with horseradish peroxidase (Abcam) for 1 h at room temperature. DAB solution (Dako North America) was then applied for 5 min, and the cells were counterstained with hematoxylin. DAB positive areas in pellets were quantified using Image J.

Statistical analysis

Statistical analysis was performed using SPSS (IBM Corp., Chicago, IL, USA). Paired-t tests were used for comparison between two groups. One-way analysis of variance, followed by Tukey's multiple-comparisons test, was used for comparison between multiple groups. All p values were one-sided, and a p-value

249 < 0.05 was considered statistically significant.

250

251 **Results**

252 **SA-β-gal staining**

253 An overview of our experimental design is shown in Fig. 1. We pretreated
254 OA synovial MSCs with 0.1% DMSO or 20 μM ABT-263 for 1 day and then
255 performed SA-β-gal staining to evaluate the proportions of senescent cells. The
256 optimal concentration of ABT-263 had been determined in a preliminary study
257 using cells from one donor (Supplementary Fig. 1). Phase contrast and brightfield
258 images showed the presence of flattened and enlarged cells in the untreated
259 control group, and those cells showed positive staining for SA-β-gal staining (Fig.
260 2a). By contrast, the culture treated with ABT-263 showed few flattened and
261 enlarged cells, and the percentage of SA-β-gal-positive cells was significantly
262 lower than in the control group (Fig. 2b, $p = 3.8 \times 10^{-5}$).

263

264 **Apoptosis assay**

265 Early- and late-stage apoptosis was assessed by annexin V/propidium
266 iodide (PI) staining in control OA synovial MSCs and in OA synovial MSCs treated

267 with ABT-263 (Fig. 3a). The MSCs treated with ABT-263 underwent significantly
268 greater levels of early-stage apoptosis ($p = 8.0 \times 10^{-4}$), late-stage apoptosis ($p = 3.2 \times 10^{-5}$), and total apoptosis ($p = 2.5 \times 10^{-4}$) (Fig. 3b). Co-staining for SA-β-gal
270 and cleaved caspase-3, a molecule with a known pivotal role in the apoptotic
271 cascade [19], revealed no staining for caspase-3 in the SA-β-gal–positive cells of
272 the control group, whereas the cells in the ABT-263 group were positive for
273 cleaved caspase-3 (Fig. 3c).
274
275 **Colony-forming ability**
276 Colony-forming ability was assessed by culturing 100 pretreated cells for
277 14 days. Representative images of the colonies stained with crystal violet are
278 shown in Fig. 4a. The colony numbers were significantly greater in the ABT-263
279 group than in the control group (Fig. 4b, $p = 1.5 \times 10^{-3}$). The colony diameters
280 were also significantly larger in the ABT-263 group than in the control group (Fig.
281 4c, $p = 9.7 \times 10^{-4}$). Histogram distributions of the colony diameters showed a shift
282 toward larger colonies in response to ABT-263 pretreatment (Supplementary Fig.
283 2). Most colony-forming cells were small and spindle-shaped, both in the control
284 and the ABT-263 groups (Fig. 4d). However, the few flattened and enlarged cells

that did not form colonies were observed predominantly in the control group (Fig. 4e).

Cell surface antigen phenotype

Flow cytometry analyses demonstrated an almost 100% mean rate of positivity for MSC markers such as CD44, CD73, CD90, and CD105 in both the control and ABT-263 groups (Fig. 5a), but the difference in the CD90 positive rate between the control and ABT-263 groups was statistically significant ($p = 9.0 \times 10^{-3}$) (Fig. 5b). The mean rates of positivity for the hematopoietic markers CD34 and CD45 were 11.9% in the control group and 3.3% in the ABT-263 group for CD34 and 0.2% in the control group and 0.1% in the ABT-263 group for CD45. The positive rate for CD34 was significantly lower in the ABT-263 group than in the control group ($p = 0.016$).

Adipogenic and osteogenic potential

The adipogenic and osteogenic potential of single-cell derived colonies was investigated by culturing 100 pretreated cells in growth medium for 14 days to allow colony formation, followed by culture in induction medium for 21 days.

303 Macroscopic images of oil red O and subsequent crystal violet staining are shown
304 in Fig. 6a. Observation by light microscopy confirmed that pretreatment with ABT-
305 263 enhanced oil red O staining, indicating increased lipid production by the cells
306 (Fig. 6b). Quantitative analysis showed that the percentage of oil red O-stained
307 colonies was significantly higher in the ABT-263 group than in the control group
308 (Fig. 6c, $p = 6.4 \times 10^{-4}$).

309 Macroscopic images of the cells stained with alizarin red and subsequent
310 crystal violet staining are shown in Fig. 6d. The cells in both the control and the
311 ABT-63 groups underwent calcification (Fig. 6e). No significant difference was
312 detected in the proportion of alizarin red-stained colonies between the control and
313 ABT-263 groups (Fig. 6f, $p = 0.070$).

314

315 **Chondrogenic potential**

316 Pellets of pretreated cells were cultured for 21 days in chondrogenic
317 induction medium. The cells formed larger pellets after chondrogenic induction
318 when pretreated with ABT-263 compared to the control cells (Fig. 7a). The mean
319 pellet wet weight from the control and ABT-263 groups were 1.9 mg and 4.8 mg,
320 respectively, confirming a greater pellet mass in the ABT-263 group than in the

control group ($p = 0.052$) (Fig. 7b).

Biochemical analyses revealed significantly increased GAG amounts ($p = 9.4 \times 10^{-3}$) and a higher GAG/DNA ratio ($p = 0.013$) in the ABT-263 group than in the control group; however, no significant difference was found in the DNA amounts between the two groups (Fig. 7b). Safranin o staining was more intense in the pellets from the ABT-263 group than from the control group (Fig. 7c).

Immunohistochemistry of chondrogenic pellets

The chondrogenic pellets were immunostained with senescence markers (p16 and p21) and SASP markers (MMP-13 and IL-6). Representative images and the ratios of the stained areas are shown in Fig. 8. The percentages of p16, p21, MMP-13, and IL-6 positive areas group were significantly lower in the ABT-263 group than in the control group ($p = 2.1 \times 10^{-3}$, 9.8×10^{-3}, 5.0×10^{-4}, and 2.5×10^{-3}, respectively).

Discussion

In the present study, we demonstrated that senescent cells showing positive staining for SA-β-gal in human synovial MSC samples obtained from OA

patients were effectively removed by pretreatment with 20μM ABT-263. Our apoptosis assay revealed that ABT-263 treatment induced both early- and late-stage apoptosis in the senescent OA synovial MSCs. In addition, the SA-β gal–positive cells expressed cleaved caspase-3 after the treatment. These results indicate that ABT-263 could selectively kill senescent cells present in OA synovial MSC samples by inducing apoptosis. Previous studies have shown that senescent cells in human chondrocyte and bone marrow derived MSC samples showed dose-dependent and statistically significantly decreases in the numbers of SA-β-gal–positive cells when treated with ABT-263 [18, 20]. Similar effects of ABT-263 were also observed in our study. ABT-263 had no adverse effects on the function of non-senescent MSCs in the present study, but a previous report indicated that ABT-263 had a cytotoxic effect on bone marrow MSC-derived osteoblasts both in vitro and in vivo [21].

Our colony formation assays indicated that the number and diameter of colonies were significantly greater in the ABT-263 group than in the untreated control group. The control group also contained a few colonies with flattened and enlarged cells, indicative of senescent cells, which are generally growth-arrested and have enlarged and flattened morphologies [22, 23]. Therefore, our results

suggested that senescent cells that have poor colony-forming abilities were eliminated by the ABT-263 treatment, leaving MSCs with high colony formation potential in the final culture.

Most cells in both groups showed positive immunostaining for CD44, CD90, and CD105, despite the successful clearing of senescent cells by the ABT-263 treatment. Consequently, none of these can be considered a candidate for use as a specific marker for senescent cells. On the contrary, the mean positivity for CD34 (MSC negative marker) was significantly decreased by ABT-263 treatment, from 11.9% in the control group to 3.3% in the ABT-263 group. A previous report showed that CD34-positive synovial fibroblasts from OA and rheumatoid arthritis tissues release high levels of inflammatory cytokines [24]. Similar characteristics are also observed in senescent cells; therefore, investigating the relationship between CD34 expression and cellular senescence in the synovium would be an interesting future study.

In terms of multipotency, we noted a significant improvement in adipogenic potential in cells treated with ABT-263, but the osteogenic potential did not improve. Wagner et al. demonstrated a deterioration in adipogenic potential in human MSCs with increasing passage number, but did not see an

equivalent decline in osteogenesis [25]. However, Bertolo et al. reported opposite findings [26]. Therefore, no consensus exists regarding the adipogenic and osteogenic capacities of senescent MSCs. Regarding chondrogenesis, we observed that the synovial MSCs pretreated with ABT-263 formed cartilaginous pellets rich in GAGs and showed decreased expression of senescence markers and SASP factors. Therefore, these cells are expected to induce better cartilage regeneration than untreated cells by producing more cartilage matrix and releasing fewer of the catabolic factors that deteriorate the functions of the surrounding cells. Our study had one significant limitation, as we did not investigate the in vivo therapeutic effect of ABT-263–treated MSCs. Further research using animal models of cartilage/meniscus injury or OA is needed to verify our results.

MSCs are a heterogeneous population, so purification is sometimes carried out to isolate true stem cells [27]. MSCs are typically purified by fluorescence-activated cell sorting (FACS) [28], but the lack of specific surface markers for senescent cells limits this approach [29]. We propose that the purification of MSCs could be improved by administration of a senolytic agent, such as ABT-263. Care would be needed when using ABT-263 in terms of

cytotoxic effects; however, when compared with FACS, ABT-263 pretreatment is easy, inexpensive, and requires no special equipment. Senescence of various MSC types is also known to be accelerated during aging of individuals and during in vitro serial passaging of MSCs in culture [25, 30]. ABT-263 pretreatment could also be beneficial in these cases.

Conclusions

Pretreatment with ABT-263 selectively eliminated senescent cells in synovial MSCs derived from OA patients. The pretreated MSCs consistently showed higher colony formation, adipogenic, and chondrogenic abilities, and decreased SASP expression. These results indicate that ABT-263 could be a useful senolytic drug to improve the function of synovial MSCs obtained from OA patients and could hold promise for the development of effective cell-based OA therapy.

Abbreviations

α-MEM: α-modified essential medium; DAB: Diaminobenzidine; DMSO: Dimethyl sulfoxide; FACS: Fluorescence-activated cell sorting; GAG: Glycosaminoglycan;

ITS: Insulin-transferrin-selenium; IL-6: Interleukin-6; MRI: Magnetic resonance imaging; MMP-13: Matrix metalloprotease-13; MSCs: Mesenchymal stem cells; OA: Osteoarthritis; PBS: Phosphate buffered saline; PI: Propidium iodide; SASP: Senescence-associated secretory phenotype; SA-β-gal: Senescence associated β-galactosidase

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Research Ethics Committee of Tokyo Medical and Dental University, and written informed consent was obtained from all donors.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YM provided ideas, performed all experiments, and wrote the manuscript. KE designed the study, provided ideas, organized the data, and completed the manuscript. KK provided ideas, contributed to acquisition of data, and revised the manuscript. IS provided ideas and revised the manuscript. All authors read and approved the submitted draft of the paper.

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545

546 **Figure legends**

547 **Fig. 1. Scheme of the experiments.** Synovial mesenchymal stem cells (MSCs)
548 from osteoarthritis (OA) patients were treated with 0.1% dimethyl sulfoxide
549 (DMSO) or 20 μM ABT-263 in DMSO for 24 h. After the treatment, the cells were
550 expanded for another 6 days and used for the experiments. Apoptosis assays
551 were performed immediately after the treatment.

552

553 **Fig. 2 Senescence-associated beta galactosidase (SA-β gal) staining.** (a)
554 Phase contrast and brightfield images. (b) The ratio of SA-β gal–positive cells in

555 control and ABT-263 group.

556

557 **Fig. 3. Apoptosis assay.** (a) Flow cytometry analysis of Annexin V/propidium
558 iodide (PI). Dot plots show the percentages of viable (Q4), early apoptotic (Q3),
559 and late apoptotic (Q2) cells. (b) The ratio of early, late, and total apoptotic cells
560 in the control and ABT-263 groups. (c) Co-staining of senescence-associated
561 beta galactosidase (blue) and cleaved caspase-3 (brown) after the treatment with
562 ABT-263.

563

564 **Fig. 4. Colony-forming ability.** (a) Representative images of colonies stained
565 with crystal violet (4 replicate dishes per donor). (b) Colony numbers per dish in
566 the control and ABT-263 groups. (c) Diameter of colonies in the control and ABT-
567 263 groups. (d) Representative image of colony-forming cells. (e) Representative
568 image of flattened and enlarged cells that did not form colonies.

569

570 **Fig. 5. Surface antigen expression.** (a) Representative flow cytometry
571 histograms. Red solid lines indicate the control group and green solid lines
572 indicate the ABT-263 group. Dotted lines show the isotype control. **(**b) The

573 expression rate of each surface antigen. Values are shown as mean ± SD from 5
574 donors.
575
576 **Fig. 6. Adipogenic and osteogenic differentiation.** (a) Representative images
577 of oil red O and crystal violet (CV) staining (3 replicate dishes per donor). (b) The
578 percentage of oil red O-stained colonies in the control and ABT-263 groups. (c)
579 Representative images of alizarin red and CV staining (3 replicate dishes per
580 donor). (d) The percentage of alizarin red-stained colonies in the control and ABT-
581 263 groups.
582
583 **Fig. 7. Chondrogenic differentiation.** (a) Gross appearance of chondrogenic
584 pellets (5 or 6 replicate pellets per donor). (b) Pellet wet weight, DNA
585 quantification, glycosaminoglycan (GAG) quantification, and GAG/DNA ratio (3 or
586 4 replicate pellets per donor). (c) Representative images of safranin O staining of
587 chondrogenic pellets.
588
589 **Fig. 8. Immunohistochemistry of senescence markers in chondrogenic**
590 **pellets.** Representative images and positive ratios of (a) p16, (b) p21, (c) MMP-

591 13, and (d) IL-6 immunostaining in the control and ABT-263 groups.

Figures

Figure 1

Scheme of the experiments. Synovial mesenchymal stem cells (MSCs) from osteoarthritis (OA) patients were treated with 0.1% dimethyl sulfoxide (DMSO) or 20 μM ABT-263 in DMSO for 24 h. After the treatment, the cells were expanded for another 6 days and used for the experiments. Apoptosis assays were performed immediately after the treatment.

Fig. 2

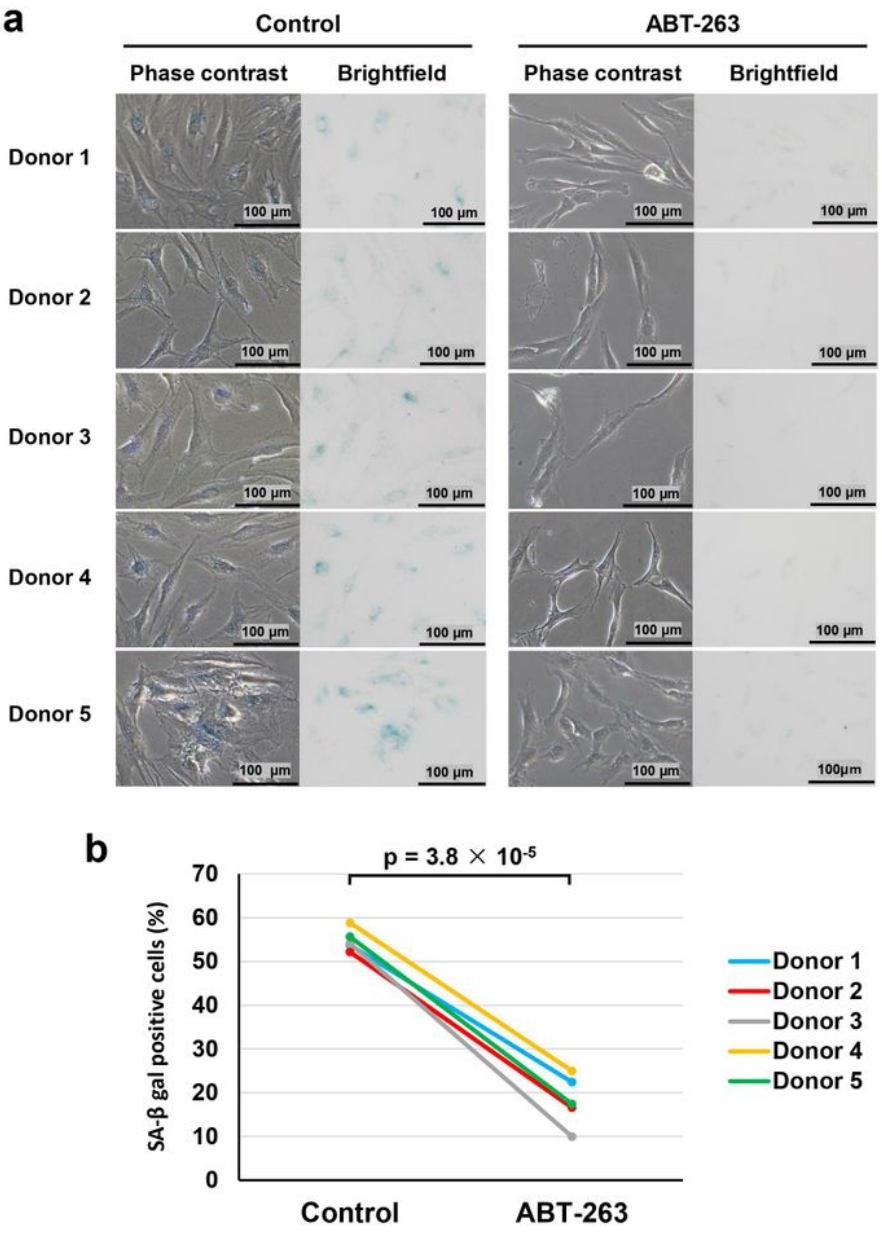

Figure 2

Senescence-associated beta galactosidase (SA-β gal) staining. (a) Phase contrast and brightfield images. (b) The ratio of SA-β gal–positive cells in control and ABT-263 group.

Fig. 3

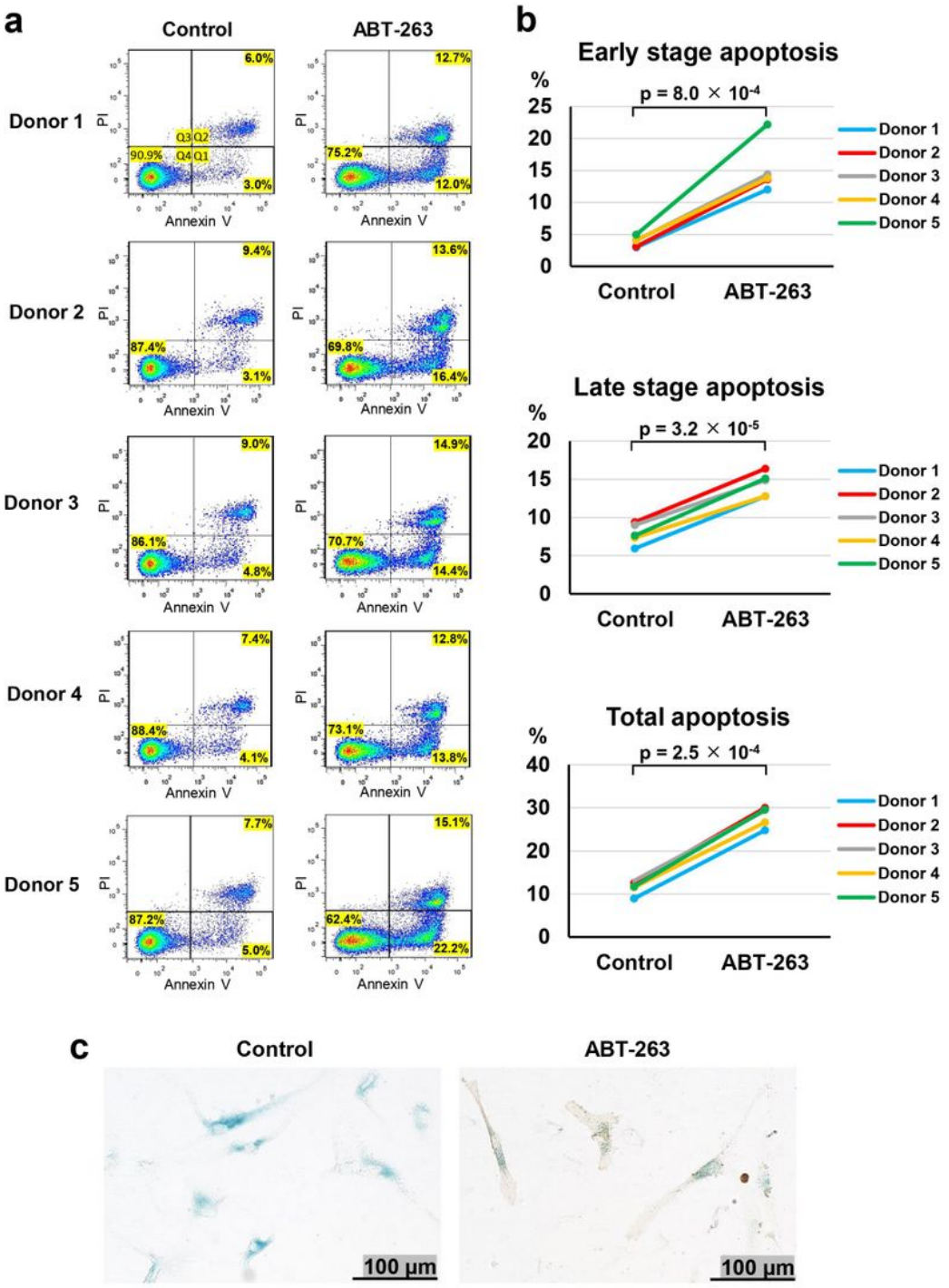

Figure 3

Apoptosis assay. (a) Flow cytometry analysis of Annexin V/propidium iodide (PI). Dot plots show the percentages of viable (Q4), early apoptotic (Q3), and late apoptotic (Q2) cells. (b) The ratio of early, late, and total apoptotic cells in the control and ABT-263 groups. (c) Co-staining of senescence-associated beta galactosidase (blue) and cleaved caspase-3 (brown) after the treatment with ABT-263.

Fig. 4

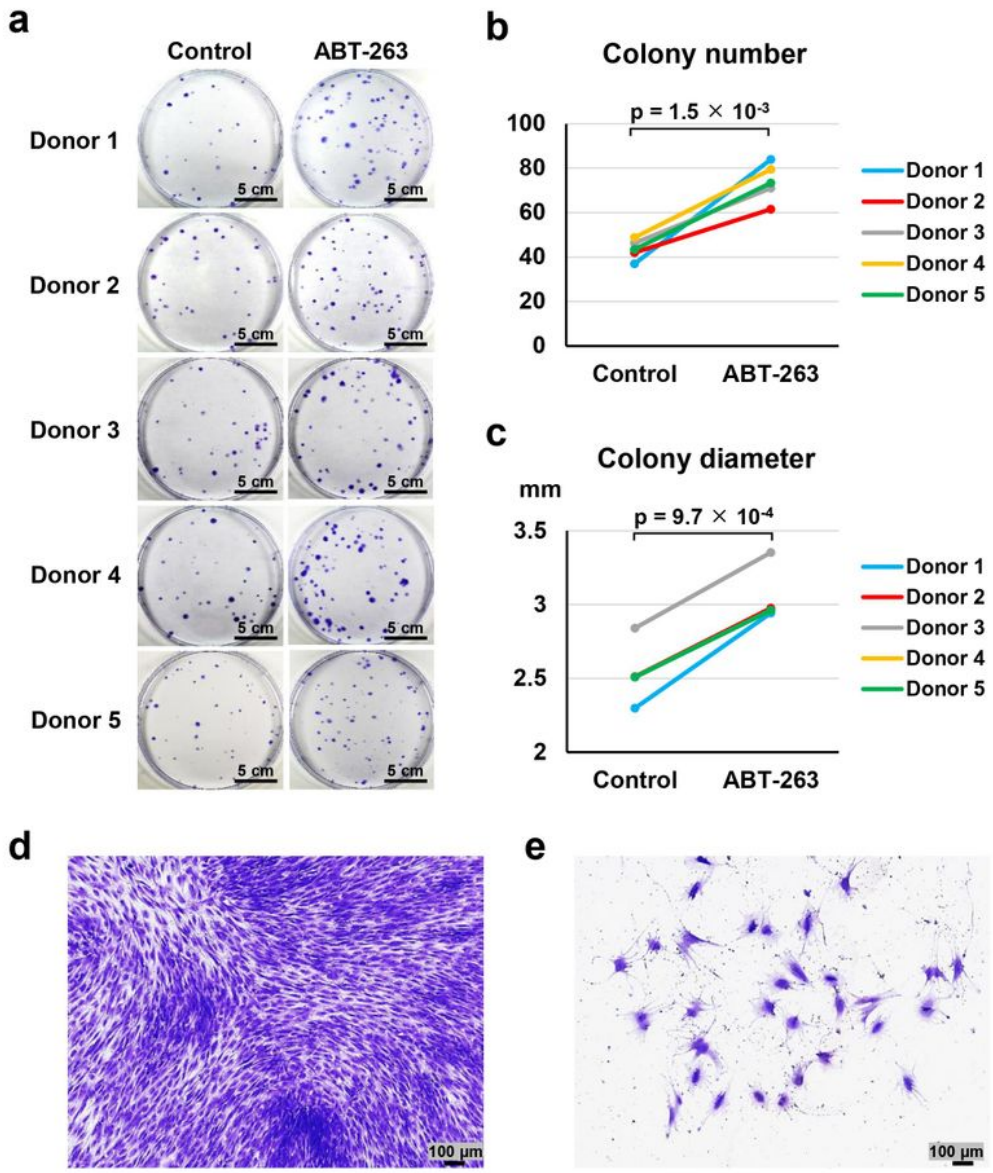

Figure 4

Colony-forming ability. (a) Representative images of colonies stained with crystal violet (4 replicate dishes per donor). (b) Colony numbers per dish in the control and ABT-263 groups. (c) Diameter of colonies in the control and ABT-263 groups. (d) Representative image of colony-forming cells. (e) Representative image of flattened and enlarged cells that did not form colonies.

Fig. 5

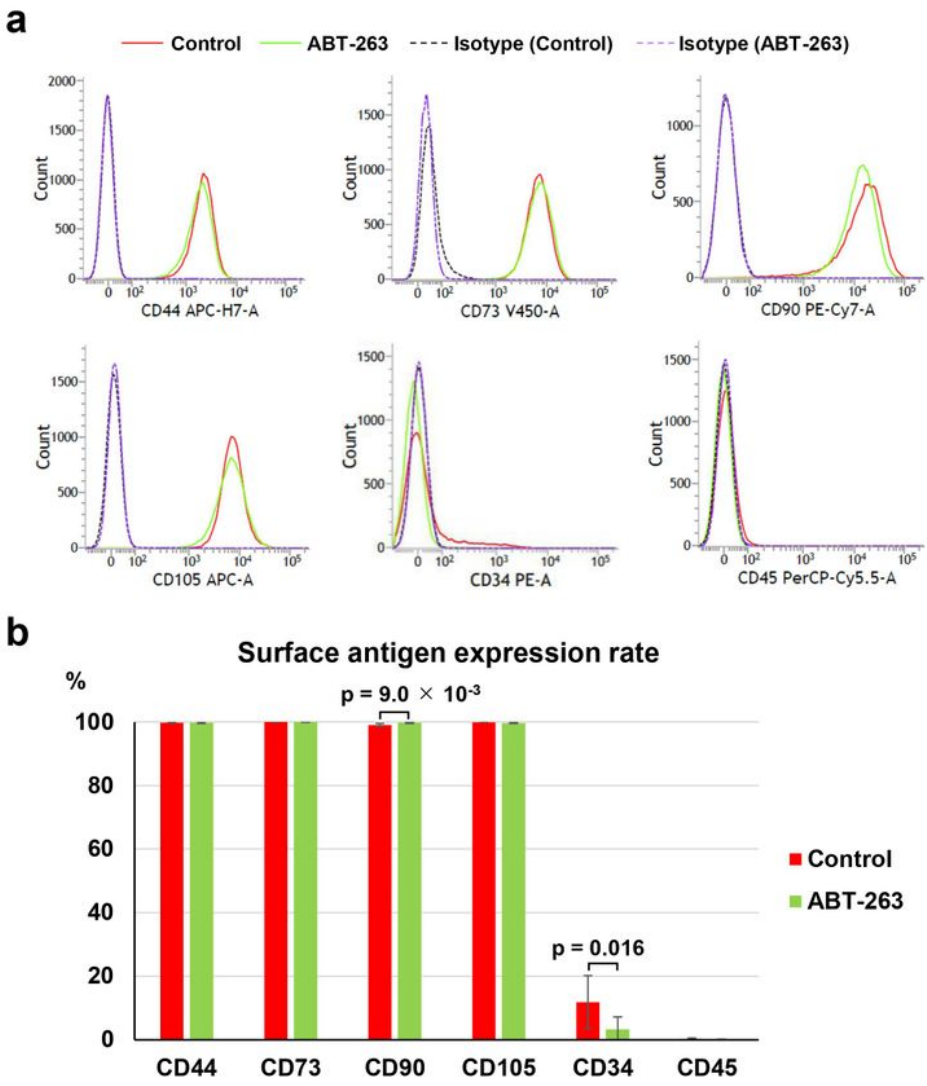

Figure 5

Surface antigen expression. (a) Representative flow cytometry histograms. Red solid lines indicate the control group and green solid lines indicate the ABT-263 group. Dotted lines show the isotype control. (b) The expression rate of each surface antigen. Values are shown as mean ± SD from 5 donors.

Fig. 6

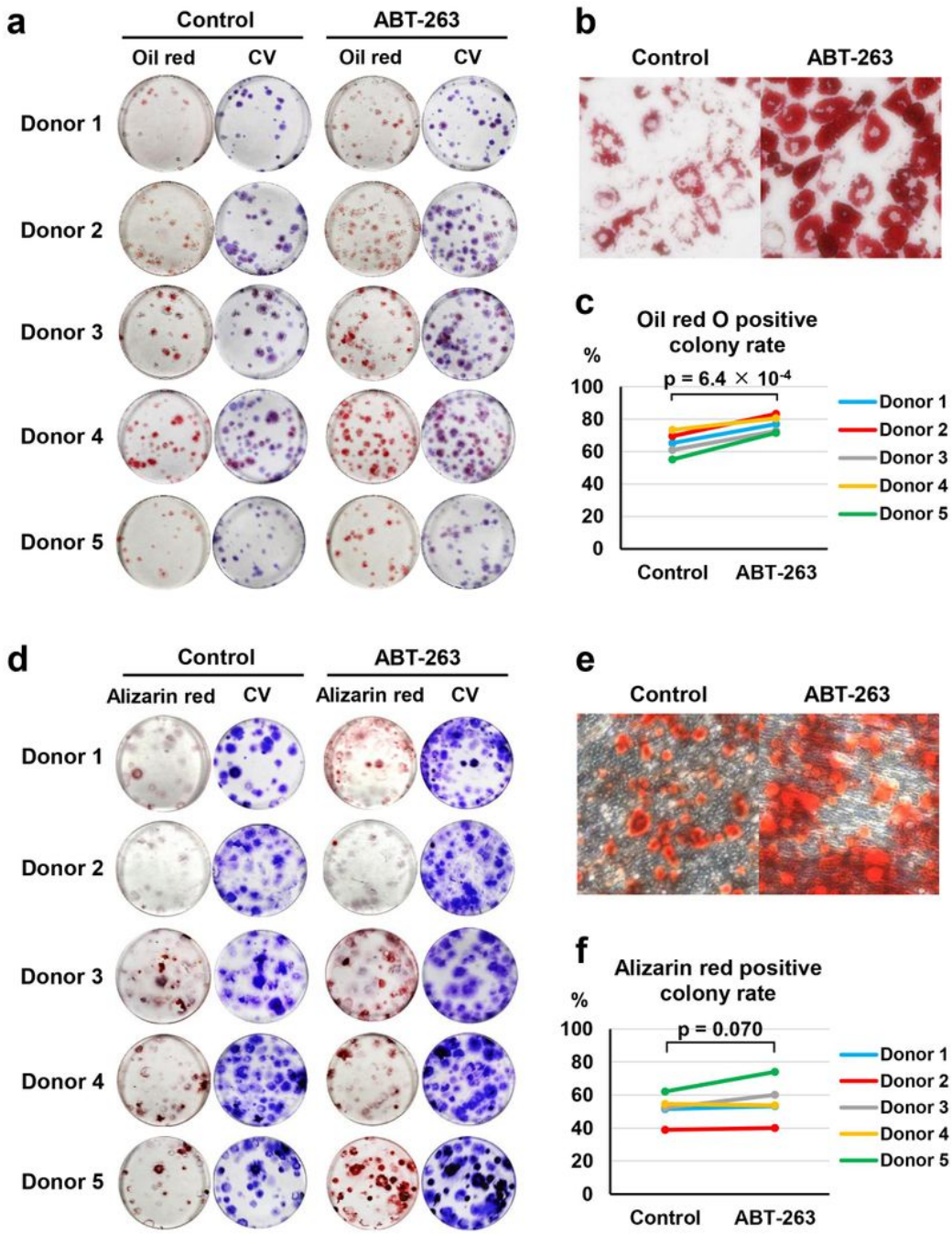

Figure 6

Adipogenic and osteogenic differentiation. (a) Representative images of oil red O and crystal violet (CV) staining (3 replicate dishes per donor). (b) The percentage of oil red O-stained colonies in the control and ABT-263 groups. (c) Representative images of alizarin red and CV staining (3 replicate dishes per donor). (d) The percentage of alizarin red-stained colonies in the control and ABT-263 groups.

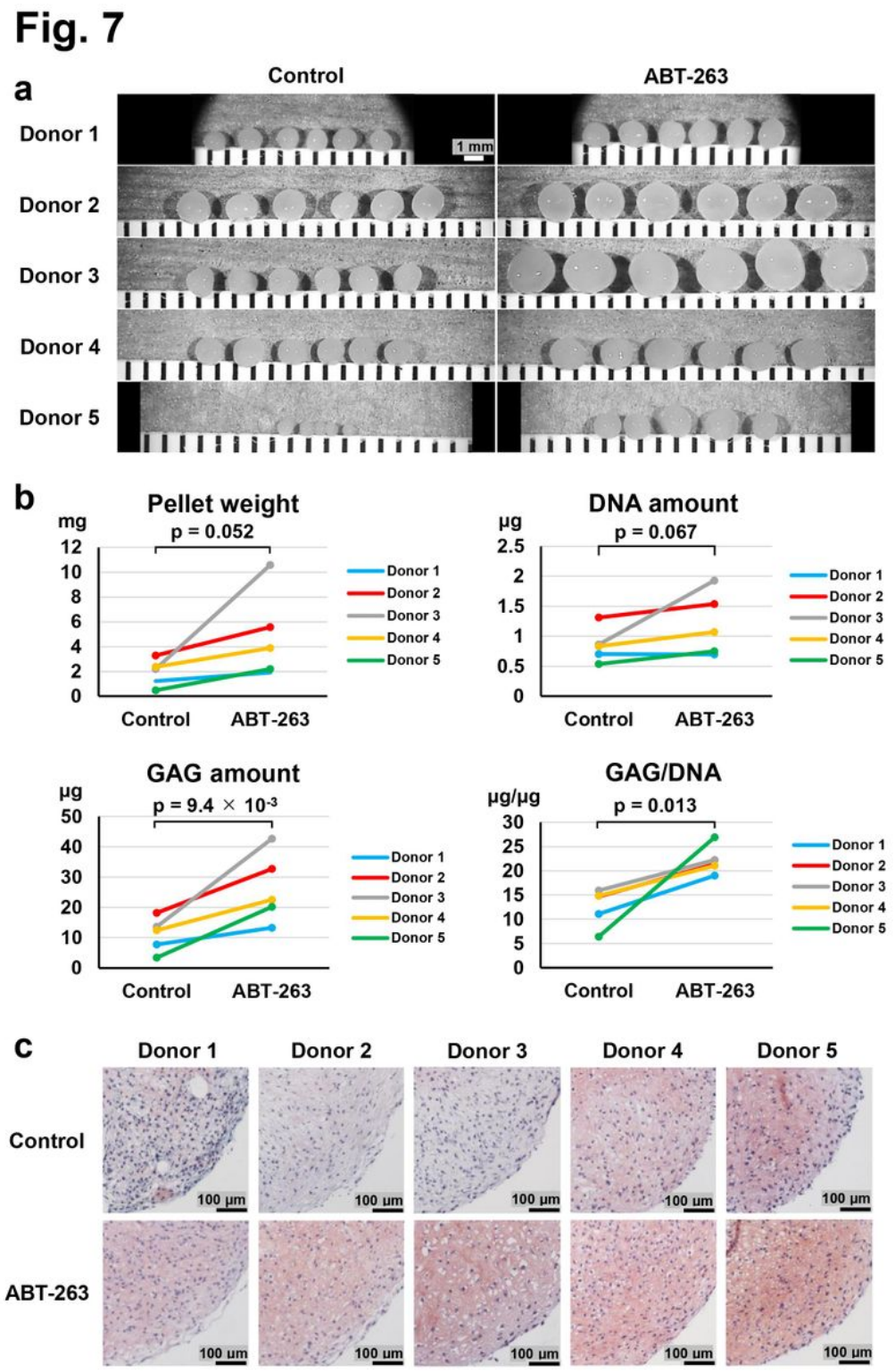

Figure 7

Chondrogenic differentiation. (a) Gross appearance of chondrogenic pellets (5 or 6 replicate pellets per donor). (b) Pellet wet weight, DNA quantification, glycosaminoglycan (GAG) quantification, and GAG/DNA ratio (3 or 4 replicate pellets per donor). (c) Representative images of safranin O staining of chondrogenic pellets.

Fig. 8

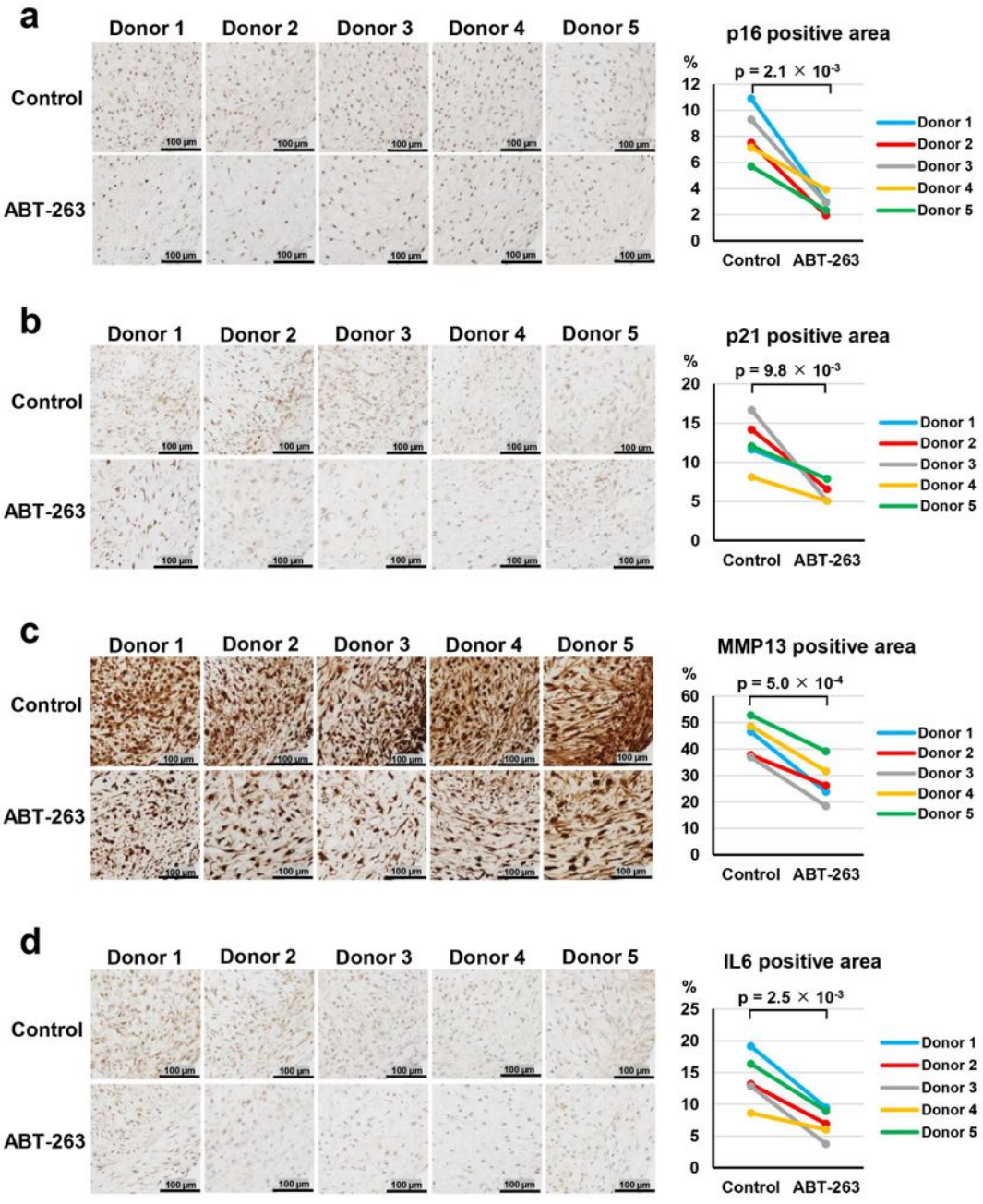

Figure 8

Immunohistochemistry of senescence markers in chondrogenic pellets. Representative images and positive ratios of (a) p16, (b) p21, (c) MMP- 13, and (d) IL-6 immunostaining in the control and ABT-263 groups

Supplementary Files

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- SCRTABT263SupplementalFigures220228.pdf