SUPPLEMENTARY INFORMATION

Muscle-derived stem/progenitor cell dysfunction limits healthspan and lifespan in a murine progeria model

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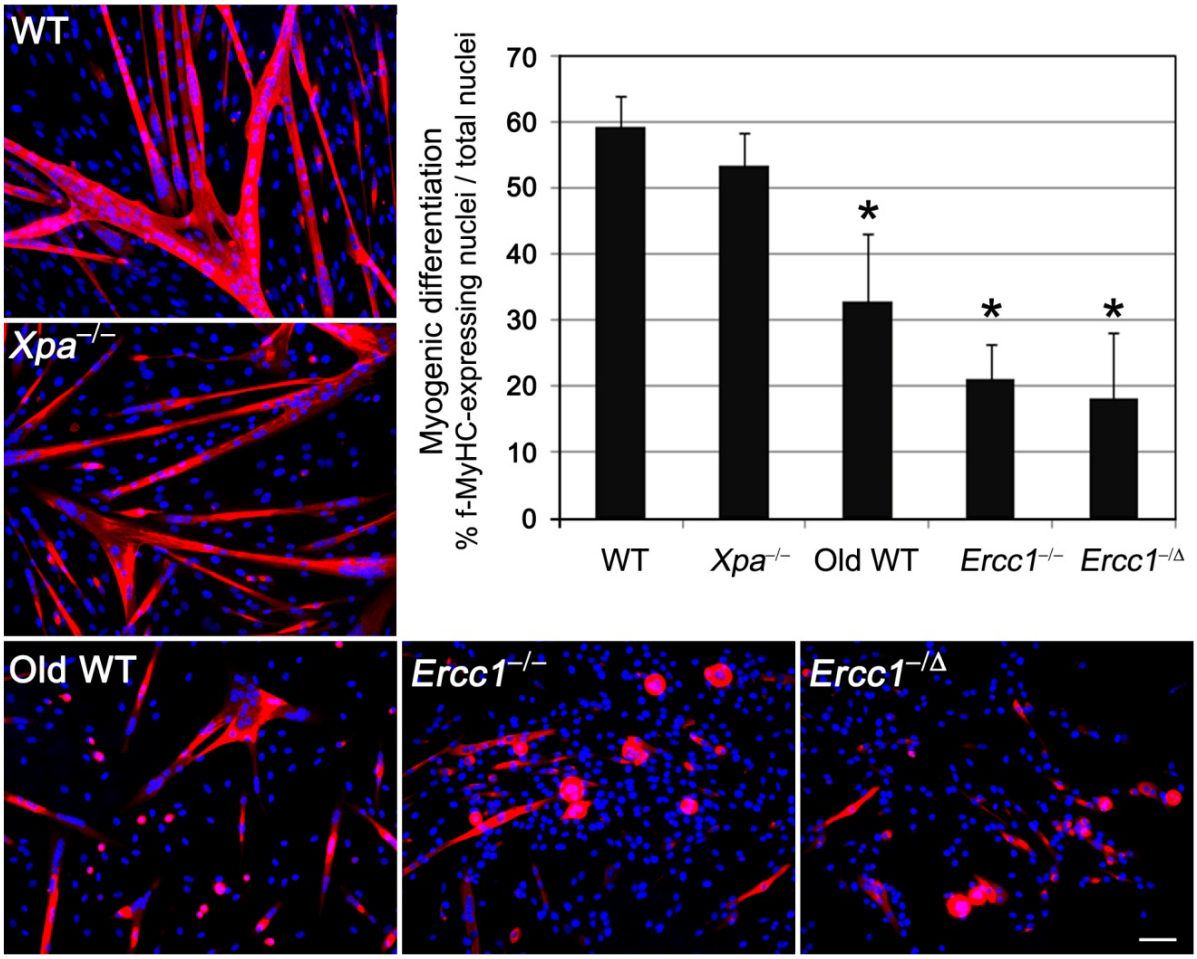
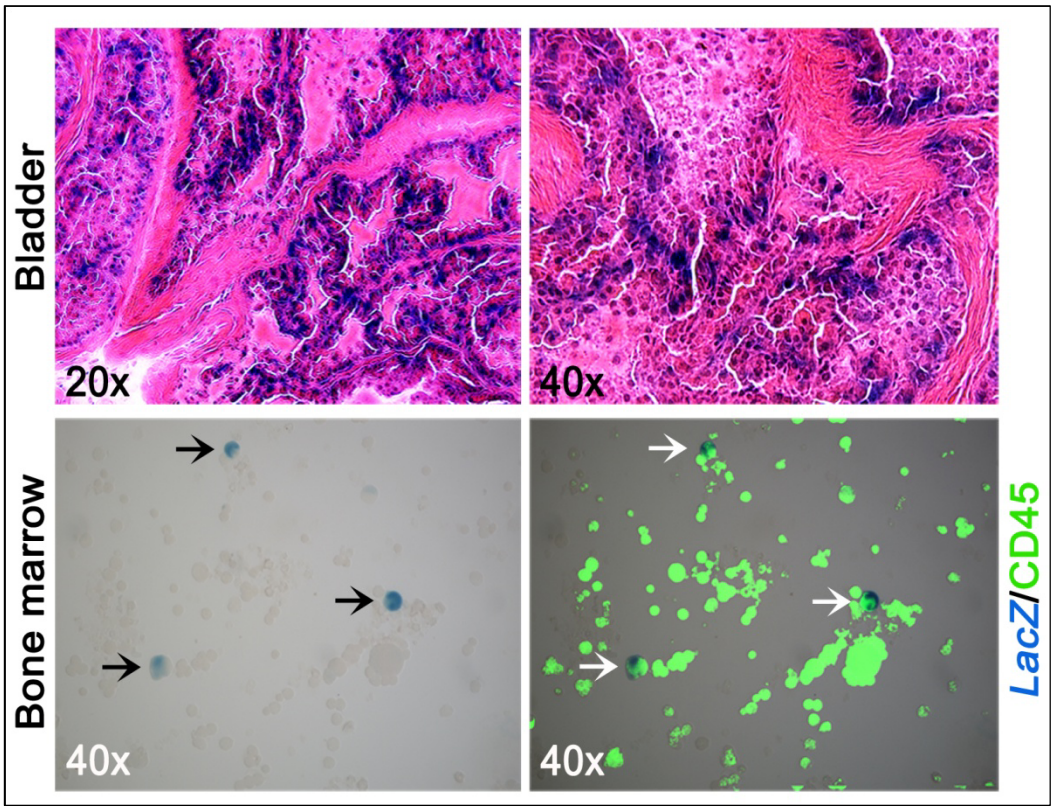
SUPPLEMENTARY FIGURES

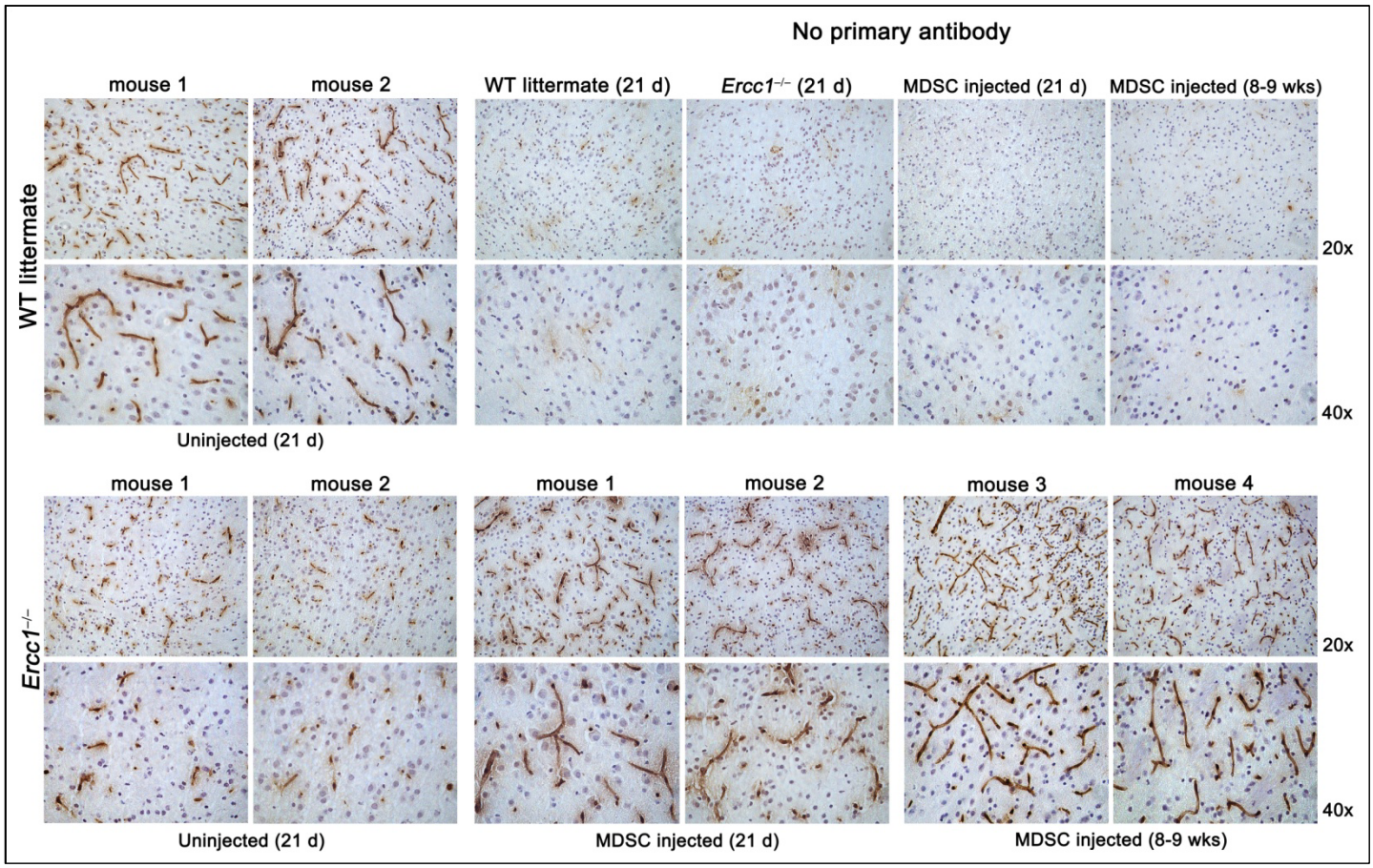
Supplementary Figure S1. In vitro myogenic differentiation of individual MDSPCs (clones). Shown are representative images of clonal myogenic differentiation of MDSPCs isolated from young and old WT mice as well as XPA- and ERCC1-deficient mice. After measuring division time (Supplementary Table S1), outgrowth from individual clones were switched to differentiation media (n = 3-7 per genotype). After 2-3 days, cells were immunostained for the terminal myogenic marker, f-MyHC (red). Scale bar = 100 μm. Also shown is the quantification of 50 images. Plotted are the average ± s.d. Myogenic differentiation [% of cells (DAPI, blue) expressing f-MyHC (red)] was significantly reduced in old WT, *Ercc1*$^{-/-}$ and *Ercc1*$^{-/\Delta}$ MDSPCs clones relative to young WT and *Xpa*$^{-/-}$ (*P < 0.001, Kruskal-Wallis ANOVA on ranks). These data demonstrate a myogenic differentiation defect in clonal MDSPCs isolated from old WT and progeroid mice, consistent with the cell population studies in Figure 1d-e.



Supplementary Figure S2: Determination of the site for donor cell engraftment after transplantation of progeroid *Ercc1*$^{-/-}$ mice with young WT-MDSPCs. 2-4 x 10^5 young WT-MDSPCs expressing nuclear *LacZ* per gram body-weight were injected IP into *Ercc1*$^{-/-}$ mice to determine the sites of engraftment. Mice were injected at 12 days of age and euthanized 8-9 days later (n = 4) or injected at 17-19 days of age and tissues harvested at the end of their lifespan (4-9 wks of age; n = 4). Fourteen organs/tissues were isolated, sectioned and stained with X-gal to identify donor cells (see Supplementary Table S4 for a summary). Donor cells (*LacZ*+ stained blue) were detected in the pancreas, liver, spleen, and kidney of all host animals and in the lung, esophagus, thymus and ureter of at least one mouse (Fig. 5). Large numbers of donor cells were also detected in the bladder of one mouse (below, top row seen at 20X and 40 X magnifications). Donor cells were also detected in the bone marrow of recipient mice. The bottom row depicts brightfield image (left) of cells isolated from the bone marrow of the long bones (hindlimb) depicting *LacZ*+ donor cells (black arrows). The right panel is a fluorescent image of the same sample illustrating co-localization (white arrows) of the *LacZ*+ donor cells with leukocyte marker CD45 (green).



Supplementary Figure S3. Progeroid *Ercc1*$^{-/-}$ mice treated with MDSPCs showed dramatically improved neovascularization in the cerebral cortex compared to untreated mutant animals. *Ercc1*$^{-/-}$ mice were administered intraperitoneally with 2-4 x 10^5 MDSPCs per gram of body weight at 17 days of age and tissues harvested 1-9 wks later (same animals as in Fig. 5). Tissue sections of the cerebral cortex were immunostained for the endothelial marker CD31 to identify microvasculature. Shown are representative images from two mice per group. Untreated *Ercc1*$^{-/-}$ mice (lower left panel) showed a significantly decreased vascular area (1.2%) in brain compared to WT littermates (2.5%, upper left panel) at 21 days of age. Progeroid mice injected with young WT-MDSPCs showed significantly improved neovascularization in the brain at 21 days (2.2%) and 8-9 wks (3.2%, lower right panel) post-injection with vascular areas indistinguishable from age-matched WT mice (3.1%, $P < 0.05$, Dunn's test).



SUPPLEMENTARY TABLES

Supplementary Table S1. The division time of individual MDSPCs measured while growing in the cell population or as isolated clones. To confirm that the population doubling time (PDT) accurately represents the proliferation rate of MDSPCs and is not simply the average of the heterogeneous population, we measured the cellular division time (DT) of individual cells. The DT of single cells in the population and single clones isolated from the population (n = 3-7 per genotype) were compared. Individual cells from preplate 6 were seeded in 96-well plates using FACSAria cytometer. Five days after plating, the DT of individual cells in each well was monitored for 72 hrs using the Live Cell Imaging. Using the images acquired, we calculated the time between the cytokinesis events. Reported is the average ± s.d. for each genotype. There was no significant difference between the population and clonal DT. See attached representative movies of progeroid MDSPCs (Supplementary Movie 1) and WT-MDSPCs (Supplementary Movie 2).

	WT (21d)	***Ercc1*$^{-/-}$ (21 d)**	**WT (19 wks)**	***Ercc1*$^{-/\Delta}$ (19 wks)**	**Old WT (2 yrs)**	***Xpa*$^{-/-}$ (21 d)**
Population	13 ± 2	19 ± 4	15 ± 3	22 ± 5	16 ± 6	15 ± 4
Single clone	11 ± 1	18 ± 2	14 ± 2	20 ± 2	16 ± 4	14 ± 2

Supplementary Table S2. Comparison of the total area of skeletal muscle regeneration following cardiotoxin injection into the gastrocnemius muscle of mice of the various genotypes.

Genotype	n	Median area of regeneration (μm^2)
WT (8 wks)	3	6708413
Ercc1$^{-/\Delta}$ (8 wks)	3	5250861
WT (18-21 wks)	5	2787460
Ercc1$^{-/\Delta}$ (18 wks)	2	4570307
WT (3 yrs)	3	2698447

P = 0.363 (Kruskal-Wallis One Way Analysis of Variance on Ranks difference).

Supplementary Table S3. Summary of the MDSPC populations transplanted into progeroid *Ercc1*$^{-/-}$ mice to evaluate the impact of stem cells on lifespan. All donor cells were injected into 17 day-old f1 C57BL/6;FVB/n *Ercc1*$^{-/-}$ mice of the opposite sex as the cell line injected.

Donor cell line name	Genotype	Age	Cell Sex	Strain	Source of mice	Passage number	# of mice injected	Median lifespan (days)	Average Lifespan (days)	s.e.m.
ft*	WT	3 wks	F	C57BL/6J	Jackson	20-25	6	66	66	2.7
A	WT	14 d	M	f1 C57BL/6;FVB/n	bred	15-20	4	66	67	2.0
C	*Ercc1*$^{-/-}$	3 wks	F	f1 C57BL/6;FVB/n	bred	20-22	6	26	37	7.2
D	WT	2 yrs	M	f1 C57BL/6;FVB/n	bred	20-22	6	25	26	1.1
MEF	WT	E13.5	u	f1 C57BL/6;FVB/n	bred	5	6	25	38	9.6
PBS	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	10	21	36	7.5

n.a.: not applicable, u: unknown, MEF: Primary mouse embryonic fibroblast
*This cell line was used to inject *Ercc1*$^{-/\Delta}$ mice (Figure 4b).

Supplementary Table S4. The tissues from *Ercc1*$^{-/-}$ mice administered labeled MDSPCs IP that were analyzed for the presence of *LacZ*+ donor cells. Listed are the organs/tissues collected, the number of mice analyzed, the average number of sections analyzed per tissue, and the frequency with which donor cells were detected.

Organ/Tissue/Cell	Approximate number of sections analyzed per mouse	Number of mice from which tissues were analyzed	Number of mice in which *LacZ*+ cells were detected
Pancreas	16	8	8
Liver	11	8	8
Kidney	27	8	8
Spleen	20	8	8
Lung	12	8	1
Heart	39	3	0
Thymus	14	8	2
Bladder/ureter	31	1	1
Brain	49	6	0
Skeletal muscle	25	4	0
Spinal cord	79	2	1
Lymph nodes	38	6	0
Esophagus	18	1	1
Bone marrow	3.4×10^7 cells	8	4

Supplementary Table S5. The primers used to measure expression of stem/progenitor surface markers and markers of differentiation.

Target gene	Marker of	Forward	Reverse	Amplicon length (bp)
Sca-1	Stem/progenitor cell	CCTAGTGTGTGCTGCAGAAAGAGC	GAGGAAGTCTTCACGTTGACG	243
CD34	Stem/progenitor cell	GCAGCTTTGAGATGACATCACC	CTCAGCCTCCTCCTT TTCACA	280
Desmin	Myogenic	AACCTGATAGACGACCTGCAG	GCTTGGACATGTCCATCTCCA	258
Myogenin	Myogenic	CTACAGGCCTTGCTCAGCTC	AGATTGTGGGCGTCTGTAGG	200
MyHC	Myogenic	GAATGACGGACGCCCAGATG	ACTGGCAGCCACTTGTAGGG	400
Col-I	Osteogenic	CCTGAGTCAGCAGATTGAGAACA	CCAGTACTCTCCGCTCTTCCA	115
Col-II	Chondrogenic	TCTGGTAAAGAAGGCCCTGTG	GTCCAGGGAATCCGATGTTG	106
PPARγ	Adipogenic	Qiagen Inc. Nm_011146 (QT00100296)		144
β-actin	Loading Control	AAGAGCTATGAGCTGCCTGA	TGGCATAGAGGTCTTTACGG	111

SUPPLEMENTARY METHODS

RT-PCR

RNA was extracted from 5 x 10^5 cultured MDSPCs using Trizol (Sigma-Aldrich) and an RNeasy Mini kit (Qiagen Inc.). RNA amount was quantified with a Nano-Quant (Tecan, Austria) and a total of 1 ug RNA was reverse-transcribed to cDNA using the SuperScriptTM III reverse transcriptase (Invitrogen) according to manufacturer's instructions. Then, cDNA was mixed with GoTaq polymerase, dNTPs and green GoTag reaction buffer (Promega) and amplified on the Vapo protect PCR system (Eppendorf). PCR was performed with Taq polymerase (Invitrogen) per the manufacturer's instructions for 30 cycles at 58°C annealing temperature and the PCR products were separated by electrophoresis on 2% agarose gels. β-actin was used as a loading control. The primers used to measure the expression of various differentiation markers are listed in Supplementary Table S5.

Animal husbandry

All animal experiments were performed with the approval of the University of Pittsburgh Institutional Animal Care and Use Committee. The mice were housed in individually ventilated micro-isolator caging (IVC) systems. Each IVC unit has HEPA-filtered intake and exhaust. Cages are 11" X 8" X 5" polycarbonate cages housing 4-5 adult mice. Forced ventilation IVC racks are maintained in positive pressure mode. Immunocompromised animals are housed in autoclaved cages. All rodent caging is sanitized every 7 days. The mice are fed ad libitum a standard chow from Purina Laboratory which is gamma-irradiated. Water is also provided ad libitum via bottles and sipper tubes. The water is pre-treated with sodium hypochlorite to a concentration of 5-7 parts per million (ppm) provided via a chemical proportioner then autoclaved. The light/dark cycle is set at 12/12 at 40-60 ft. cds. Cage enrichment includes nestlets and group housing. The barrier facility in which the mice were housed is specified-

pathogen free rodent facility, monitored quarterly via sentinel animals. Animals are handled exclusively in a laminar hood by personnel wearing head to toe protective clothing and gloves. Clidox disinfection is used between handling individual cages.

Cardiotoxin injection

For cardiotoxin experiments, 20 µl of 2 µM cardiotoxin (Sigma-Aldrich) dissolved in PBS was injected into the gastrocnemius muscle of mice: 3 old WT (3 yr-old), 5 WT (18 wk-old), and 3 *Ercc1*$^{-/\Delta}$ (18 wk-old) mice. Two weeks later, the gastrocnemius muscles were harvested, frozen in 2-methylbutane pre-cooled in liquid nitrogen, and cryostat-sectioned (8 µm). Sections were immunostained for dystrophin and fluorescent images were captured using a Nikon Eclipse E800 microscope equipped with a Q imaging Retiga Exi digital camera using Northern Eclipse software (v. 6.0; Empix Imaging, Inc.). To perform dimensional analysis of dystrophin-positive myofibers, the images were thresholded using this computer software to distinguish the immunofluorescence signal from the back-ground noise signal, and then to determine the number and area of fibers and to provide quantitative measurements of the number of pixels occupied by each individual fiber. The fiber area distribution of >1000 centronucleated individual myofibers per group was measured by determining the total number of pixels occupied by each fiber and converted to square micrometers with the software. Finally, the cross sectional area of, and the distribution of the fiber areas was plotted[60, 61].

To reveal areas of fibrosis in regenerating muscles, sections were stained with Masson's Trichrome (Kit, K7228; IMEB Inc.), which stains collagen blue, muscle fibers red, and nuclei black. Quantitation of the fibrotic area was done on n = 13 images per mouse using the Northern Eclipse software (v. 6.0; Empix Imaging, Inc.).

Transplantation

To test the myogenic potential of MDSPCs in vivo (Fig. 1f-g), 3 x10^5 WT-MDSPCs (pp6) isolated from two 19 week-old male *Ercc1*$^{-/\Delta}$ mice and two of their WT littermates were injected into the gastrocnemius muscle of 9 week-old female *mdx*/SCID mice (n = 8 mice, 4 mice/cell population). *Mdx* is a mouse model of Duchenne Muscular Dystrophy, with profound muscle degeneration due to lack of dystrophin expression. The mice were also immunocompromised (SCID). Two weeks after transplantation, the mice were euthanized and the injected gastrocnemius muscles were isolate and cryopreserved for sectioning. Muscle sections were immunostained with dystrophin to identify regenerated myofibers by donor cells.

To test the ability of MDSPCs to rescue aging phenotypes in progeroid mice after IP administration (Fig. 4 and Supplementary Table S3), 2-4 x 10^5 MDSPCs per gram body-weight were injected into the peritoneal cavity of 17 day-old *Ercc1*$^{-/-}$ mice. Transplantations were performed in a sex-mismatched manner such that the donor cells were of the opposite sex from the host. A littermate mutant animal was injected with vehicle only (PBS). *Ercc1*$^{-/-}$ mice were allowed to live until "natural" death to determine the effect of MDSPCs on lifespan. In an identical experiment, littermate mutant animals were injected with the same quantity of early passage WT primary mouse embryonic fibroblasts (MEFs) or vehicle only. For the evaluation of the impact of MDSPCs on healthspan, 6-7 wk-old *Ercc1*$^{-/\Delta}$ mice were injected IP with 2-4 x 10^5 MDSPCs per gram body-weight. The injection was repeated 6 wks later. The weight and the age-at-onset of spontaneous progeroid symptoms were assessed bi-weekly by an investigator blinded to the treatment. The age at onset of each symptom was averaged within a treatment group. Dystonia, a sign of neurodegeneration[62], is measured as an abnormal response to tail suspension (a clasping rather than splaying reflex)[63]. Kyphosis, a sign of osteoporosis[34, 64], was measured by observing the curvature of the spine compared to wild-type littermates. Ataxia, a sign of cerebellar neurodegeneration, was measured by evaluating the animal's gait and ability to maintain their posture when reared on hind limbs to groom[65]. Priapism and incontinence are

only observed in male mice. Lethargy was scored as positive when the mice were slow to rise when startled by moving their cage or attempting to pick them up. Sarcopenia was scored as positive when the mice showed proximal hind-limb wasting. The Aging Score is an overall measurement of the quality of life or healthspan and reflects the fraction of symptoms that occurred later in mice treated with MDSPCs relative to littermate mutant animals that received vehicle only. The aging score was determined as follows. Mice were studied as littermate pairs, where one mutant mouse was treated with the test cells and the other was treated with vehicle only (PBS). For each symptom, the mouse in which the symptom was delayed received a score of +1 and the littermate got a score of 0. If the symptoms occurred simultaneously in both mice, both mice received a score of 0. The sum of the scores for each animal was divided by the number of symptoms measured in that pair of mice (8 for males; 6 for females) to determine the fraction of symptoms delayed. This number was plotted for both animals in each littermate pair then the average for each treatment group was determined to give the aging score. The symptoms evaluated included kyphosis, dystonia, trembling, ataxia, priapism and urinary incontinence, muscle wasting (sarcopenia) and reduced spontaneous activity (lethargy) due to frailty.

To determine the site of MDSPC cell engraftment after IP administration (Fig. 5, Supplementary Figure S2), *Ercc1*$^{-/-}$ mice were injected IP at 12 days of age with MDSPCs expressing *LacZ* and euthanized 8-9 days later (n = 4) or injected at 17-19 days of age and tissues harvested at the end of their lifespan (4-9 wks of age; n = 4). The reporter MDSPCs were created by transducing them with a retroviral vector containing *LacZ* with a nuclear localization sequence[21]. Fourteen organs/tissues were isolated from each of the transplanted mice (see Supplementary Table S4), frozen in 2-methylbutane pre-cooled in liquid nitrogen, cryostat-sectioned (7 μm), and stained with X-gal to identify donor cells as well as hematoxylin and eosin to reveal tissue architecture. To isolate hematopoietic cells from the bone marrow, the femurs of each animal were dissected, muscle removed and the bone marrow was flushed from

the long bones using PBS. Red blood cells were removed using Red Blood Cell Lysing Buffer (Sigma-Aldrich). The cell suspension was centrifuged, counted and 2-3 x 10^5 cells in PBS (5 µl) were smeared over Precleaned Superfrost Plus (VWR) slides, air dried overnight, and stained for *LacZ* expression using X-gal (see the Histology section of Material and Methods). The sections were then stained with CD45 conjugated to FITC (1:300, BD Pahrminogen) for 20 min and evaluated by brightfield and fluorescence microscopy to detect CD45+ hematopoietic cells expressing *nLacZ*.

To determine if MDSPCs injected IM contribute to host muscle and vasculature (Fig. 8), 3 x 10^5 *LacZ*+ donor WT-MDSPCs were transplanted into the gastrocnemius muscles of 12-17 day-old *Ercc1*$^{-/-}$ mice. Mice were sacrificed 4-5 days post-transplantation, and the gastrocnemius muscles (n = 10) were harvested, frozen in 2-methylbutane pre-cooled in liquid nitrogen, and cryostat-sectioned (8 µm). For co-localizing blood vessels and *nLacZ*, sections were fixed with 4% PFA (paraformaldehyde) stained for rat anti-CD31 (1:300, BD Pharmingen) using Vectastain® *Elite* ABC kit (Vector Laboratories). Slides were developed using the peroxidase 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories). For brain, endogenous avidin/biotin was inhibited using a Vector Blocking kit (Vector Laboratories). Primary antibody was incubated overnight at 4°C in 10% rabbit serum containing 0.1% Triton in 1% BSA, and washing buffer used throughout the staining procedure was 0.1% Tween 20 in PBS.

SUPPLEMENTARY REFERENCES

60. Jankowski, R.J., Deasy, B.M., Cao, B., Gates, C. & Huard, J. The role of CD34 expression and cellular fusion in the regeneration capacity of myogenic progenitor cells. *J. Cell Sci.* **115**, 4361-4374 (2002).

61. Lavasani, M., Lu, A., Peng, H., Cummins, J. & Huard, J. Nerve growth factor improves the muscle regeneration capacity of muscle stem cells in dystrophic muscle. *Hum. Gene Ther.* **17**, 180-192 (2006).

62. Brooks, P.J. DNA repair in neural cells: basic science and clinical implications. *Mutat. Res.* **509**, 93-108 (2002).

63. Liou, Y.C. *et al.* Role of the prolyl isomerase Pin1 in protecting against age-dependent neurodegeneration. *Nature* **424**, 556-561 (2003).

64. Ferguson, V.L., Ayers, R.A., Bateman, T.A. & Simske, S.J. Bone development and age-related bone loss in male C57BL/6J mice. *Bone* **33**, 387-398 (2003).

65. Crawley, J.N. *What's wrong with my mouse?*, (New York: Wiley-Liss, 2000).