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ORIGINAL PAPER

Mitochondrial fission protein 1 up-regulation ameliorates senescence-related endothelial dysfunction of human endothelial progenitor cells

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Abstract

Background We investigated the contribution of mitochondrial dysfunction to the senescence of human endothelial progenitor cells (EPCs) expanded in vitro and the underlying molecular mechanism.

Methods and results Serial passage increased cell doubling time and those cells reaching the doubling time for more than 100% were defined as senescent EPCs, of which the activity of therapeutic angiogenesis was attenuated in mouse ischemic hindlimbs. The senescent cells, in medium free of glucose and bicarbonate, showed impaired activity in migration and tube formation. Flow cytometry indicated increased content of reactive oxygen species, mitochondria, and calcium, while bioenergetic analysis showed increased oxygen consumption and reduced ATP content. Examination of mitochondrial network showed that senescence increased the length of the network and ultrastructure analysis exhibited elongated mitochondria. Immunoblotting of the senescent EPCs demonstrated decreased expression level of fission protein1 (Fis1). In rat EPCs, the Fis1 level was decreased in the animals aged 24 months or older, compared to those of 3 months. Silencing of Fis1 in the young EPCs using Fis1-specific siRNA leads to appearance of phenotype resembling those of senescent cells, including elevated oxidative stress, disturbed mitochondrial network, reduced mitochondria membrane potential, decreasing ATP content, lower proliferation activity, and loss of therapeutic potential in ischemic hindlimbs. Fis1 over-expression in senescent EPCs reduced the oxidative stress, increased the proliferation, and restored the cobble stone-like morphology, senescence, bioenergetics, angiogenic potential, and therapeutic activity.

Conclusion In human EPCs, down-regulation of Fis1 is involved in mitochondrial dysfunction and contributes to the impaired activity of EPCs during the senescence process. Enhanced expression of Fis1 in senescent EPCs restores the youthful phenotype.

Keywords Angiogenesis · Endothelial progenitor cells · Mitochondrial fission protein 1 · Senescence

Abbreviations

DCFDA	2′,7′-dichlorofluorescin diacetate
DHE	Dihydroethidine
Drp1	Dynamin-related protein 1
EPCs	Endothelial progenitor cells
Fis1	Fission protein 1
MFN	Mitofusin
mtDNA	Mitochondrial deoxyribonucleic acid
MTR	MitoTracker Red CMXRos
NAO	Nonyl acridine orange
OPA1	Optic atrophy 1
OCR	Oxygen consumption rate
PBMCs	Peripheral blood mononuclear cells
ROS	Reactive oxygen species
VDAC	Voltage-dependent anion channel
SA-β-Gal	Senescence-associated β-galactosidase

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Introduction

Preclinical and clinical data have shown that in ischemic organs implantation of exogenous or autologous vascular progenitor cells, including endothelial progenitor cells (EPCs), promotes neovascularization and improves the function [1]. In humans, EPCs can be collected from peripheral blood and expanded in vitro [2]. However, during the aging process, the repair mechanism via progenitor cells declined. Clinical investigation confirmed that the number and the function of EPCs reduced in aged people [3, 4]. Similarly, during in vitro expansion of progenitor cells, the proliferation and function of the cells declined after certain passages and the cells exhibited a senescent phenotype [5]. Since an adequate amount of functional EPCs is prerequisite for cell therapy, understanding the aging process of EPCs during in vitro expansion is important for developing strategies to preserve the angiogenic potential of the cells.

Although multiple factors were found involved in the aging process of cells, a major contributor is oxidative stress [6]. Reactive oxygen species (ROS), primarily produced in the mitochondria, are intracellular metabolites of oxygen molecule which have high reactivity. Increasing studies have extended the oxidative stress theory to the functional integrity of mitochondria which mediates the mechanisms of age-related conditions [7]. Aging process impairs the homeostasis of mitochondrial dynamics and leads to abnormal mitochondrial structure [8–10]. The structure dynamics, which reflect the balance of fusion and fission, are associated with the cellular requirement of energy and metabolism [11]. The fusion and fission proteins have been shown to regulate the distribution of mitochondria as well as repair of damaged mitochondria [12, 13].

However, it is still unclear about the mechanisms beneath oxidative stress and bioenergetic deficiency in the progress of progenitor cells senescence and how such the decline of mitochondrial function in EPCs affects the reparative effect of EPCs. To clarify these issues, we investigated the senescence-related proteins in human EPCs expanded in vitro and then analyzed the biological behavior of EPCs. We demonstrated the impact of mitochondrial fission proteins in the progression of EPC senescence.

Materials and methods

Isolation of human CD34-positive endothelial progenitor cells (EPCs)

This investigation conforms with the principles outlined in the Declaration of Helsinki for use of human tissues. Ethical approval was granted by the Institutional Review Board of the Mackay Memorial Hospital, Taipei, Taiwan (reference number: MMH-I-S566). Informed consent was obtained from healthy donors before the collection of 80 mL peripheral blood (29 males, aged 24.1 ± 2.6 years; 34 females, 29.4 ± 5.9 years). Isolation, maintenance, and examination of EPCs were as previously reported [14]. EPC characterization was shown in Supplementary Fig. 1. The peripheral blood mononuclear cells (PBMCs) were fractionated from other blood components by centrifugation on Ficoll-Paque™ plus (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. CD34-positive progenitor cells were obtained from the isolated PBMCs using CD34 MicroBead kit and MACS™ Cell Separation System (all from Miltenyi Biotec, Bergisch Gladbach, Germany). Human CD34-positive EPCs were maintained in basal MV2 with growth supplement (annotated complete medium; both from PromoCell, Heidelberg, Germany) consisting of endothelial basal medium, 20% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, and heparin. 1×10^6 cells/cm^2 were seeded on fibronectin-coated dish (BD Biosciences, San Jose, California, USA) and maintained in 37 °C incubator under a humidified 95% air and 5% CO_2 atmosphere. Under daily observation, first medium change was performed 3 days after plating. Thereafter, media were changed every 3 days. For fluorescence quantification, labeled EPCs were examined with FACSCalibur™ flow cytometer and analyzed using CellQuest™ software (all from BD Biosciences).

Cell culture, obtainment of senescent EPCs, transfection of short interfering RNA, lentivirus transduction of Fis1, and analysis

The cultures were seeded onto 1% gelatin-coated plasticware or 2% gelatin-coated glass coverslips and maintained at 37 °C under a humidified 95% air and 5% CO_2 atmosphere. EPCs were grown in MV-2 complete medium and serially passaged until the proliferation time was more than two-fold. Chemically synthesized Fis1-specific short interference RNA (siRNA, using Stealth RNAi modification, Invitrogen, Carlsbad, California, USA) was delivered into cells using basal medium in the presence of cationic lipid reagent (LipofectAMINE 2000, Invitrogen). Non-sense siRNA was used as a transfection control (NSi). The sequences were listed in Supplementary Table 1. After 5.5-h treatment, medium containing the siRNA mixture was replaced with fresh culture medium, according to the experimental protocols. For Fis1 over-expression (NM_016068.2), cumate-plenti-SV40-GFP lentivirus vector and pAAV-TetOne vector were used (from Applied Biological Materials Inc, Richmond, Canada and Clontech Laboratories, Mountain View, California, USA,

respectively). For lentivirus, at the day of transduction, culture medium was replaced with complete medium supplemented with 8 μg/ml polybrene and virus was added at the indicated multiplicity of infection (MOI). After 48 h incubation, medium containing virus/polycation complex was replaced with fresh medium in the presence of indicated concentration of cumate (Applied Biological Materials Inc).

Cells were processed for western blotting, real-time polymerase chain reaction, flow cytometry, cell proliferation examination, senescence-associated β-galactosidase staining, ATP measurement, ADP/ATP ratio, bioenergetic analysis, and electron microscopy, as described in Supplementary Materials and Methods. For functional analysis, including angiogenesis, migration, and proliferation [15, 16], cells were additionally incubated in conditioned medium with indicated concentration of serum supplement (conditioned MV2, glucose and sodium bicarbonate free, PromoCell), which reflect the lower perfusion and reduced pH value to mimic the environment of ischemia in vitro [17]. To examine the protein localization, cells were fixed with 2% paraformaldehyde for 10 min, washed with PBS, followed by incubation with specific antibodies, counterstaining with bisbenzimide (18.7 μmol/l) for 15 min, washed with PBS again, and then mounted using 60% glycerol (v/v). Antibodies, fluorescence indicators, and primers used in this study were listed in Supplementary Tables. Images were examined by a microscope (DM6000 B, Leica, Wetzlar, Germany) with 63×/1.32 or 100×/1.32 aperture objectives and the images were scanned by a confocal microscope (TCS SP5, Leica) at room temperature. For analysis of mitochondrial structure, images were acquired by a CCD camera.

In vivo angiogenesis model

This animal investigation conforms with the policies in the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (revised 2011). The animal use protocol had been reviewed and approved by the Institutional Animal Care and Use Committee in the Mackay Memorial Hospital, Taipei, Taiwan (reference number: MMH-A-S-100-21). Hindlimb ischemic mice were used to evaluate the impact of Fis1 manipulation on the therapeutic potential of EPC [14, 18]. Briefly, 8-week BALB/c female athymic nude mice, weighing 18 to 22 g, were randomized into 3 or 4 groups. Mice were anesthetized by intramuscular ketamine (60 mg/kg) combined with Rompun (xylazine, 7 mg/kg). The physiological conditions were examined using vital signs monitor (BioPac, Goleta, California, USA). The right femoral artery and vein were ligated and cut, from just above the deep femoral artery to the popliteal artery and vein. Twenty four hours after operation, 2×10^5 human late EPCs in 50 μL PBS were injected into the hindlimb intramuscularly. Animals were sacrificed by intraperitoneal injection of overdose ketamine (120 mg/kg) and calf muscles were dissected transversally and prepared for immunofluorescence microscopy and histology examination at 21 days. The structure of capillary and myotube were examined by Bandeiraea simplicifolia (BS-1) lectin stain and anti-laminin antibody (1:100, AB2034, Chemicon, Temecular, California, USA), respectively. All samples were examined using the Leica microscope with a 40×/1.25 aperture oil objective and recorded by confocal microscopy.

Laser-Doppler perfusion imaging (LDPI)

Detection of hindlimb subcutaneous blood flow was performed using a Laser-Doppler Imager (Moor Instruments, Millwey, UK) as previously described [14, 15]. Mice were anesthetized with intraperitoneal injection of Zoletil (80–100 mg/kg) and placed on a heater at 37 °C before scanning. The region of interest included the thigh, leg, and foot. Perfusion analyses were performed sequentially at 24 h after surgery (just before the injection of EPC) and at 7, 14, and 21 days after the injection. The calculated perfusion was expressed as a ratio of right (ischemic) to left (normal) hindlimb.

Mitochondrial structure analysis

We examined mitochondrial structure of EPCs by MitoTracker Red CMXRos (MTR) staining with modified methods [10, 19]. Briefly, Cells were seeded onto 2% gelatin-coated glass coverslips and stained with 25 nM MTR for 1 h at 37 °C followed by PBS washing, 10 min 4% (w/v) paraformaldehyde fixation, counterstaining with bisbenzimide for 15 min, washing again with PBS, and then mounted. Images were captured immediately by the Leica microscope with a 100×/1.40 aperture oil objective and the images were captured by a CCD camera (DC 300F, Leica) at room temperature. To characterize the structure, uncompressed images of Cy3 and UV channels were merged and imported to software QWIN (Leica). Images were randomly assigned to two independent researchers for analysis. In each cell (event), a straight line that ran through the vertex of nucleus and reached perimeter of mitochondria network was marked and the length measured, and then normalized to the transverse diameter of nucleus. Calculation of 127 control cells, which derived from at least three independent individuals, yielded an average ratio about 1.2 and this value was set as the cut-off to distinguish normal and elongated mitochondrial network.

Data analysis

Data, expressed as mean ± SD, were analyzed using Chi square, one-way ANOVA, or Student's *t* test. When P value < 0.05, it is considered statistically significant.

Results

Expression profile, therapeutic potential, and cellular indices of senescent EPCs

Senescent EPCs were defined as an increase of cell doubling time for more than 100% of the control (young) cells, which were less than passage 8. SA-β-Gal assay showed that absorbance was increased for more than 130%. However, CD31 and CD34 were minimally changed between control and senescent cells. Also, the level of CD45 was negative in the senescent cells (Supplementary Fig. 2a, b). Analysis of protein markers demonstrated that senescence increased the expression level of manganese superoxide dismutase (MnSOD), non-phosphorylated connexin43 (Cx43), cyclin-dependent kinase inhibitor 2A (p16) and 1 (p21) (respectively 32%, 49%, 71%, and 86% increment) but decreased the content of sirtuin 1 (SirT1), Ki67, and proliferating cell nuclear antigen (PCNA) (respectively 47%, 63%, and 46% decrement), while catalase, GPX, Cx43, and PGC 1-α remained minimally changed (Supplementary Fig. 3). The telomere length was found decreased in the senescent cells (Supplementary Fig. 4). Interestingly, there was insignificant difference of the activity of migration and tube formation between the control and senescent EPCs in normal culture medium (MV2). However, the activity of angiogenesis and migration exhibited a remarkable reduction (tube formation, 39%; migration, 45% decrement) in conditioned medium which mimicked the low perfusion environment in ischemic tissue (Fig. 1a). To evaluate the angiogenic potential in vivo, control and senescent EPCs were intramuscularly injected into nude mice ischemic hindlimbs and then the perfusion was examined along the following 21 days. The results showed worse limb outcomes (Fig. 1b) and perfusion of the ischemic limb (perfusion ratio respectively on days 14 and 21, PBS, 0.27 and 0.31; control EPC, 0.37 and 0.46; senescent EPC, 0.29 and 0.35; see Fig. 1c) in animals given senescent cells compared to those given the control cells. Immunoconfocal analysis also showed that calf muscle of mice given senescent cells had a value of capillary density lower than that of those treated with control EPCs, but higher than that of those treated with PBS (capillary density respectively for hindlimbs given PBS, 0.39; control EPCs, 0.54; senescent EPCs, 0.45; see Fig. 1d).

To understand if the cellular function profiles were changed in a senescence-dependent manner, the levels of oxidative stress and mitochondria content in control EPCs as well as senescent cells were compared (Fig. 2). Flow cytometry indicated that the capacity of ROS, examined by 2′,7′-dichlorofluorescin diacetate (DCFDA) and dihydroethidine (DHE), was up-regulated by 68% and 72%, respectively (Fig. 2a). The analysis using MitoTracker Red CMXRos (MTR) and nonyl acridine orange (NAO) stains suggested that both mitochondria content and inner membrane mass were increased (33% and 69% increment, respectively). Also, the Fluo-8 staining showed that intracellular calcium was increased (35% increment). Analysis of outer membrane protein, VDAC, and mitochondrial DNA, including mtDNA target 8294–8436 and mtCOX1, also indicated that senescence increased the mitochondrial content (Fig. 2b, c).

Senescence alters the bioenergetic profile and structure of mitochondria

The above findings suggested that functional integrity of mitochondria was involved in the senescence process of EPCs. We therefore investigated the bioenergetic profile between control and senescent EPCs. The basal oxygen consumption rate (OCR), examined using bioenergetics analyzer, was up-regulated (96% increment, Fig. 3a) in the senescent cells and the oxygen leakage, evaluated by subtracting the OCR value of cells treated with antimycin A (5 μM) from that with oligomycin (2 μM), was increased for more than 50% in the senescent cells (Fig. 3b). However, the maximal respiration was minimally changed in the senescent cells which indicated that control EPCs had more respiratory capacity (Fig. 3c). At the basal level, compared to the control cells, senescent EPCs used more oxygen but contained less ATP and had higher ADP/ATP ratio (Fig. 3d). These results indicated that the integrity of mitochondrial function was changed in the senescent cells.

Cells stained with MTR (Fig. 4a) showed that senescence was associated with alteration of the mitochondrial morphology, by elongation of the mitochondrial network (Fig. 4a, c). In addition, the overall mitochondria/nucleus length ratio was elevated by more than 54% in the senescent cells (Fig. 4b), mainly contributed by the elongation of mitochondrial network, rather than change in nucleus length. Ultrastructural study of EPCs confirmed that senescent cells contained longer mitochondria, compatible with reduction of fission activity (Fig. 4d). Further characterization of mitochondrial structure proteins suggested that in the senescent EPCs the fission 1 protein (Fis1) was decreased, as confirmed by western blotting (34% decrement, Fig. 4e). In addition, the protein levels of Drp1, OPA1, MFN1, MFN2, and Mff were insignificantly changed in the senescent cells. Phosphorylation of Drp1 at serine 637 was increased in senescent EPCs though the level of Drp1 was maintained (Supplementary Fig. 5). To investigate whether the Fis1 down-regulation occurred during the aging process in vivo, we examined the Fis1 expression level in EPCs collected

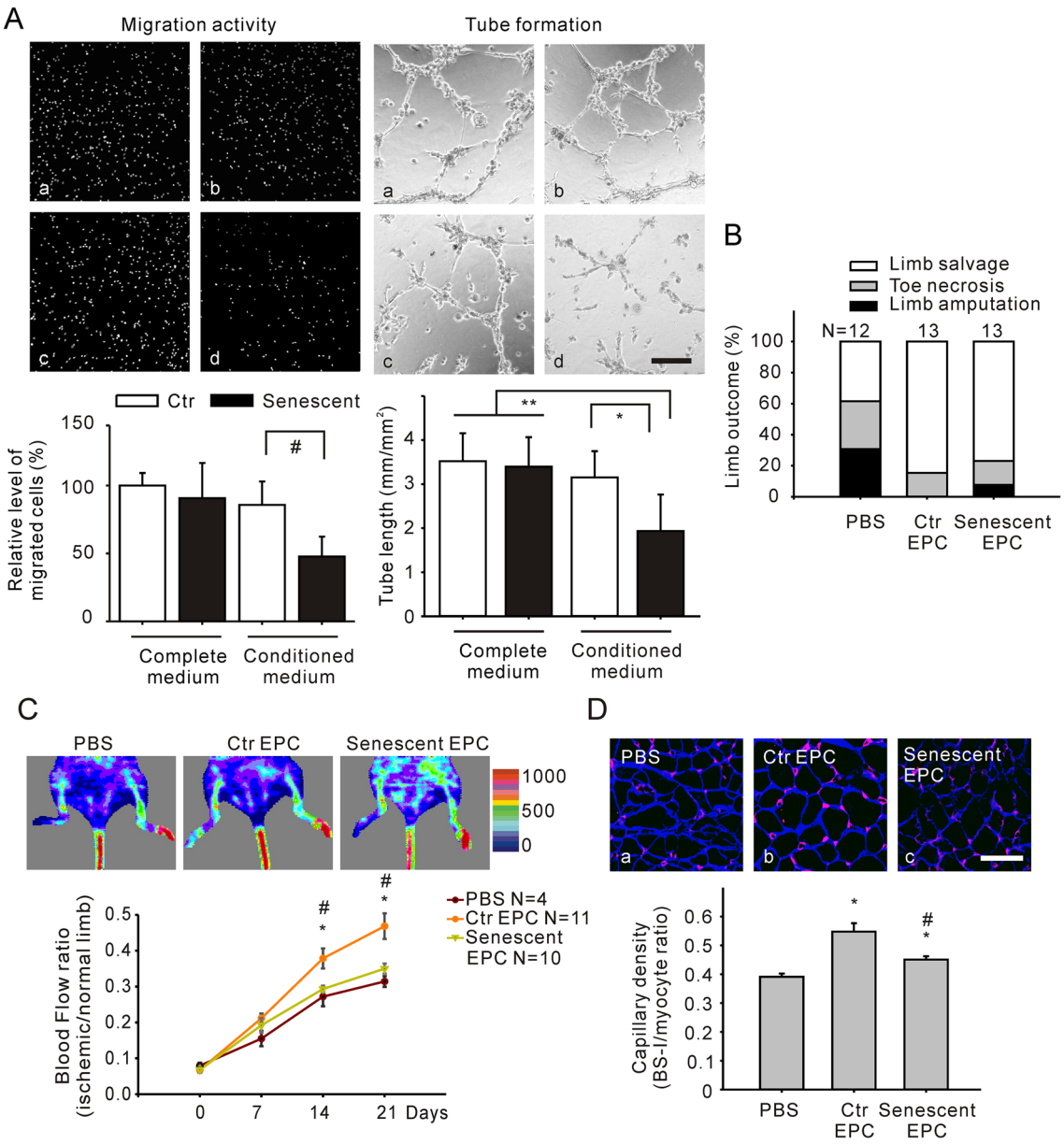

Fig. 1 Analysis of therapeutic potential of EPCs in vitro and in vivo. See text for details. Analysis of migration activity and angiogenic potential in control (young) and senescent EPCs, as evaluated by migration assay and tube formation assay (**a**). In an environment rich of glucose and sodium bicarbonate (complete medium), serial passage has little effect on the activity of migration and tube formation of the senescent cells. However, in an environment free of glucose and sodium bicarbonate (conditioned medium), both activities were remarkably reduced in the senescent cells. Each experiment was derived from EPCs of at least four individuals performed in duplicate. $^{*}P<0.05$; $^{**}P<0.01$; $^{\#}P<0.005$. Representative images of control (panel a in **a**) and senescent cells (panel b in **a**) in MV2 medium and control (panel c in **a**) and senescent cells (panel d in **a**) in conditioned medium are shown. Hindlimb ischemic mice injected with control (young) or senescent human EPCs were sacrificed at indicated time points and samples of calf muscles were applied to fluorescence microscopy (**b**–**d**). **b** Diagram of limb outcomes 21 days post operation. **c** Laser-Doppler perfusion flowmetry at different time points. * and $^{\#}P<0.05$, control (young) EPC respectively compared to senescent EPC and PBS. **d** Samples collected at 21 days post operation were stained with anti-laminin (in blue) and BS-1 lectin staining (in red). $^{*}P<0.05$, control (young) EPC and senescent EPC respectively compared to PBS. $^{\#}P<0.05$, control EPC compared to senescent EPC. Bar, 200 μm in **a**; 25 μm in **d**

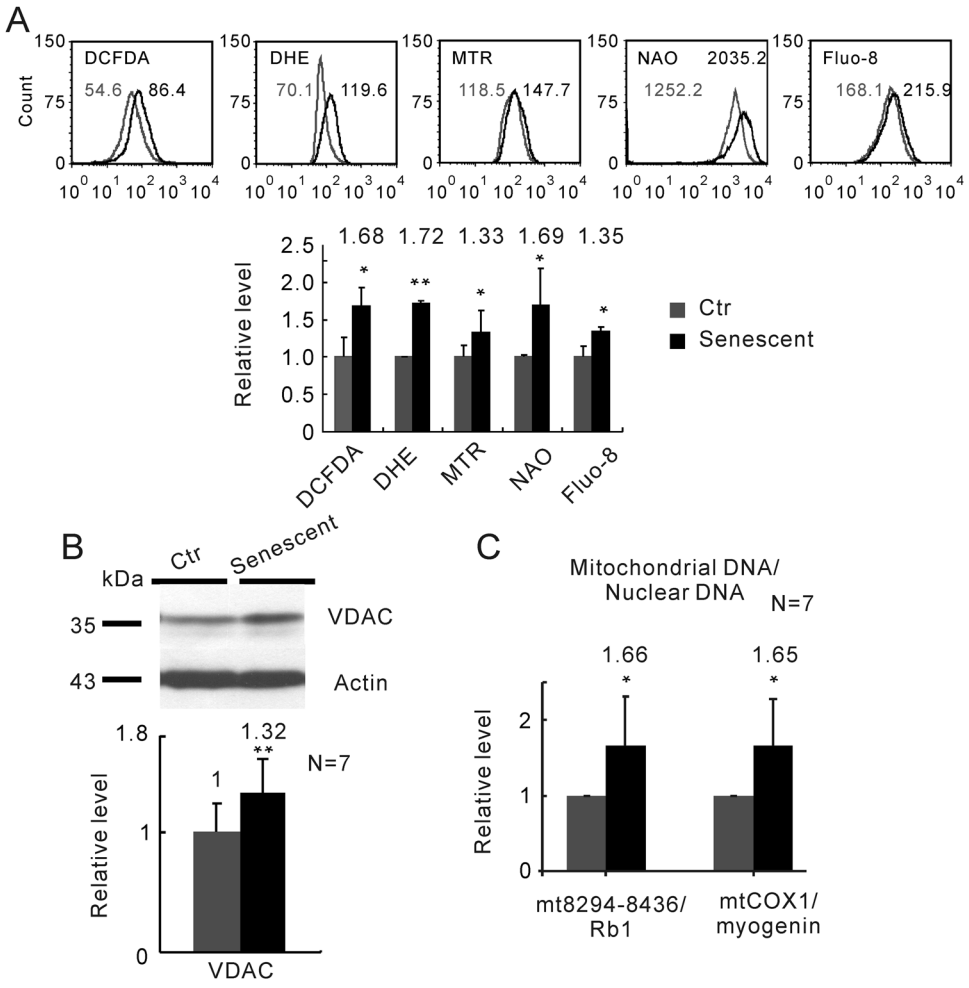

Fig. 2 Profile of oxidative stress, mitochondria, and calcium in senescent EPCs, as examined using flow cytometry (**a**), western blot (**b**), and real-time PCR (**c**) followed by analysis. Note that senescence process increases the levels of oxidative stress (DCFDA, $N=3$; DHE, $N=4$), mitochondrial content (MTR, $N=4$; NAO, $N=6$), calcium (Fluo-8, $N=3$), which reflects the disturbance of cellular function. Examination of outer membrane protein, VDAC (**b**) and mitochondrial DNA (**c**), including mtDNA target 8294–8436 and mtCOX1 also indicated that senescence increased the mitochondrial content. All $N=7$ in **b** and **c**. $^{*}P<0.05$; $^{**}P<0.01$, compared to control bar of each group

from blood of rats of different ages. Similarly, the Fis1 level was decreased in aged rat-derived EPCs (26% decrement in ≥ 24-month-olds) compared to control (young) group (3-month-olds) (Fig. 4f).

Fis1 silencing alters the mitochondrial structure and attenuates the cellular function of EPCs in vitro

To explore the biological effect of reduced Fis1 expression in the EPCs, the control cells were treated with Fis1-specific siRNA. Flow cytometry demonstrated that the transfection efficiency was above 90% in the EPCs treated with 25 nM and 40 nM AF555-labeled siRNA (Supplementary Fig. 6). The silencing potency of siRNAs specific to Fis1 was verified by real-time PCR, western blotting, and confocal microscopy and exhibited a dose- and time-dependent manner. Results showed that the Fis1 protein expression was down-regulated for more than 80% up to 240 h (Supplementary Fig. 7a–d). In addition, the protein levels of SirT1 and Drp1 were minimally changed in the transfected cells (Fig. 5a). After 72 h of transfection, mitochondria/nucleus length ratio was elongated for more than 31% (Fig. 5b) and the structure exhibited elongated pattern (panels c and d in Fig. 5c). Unlike serial passage, Fis1 down-regulation had minimal effect on the telomere length (Supplementary Fig. 8).

The function of cells treated with siRNA specific to Fis1 was checked using the flow cytometry, ADP/ATP bioluminescence, cell counter, and ischemic model (Fig. 6). The results showed that the treated EPCs (young cells) exhibited significantly decreased mitochondria membrane potential, while the mitochondria content was up-regulated (Fig. 6b, c). Two different sequences of Fis1-specific siRNA exhibited the same up-regulation of MTR signals (Fis1siRNA1, 33%; Fis1siRNA2, 24% increment). Flow cytometry further confirmed the increment of ROS in cells treated with Fis1siRNA (Fig. 6d). At 40 nM concentration of Fis1siRNA, the ATP content of the EPCs was also reduced after Fis1siRNA treatment, compared to the non-sense siRNA-treated cells (Fig. 6e). After 72 h of transfection, cells treated with 40 nM Fis1 siRNA showed lower activity of proliferation under complete medium (15% decrement, Fig. 6f). The reduction of proliferation was exaggerated in conditioned medium (compared to non-sense siRNA group, 25 nM, 24%;

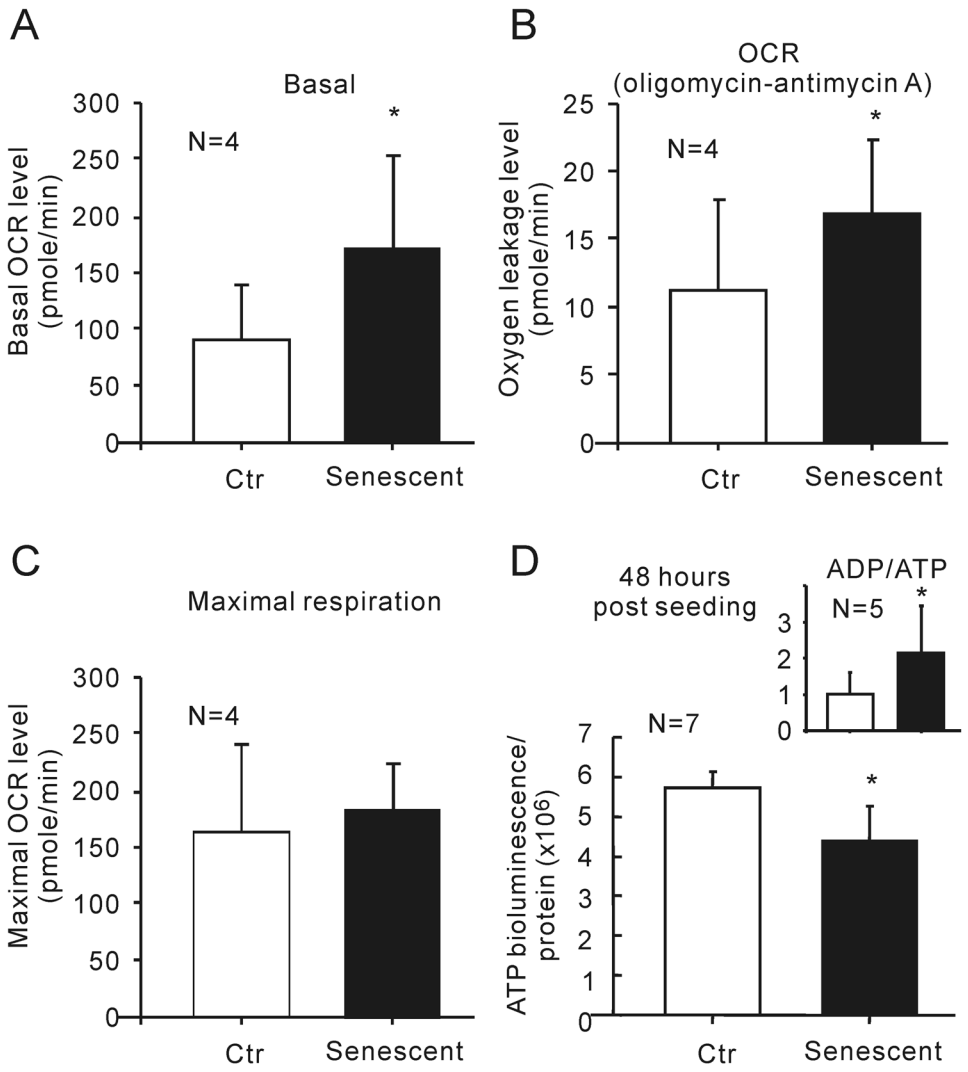

Fig. 3 Increase in basal oxygen consumption rate and leakage in senescent EPCs, as examined using bioenergetic analyzer (**a**, **b**). However, the maximal respiration rate was minimally changed in the senescent cells (**c**). Senescence reduces the ATP content of EPCs (**d**) and increased the ADP/ATP ratio (inset of **d**). See text for details. *$P < 0.05$, compared to control (young) EPCs

40 nM, 44% decrement). Compared to non-sense siRNA-treated cells, Fis1 silencing increased ADP/ATP ratio under conditioned medium (Supplementary Fig. 9). These findings showed that the phenotype of Fis1siRNA-transfected control (young) EPCs was comparable to that of senescent cells.

Down-regulation of Fis1 impairs the therapeutic potential of EPCs

To clarify if the reduction of Fis1 expression in EPCs altered the therapeutic potential, the human EPCs treated with Fis1siRNA were intramuscularly injected into nude mice ischemic hindlimbs (Fig. 6g–i). The results showed that, compared to mice given non-sense siRNA-treated EPCs, the perfusion of the ischemic limb was attenuated in animals given Fis1siRNA-treated cells (perfusion ratio respectively on days 14 and 21, PBS, 0.27 and 0.31; EPC/NSi, 0.39 and 0.45; EPC/Fis1si, 0.30 and 0.35; see Fig. 6h). Immunoconfocal analysis also showed that calf muscle of mice given Fis1siRNA-treated cells had a lower value of capillary density, compared to those given non-sense siRNA-treated EPCs (Fig. 6I).

Up-regulation of Fis1 ameliorates the cellular activities of senescent EPCs in vitro and in vivo

To understand if Fis1 up-regulation recovers the cellular function profiles in the senescent EPCs, the levels of oxidative stress, morphology, proliferation, and angiogenic potential in senescent EPCs and the cells transfected with Fis1 viruses were compared (Fig. 7 and Supplementary Fig. 10). The expression profile of Fis1 was verified by real-time PCR, western blot, and confocal microscopy. Fis1 expression level in transfected cells exhibited a dose- and time-dependent manner and in the cells treated with MOI 30 the Fis1 transcripts were increased by 51% (Supplementary Fig. 10a,

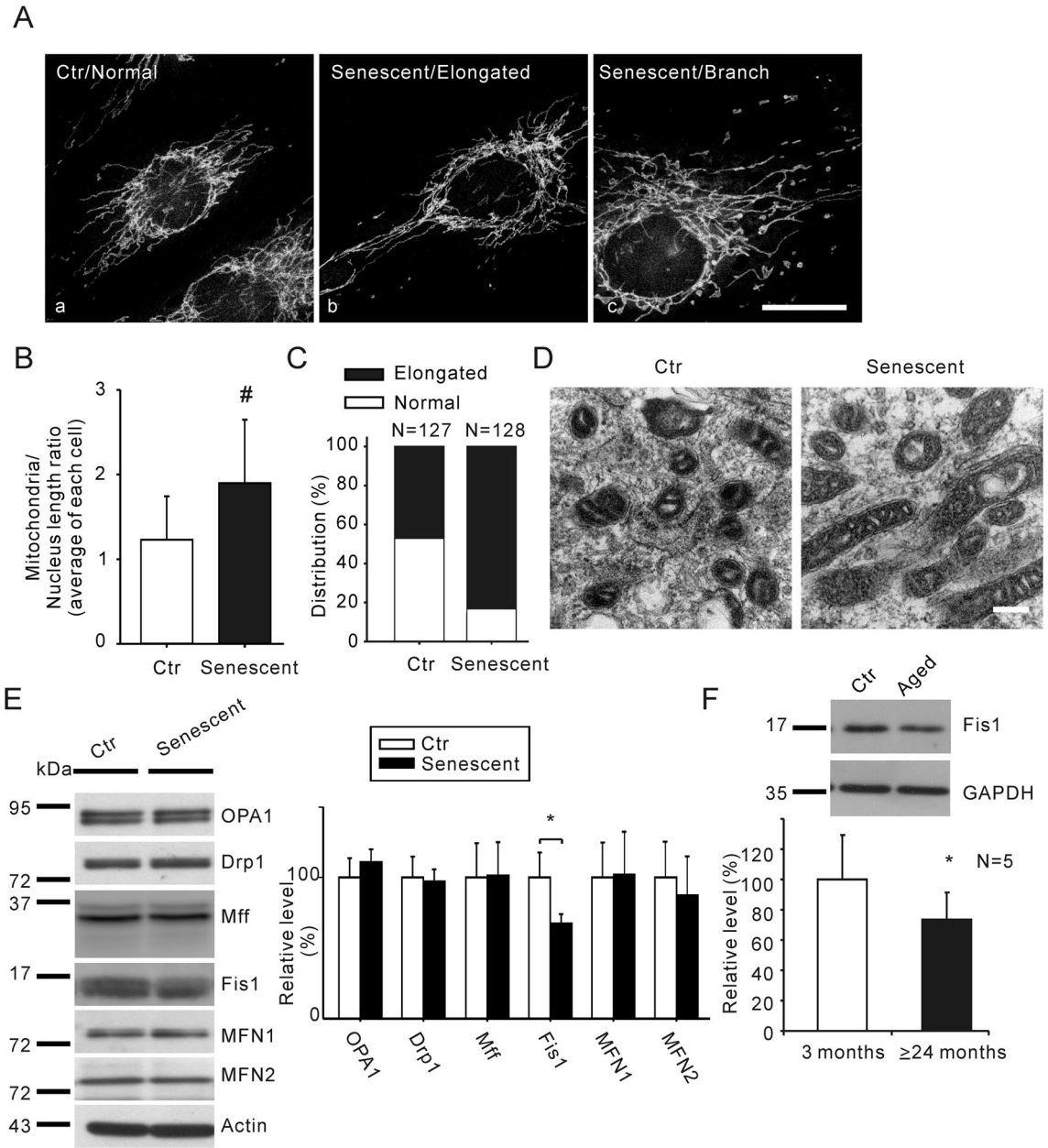

Fig. 4 Alteration in mitochondrial network, morphology, and structural protein expression profile in senescent EPCs, as examined using MitoTracker Red CMXRos staining (**a**) followed by analysis (**b**, **c**), electron microscopy (**d**), and western blotting (**e**). See text for details. Representative images of normal (panel a in **a**), elongated (panel b in **a**), and branched (panel c in **a**) mitochondrial network in control (young; $N=127$) and senescent cells ($N=128$) are shown in (**a**). The senescence process disturbs distribution profile of mitochondrial network of various morphologies (**a**, **c**) and the mitochondria/nucleus length ratio (**b**). Electron microscopy showed that mitochondria in senescent cells tended to be elongated (**d**). Examination of mitochondrial structure proteins indicated that in the senescent EPCs the Fis1 was decreased (**e**). **f** Fis1 protein level was decreased in aged (24 months) SD rat-derived EPCs compared to the cells of 3-month-old rats. $^{*}P<0.05$; $^{\#}P<0.005$, compared to control (young) EPCs in **b**, **e**, and **f**. Chi-square analysis showing significant differences ($P<0.01$) existing between the groups in **c**. Bars, 30 μm in **a**; 100 nm in **d**

b). Confocal microscopy demonstrated the increased Fis1 signal and less elongated mitochondrial network morphology compared to control (senescent) group (shown in red in Supplementary Fig. 10c, panel b).

Remarkably, Fis1 altered the morphology of senescent EPCs from extremely elongated appearance to spindle- and cobblestone-like shape after long-term maintenance (Supplementary Fig. 10d). After 144 h of transduction, cells treated with Fis1 lentivirus exhibited higher level of proliferation

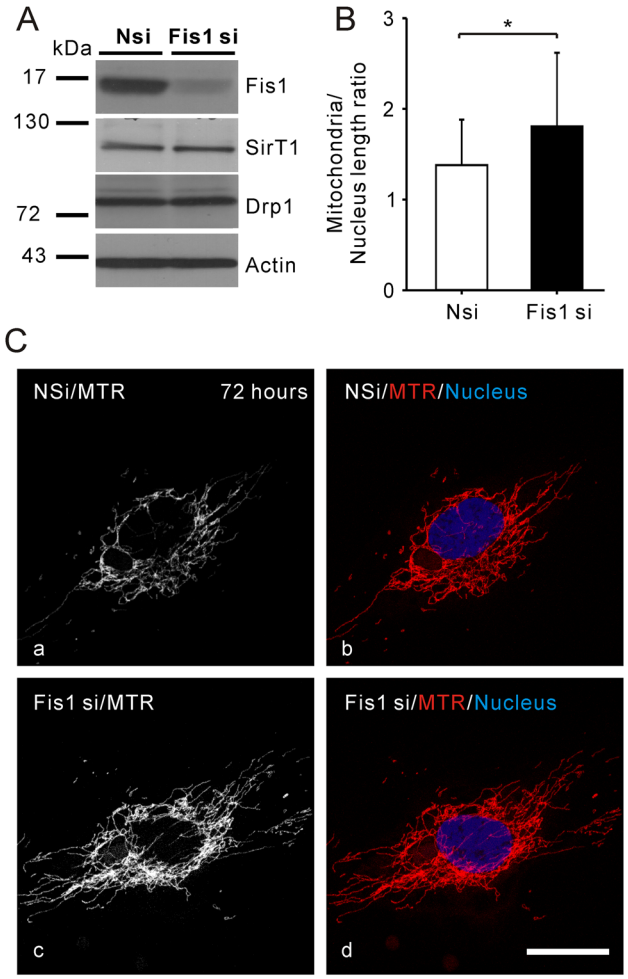

Fig. 5 Effect of Fis1 down-regulation on the mitochondria network in control (young) EPCs. Western blots of young cells post 72-h transfection with Fis1-specific siRNA (**a**). The mitochondria/nucleus length ratio post the 72-h transfection is increased (**b**, **c**). $^{*}P < 0.05$, compared to non-sense (NSi) group. Bar, 30 μm

under complete medium (compared to control group, MOI 20, 25%; MOI 30, 41% increment; Fig. 7a). Flow cytometry indicated that the capacity of ROS, examined by DHE, was down-regulated by 26% in Fis1-transduced EPCs (Fig. 7b). The activity of angiogenesis exhibited a remarkable recovery (tube length, 61%; branch point, 122% increment) in conditioned medium (Fig. 7c). Senescence marker, SA β-Gal staining was decreased in the spindle- and cobblestone-like shape cells (compared to control group, 30% decrement; Fig. 7d) and levels of ATP and ADP/ATP were improved (ATP, 14%, increment; ADP/ATP, 24% decrement, Supplementary Fig. 10e). Collectively, the presented results suggest that up-regulation of Fis1 in senescent EPCs renders the cells to exhibit youthful phenotype. To clarify if the recovery of Fis1 expression in EPCs altered the therapeutic potential, the senescent EPCs transduced with Fis1 virus were intramuscularly injected into nude mice ischemic hindlimbs (Fig. 7e). The results showed that, compared to mice given control (young) or senescent EPCs with control virus, the perfusion of the ischemic limb was improved in animals given Fis1 virus-transduced cells (perfusion ratio respectively on days 14 and 21, PBS, 0.34 and 0.40; Ctr EPC, 0.62 and 0.68; Senescent EPC, 0.43 and 0.50; Senescent EPC/Fis1, 0.55 and 0.62; see Fig. 7f). Immunoconfocal analysis also showed that calf muscle of mice given Fis1 virus-treated cells had a higher value of capillary density, compared to those senescent EPCs given control virus (Fig. 7g).

Discussion

In this study, with setting up a serial passage model of senescence process, we had clarified four related issues: (i) the impact of serial passage on the activity of human EPCs in vitro and in vivo; (ii) the phenotypic alternation of mitochondria in passage-related senescence of the EPCs; (iii) the significance of reduction of Fis1 in the EPCs, and, most importantly, (iv) the therapeutic potential of Fis1 in senescent EPCs. We showed that Fis1 is decreased in the senescent EPCs, accompanied by elevated oxidative stress, reduced content of ATP, elongated distribution of mitochondrial structure, and attenuated therapeutic angiogenesis in animal ischemic hindlimbs. We also demonstrated that reduced expression of Fis1 in young EPCs, by transfection with Fis1-specific siRNA, leads to manifestation of senescent phenotypes mentioned above. These novel findings indicated the mechanisms beneath and an important role of Fis1 in the senescence process of EPCs expanded in vitro.

Senescence with functional impairment of cultured stem cells/progenitor cells after several passages greatly limits the application for cell therapy [5]. The aging status of the senescent EPCs in the present study was supported by increased expression of cellular senescence markers SA-β-Gal staining, p16, and p21, reduced expression of proliferating markers Ki67 and PCNA [20–23], and reduced expression of SirT1, the last of which was involved in the regulation of senescence and longevity. The minimal change of PGC-1α, a metabolic regulator of mitochondrial biogenesis and respiration, may be due to the increased mitochondrial mass that partly compensated for the reduction of bioenergetic function [24]. CD markers' analysis suggested that the status of differentiation was little changed. We previously reported that silencing of Cx43 in the human EPCs impaired the angiogenic activity [15]. Although in the senescent EPCs of the present study, the total amount of Cx43 was not decreased, the non-phosphorylated form was increased. This means that phosphorylated Cx43 was decreased. Whether the reduction of phosphorylated Cx43 leads to functional impairment of the senescent EPCs required further studies.

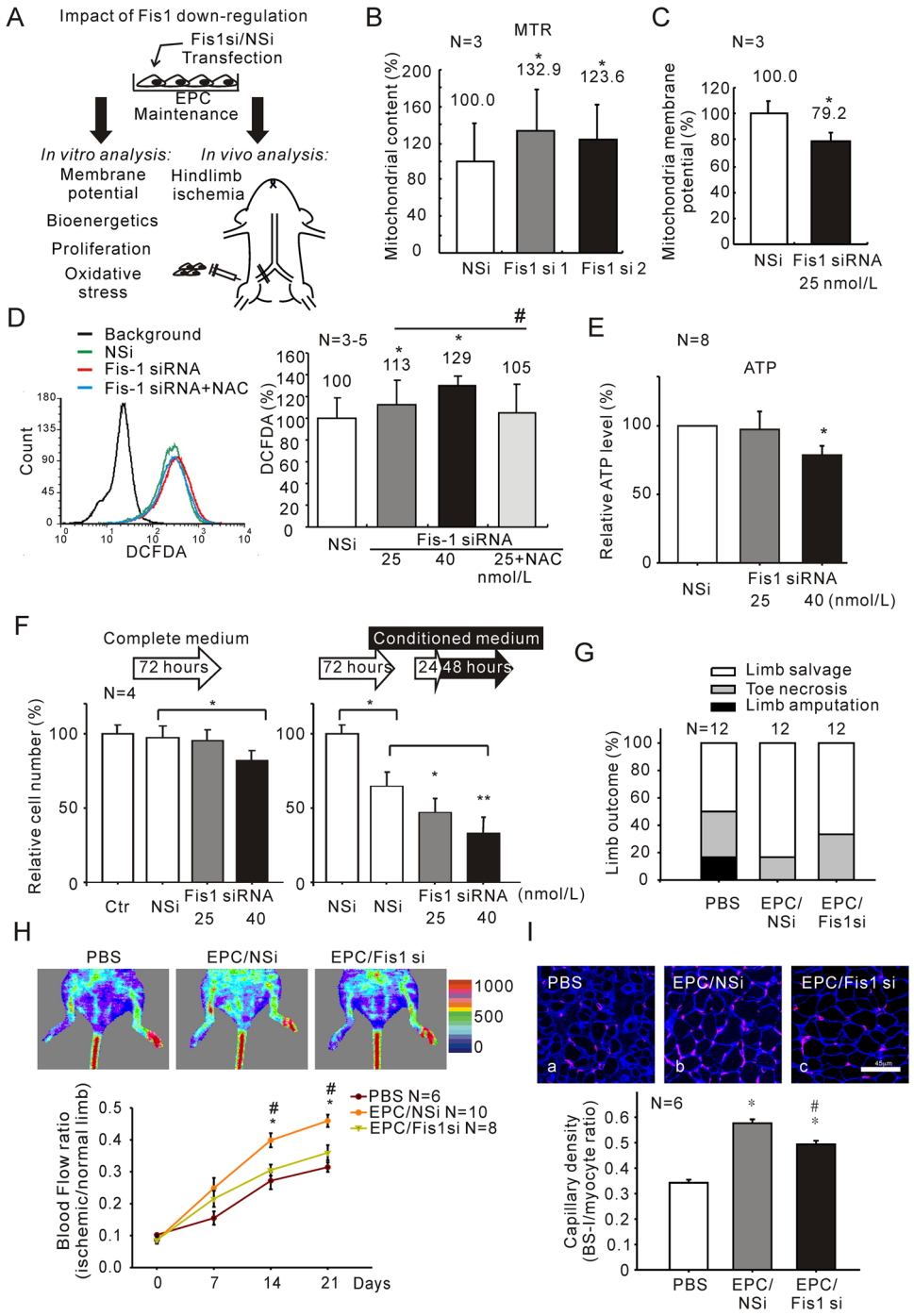

Fig. 6 Effect of Fis1 down-regulation on the function of control (young) EPCs in vitro and in vivo. Experimental design is shown in **a**. The cellular function of young EPCs after Fis1 down-regulation is similar to that of senescent EPCs, as examined using flow cytometry (**b**–**d**), ATP chemiluminescence (**e**), and cell proliferation (**f**). In experiments **b**–**e**, cells were maintained in complete culture medium. * and $^{\#}P<0.05$, compared to NSi and *N*-acetylcysteine group. For proliferation assay (**f**), transfected cells were cultured in complete medium for 72 h or initially in complete medium for 24 h and then in conditioned medium for another 48 h, respectively. $^{*}P<0.05$; $^{**}P<0.01$, compared to non-sense (NSi) bar of each group. In hindlimb ischemic mice (**g**–**i**), angiogenic potential of EPCs was attenuated post transfection with Fis1-specific siRNA. Hindlimb ischemic mice injected with young EPCs transfected with 25 nmol/l non-sense (EPC/NSi) or Fis1-specific siRNA (EPC/Fis1si) were sacrificed at indicated time points and samples derived from calf muscles were applied to fluorescence microscopy. **g** Diagram of limb outcomes at 21 days post operation. **h** Laser-Doppler perfusion flowmetry at different time points. * and $^{\#}P<0.05$, EPC/NSi respectively compared to PBS and EPC/Fis1si. **i** Samples collected at 21 days post operation were stained with anti-laminin (in blue) and BS-1 lectin staining (in red) followed by analysis of capillary-to-myocyte ratio. * and $^{\#}P<0.05$, EPC/NSi respectively compared to PBS and EPC/Fis1si. See text for details. Bar, 45 μm

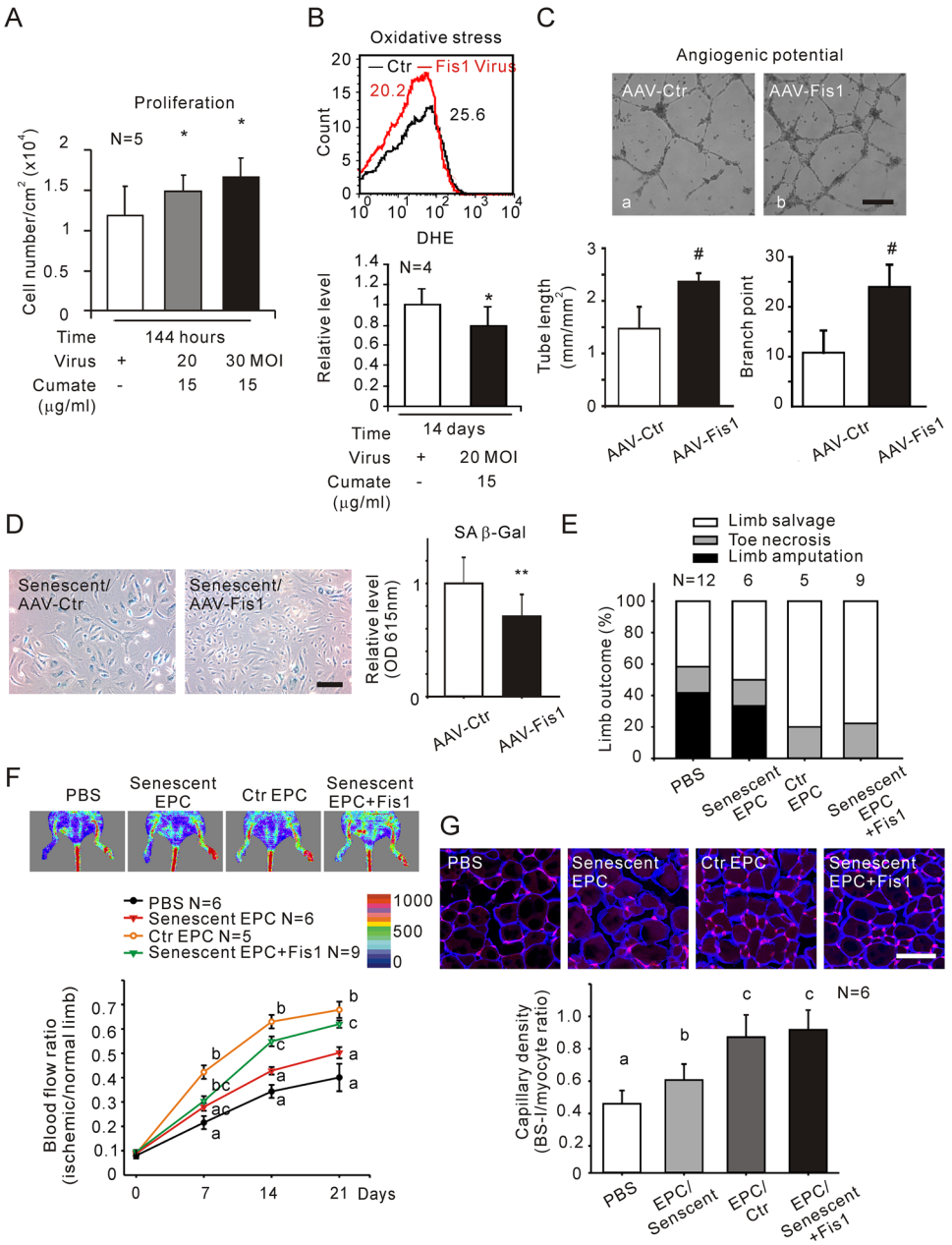

Fig. 7 Profile of Fis1 up-regulation on proliferation, cellular senescence, oxidative stress, and angiogenic activity, and therapeutic potential in Fis1-transfected EPCs, as examined using cell counting (**a**), flow cytometry (**b**), tube formation (**c**), and SA β-Gal staining (**d**) followed by analysis. Note that Fis1 up-regulation restores the senescent EPCs to youthful phenotype, including higher proliferation activity, cobble stone appearance with reduced SA β-Gal activity, lower level of oxidative stress (DHE), and higher level of angiogenic potential. In hindlimb ischemic mice (**e**–**g**), angiogenic potential of EPCs was improved by AAV-mediated Fis1 up-regulation. Hindlimb ischemic mice injected with young EPCs (EPC/Ctr), senescent EPCs, or Fis1-transduced EPCs (senescent EPC+Fis1) were examined at indicated time points and samples derived from calf muscles were collected after 21 days post injection and applied to fluorescence microscopy. **e** Diagram of limb outcomes at 21 days post operation. **f** Laser-Doppler perfusion flowmetry at different time points. **g** Samples collected at 21 days post operation were stained with anti-laminin (in blue) and BS-1 lectin staining (in red) followed by analysis of capillary-to-myocyte ratio. $*P<0.05$; $**P<0.01$; $^{\#}P<0.005$, compared to AAV control bar (senescent cells) of each group. Same letters in **f** and **g** indicate that there is no significance between groups. Bars, 500 μm in **c**; 200 μm in **d**; 25 μm in **g**

The findings of the present study that the levels of ROS, mitochondria, and calcium were increased in the senescent cells were consistent with the senescence process. However, in the senescent cells, though the oxygen consumption rate was increased, the bioenergetic capacity was attenuated [24]. The senescence process involved

biochemical and histological changes of cells, tissues, and organ systems, all ended in functional decline. Cellular aging is characterized by accumulation of cellular damage that results in growth arrest and death [25], and oxidative stress has been shown to play an essential role. For example, deficiency of antioxidant enzyme CuZnSOD was reported to impair EPCs function and lead to accelerated vascular aging [26]. However, senescence is not associated with a decline of all antioxidant enzymes [27], as shown in the present study: while catalase and glutathione peroxidase had minimal change, MnSOD was increased in the senescent cells. The reduction of ATP in senescent EPCs shown in the present study indicated decreased ATP generation, increased ATP utilization, or both. Oxidative stress also reduced ATP and further led to the alteration of energy metabolism [28, 29], both of which existed in our serial passage model.

Increasing data showed that intact mitochondrial function is crucial for the maintenance of functional progenitor cells [30]. Recent studies in the field of mitochondrial dynamics have indicated that fission and fusion are an intrinsic process that regulates aging through the regulation of cell cycle, mitochondrial metabolism, redox signaling, and apoptosis [7, 31]. Several key molecules regulate mitochondria dynamics, the dysregulation of which leads to change of mitochondrial structure and function [32–35]. However, in the present senescence model of EPCs, changes of Fis1 and inhibitory phosphorylation site of Drp1 were observed which suggest senescence attenuates the initiation and mitochondrial network of mito-fission.

Various aging cell models, like the model set up in the present study, are associated with elongated mitochondria, the development of which may reflect the adaptation to bioenergetic requirement [8, 10, 36]. Our finding that in human EPCs Fis1 down-regulation is associated with mitochondrial abnormality is consistent with a previous study examining deferoxamine-induced senescent arrest of Chang cells [37]. The reported effects of Fis1 may be related to the contribution in the mitochondrial autophagy and apoptosis [13, 38, 39]. In leukemia stem cells, Fis1 down-regulation impairs the activities of colony formation and serial engraftment and is essential for the regenerative potential and cancer cell stemness [40, 41].

Oxidative stress was reported to accelerate telomere shortening [42]. In the present study, we found that the length of telomere was shortened in senescent cells post serial passages. However, in young EPCs treated with Fis1 siRNA, the length of telomere remained stationary, though the cells otherwise exhibited senescent phenotype. Moreover, Fis1 over-expression restored the senescent cells to youthful phenotype, which exhibited higher proliferation activity, more youthful appearance, lower oxidative stress, reduced SA β-Gal staining, and recovery of therapeutic potential. This suggested that the contribution of reduced Fis1 to the senescence process of EPCs is independent of the telomere pathway.

Why in our study, the in vitro activities of tube formation and migration of senescent EPCs are maintained in normal culture medium, whereas the activity of therapeutic angiogenesis is reduced? The discrepancy may be due to the culture environment in vitro, which contains rich growth factors, nutrients, buffer, and adequate oxygen, while all of which are lacking in the ischemic tissue [17]. The remarkable reduction of migration and tube formation activity in cells maintained in conditioned medium proves this concept. Our results also consistently demonstrate that young EPCs transfected with Fis1-specific siRNA exhibit a profound reduction in proliferation activity and increase of ADP/ATP ratio under conditioned medium, which indicate Fis1 can mediate the therapeutic activity under stress.

After Fis1 transduction, the proliferation activity of senescent EPCs was improved in our study. Several studies also demonstrated that Fis1-mediated fission regulated apoptosis and proliferation in cardiomyocytes and vascular smooth muscle cells, respectively [43, 44], which indicated that mitochondrial fission–fusion is a key regulator of cell proliferation. Combining these results, cells with modest Fis1 expression contribute a more dynamic mitochondrial network for the functional integrity of bioenergetics [41]. Recent studies also indicate that in leukemic stem cells, Fis1 mediates mitophagy activity, which can compensate stress conditions to promote cell survival. With reduced Fis1 expression, cells under stress are inefficient in PINK1-activated mitophagy, resulting in further accumulation of mitochondria [41].

When all the above findings were pooled together, it became reasonable to suggest that in senescent EPCs, the down-regulation of Fis1 led to inefficiency of mitochondrial bioenergetics and subsequently impaired the angiogenic potential of the EPCs. Although microRNAs have been shown to attenuate Fis1 expression, the exact mechanism why serial passage leads to reduced level of Fis1 requires further studies [44, 45]. Moreover, the impact of Fis1 on the senescence of tumor stem cells is worth of examination and sequence identified in our study may apply to AAV-shRNA design for further investigation.

In conclusion, serial passage of human EPCs triggered senescence process and led to functional deterioration of the cells for therapeutic angiogenesis, in which perturbation of mitochondrial structure with Fis1 down-regulation is involved. Prevention of Fis1 reduction is a potential target to maintain the angiogenic potential of the EPCs against the senescence process during in vitro expansion.

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