Title: p16^{INK4A}-expressing mesenchymal stromal cells restore the senescence–clearance–regeneration sequence that is impaired in chronic muscle inflammation

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Supplementary Materials and Methods

***In situ* hybridisation analysis by PrimeFlow™ RNA Assay.** Expression of *p16INK4A*, *OCT4*, and *TERT* mRNA was evaluated using the human PrimeFlow™ RNA assay (eBioscience). Briefly, MSCs or MSCs+PE were permeabilised, and then hybridised with type 1 Human CDKN2A Alexa Fluor® 647 (Affymetrix, cat. VA1-10040), type 6 OCT4 Human Alexa Fluor® 750 (Affymetrix, cat. VA6-17268), and type 6 TERT Human Alexa Fluor® 750 (Affymetrix, cat. VA6-17270) probes. After hybridisation, cells were analysed on a FACSCanto™ II flow cytometer (BD Biosciences). Data collection and analysis were conducted using the FACSDiva™ (BD Biosciences) and Kaluza V1.5a software (Beckman Coulter).

Preparation of culture supernatant. To prepare culture supernatant from MSCs or MSCs+PE, MSCs were cultured with or without PE. After 3 days, the cells were washed with PBS and cultured with DMEM lacking serum. Twenty-four hours later, the supernatant was collected and supplemented with 20% FBS (final concentration).

In-cell enzyme-linked immunosorbent assay (In-Cell ELISA). FAPs were cultured in 96-well plates and treated with culture supernatant from MSCs or MSCs+PE in

combination with vildagliptin (a DPP-4 inhibitor) or neutralising antibodies of SerpinE1(PAI-1) for 3 days. Cultured cells were fixed in 4% paraformaldehyde for 15 min at RT, washed in PBS-T, and incubated in 2% bovine serum albumin for 60 min at RT. After washing with PBS-T, the cells were incubated at 4°C overnight with anti-$p16^{INK4A}$ (CDKN2A) antibody (Proteintech), followed by secondary antibody staining. Nuclei were stained with DAPI (1:1000; Dojindo). Fluorescence intensities of $p16^{INK4A}$ staining were measured using a microplate reader (INFINITE M1000 PRO; Tecan Trading AG, Switzerland) and normalised against DAPI staining intensity.

Characterisation of MSCs. MSC and PE-treated MSCs were cultured on 8-well chamber slides for osteogenic and adipogenic differentiation, or in 15-mL conical tubes for chondrogenic differentiation, in DMEM containing 10% FBS and 1% penicillin–streptomycin. Culture was performed at 37°C in a humidified atmosphere containing 5% CO_2. Mesenchymal stem cell functional identification kits (R&D Systems, Minneapolis, MN, USA) were used to monitor induction of differentiation. Osteocytes, adipocytes, and chondrocytes were detected by alkaline phosphatase (ALP) staining, immunofluorescence staining with anti-FABP4 antibody, and immunofluorescence staining with anti-aggrecan antibody, respectively. For flow cytometry analysis, cultured

cells were trypsinised, resuspended in PBS with 2% FBS and 5 mM EDTA, stained for 15 min in Zombie Violet Dye (BioLegend, San Diego, CA, USA) diluted 1:100 in PBS, washed, and fixed with 4% PFA. Primary antibodies were PE-conjugated anti-CD90, CD73, CD40, CD45, and IgG control (Biolegend). The cells were incubated with primary antibodies for 30 min at 4°C in the dark. Stained cells were analysed on a FACSCanto II (BD Biosciences). For colony-forming unit assays, MSCs or PE-treated MSCs were cultured on 6-well plates (4,000 cells per well) in DMEM containing 10% FBS and 1% penicillin–streptomycin. After 10 days, the cells were Giemsa stained.

Supplemental Figure legend

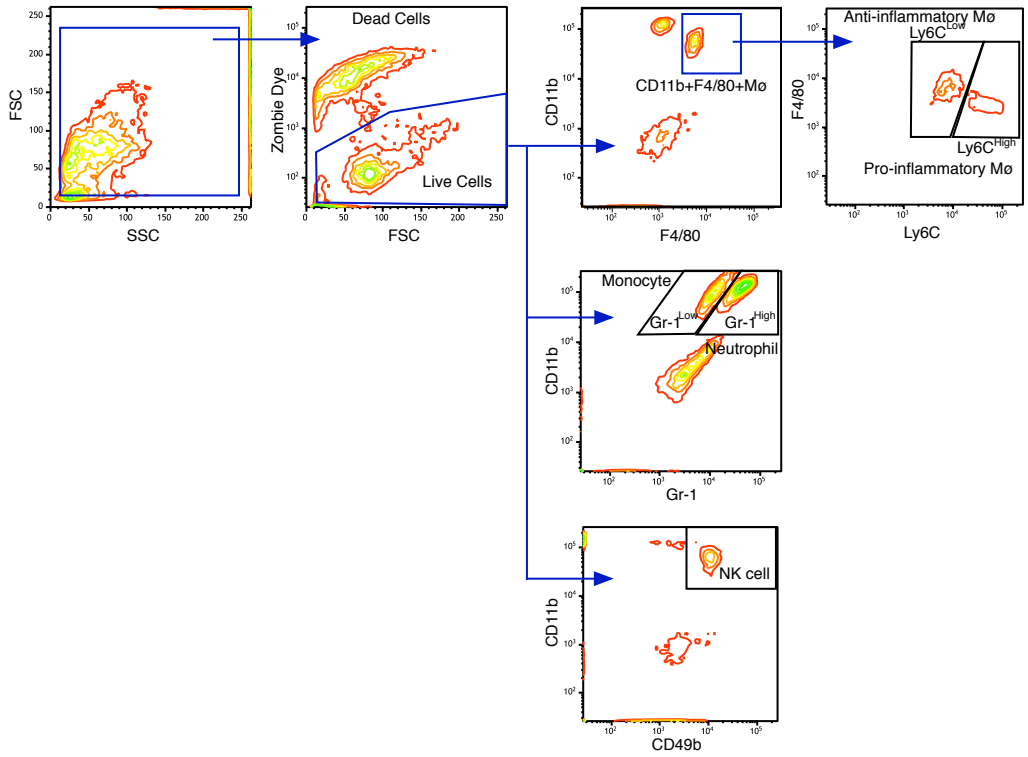


Fig. S1. Gating strategies used in flow cytometry experiments throughout the article.

Zombie dye–positive cells were gated out as dead cells.

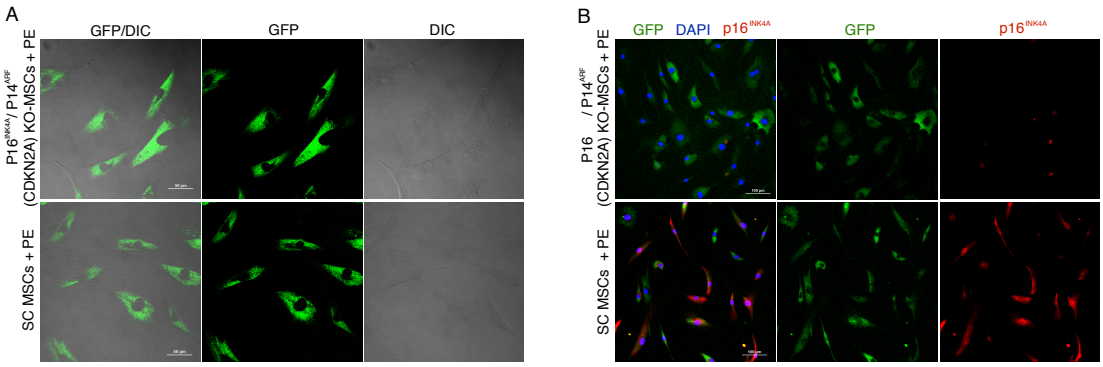


Fig. S2. Representative image of CDKN2A-KO MSCs+PE and scrambled control MSCs+PE. (a) GFP expression and differential interference contrast (DIC) microscopy and (b) GFP and p16INK4A expression in CDKN2A-KO MSCs+PE and scrambled controls.

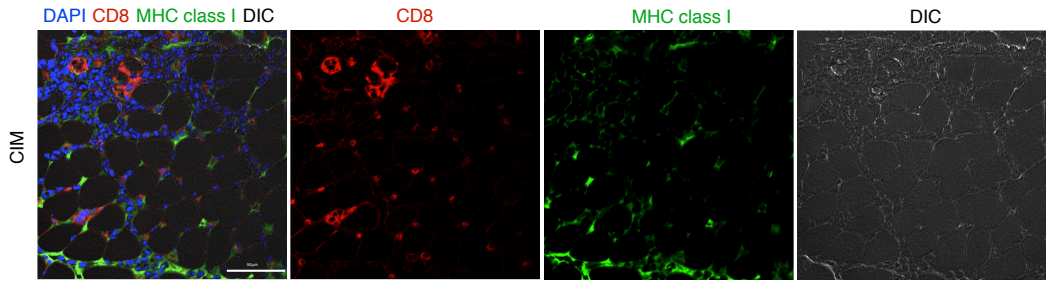


Fig. S3. Characteristics of CIM model mice. Shown are representative images of triceps surae immunostained for CD8 and MHC class 1.

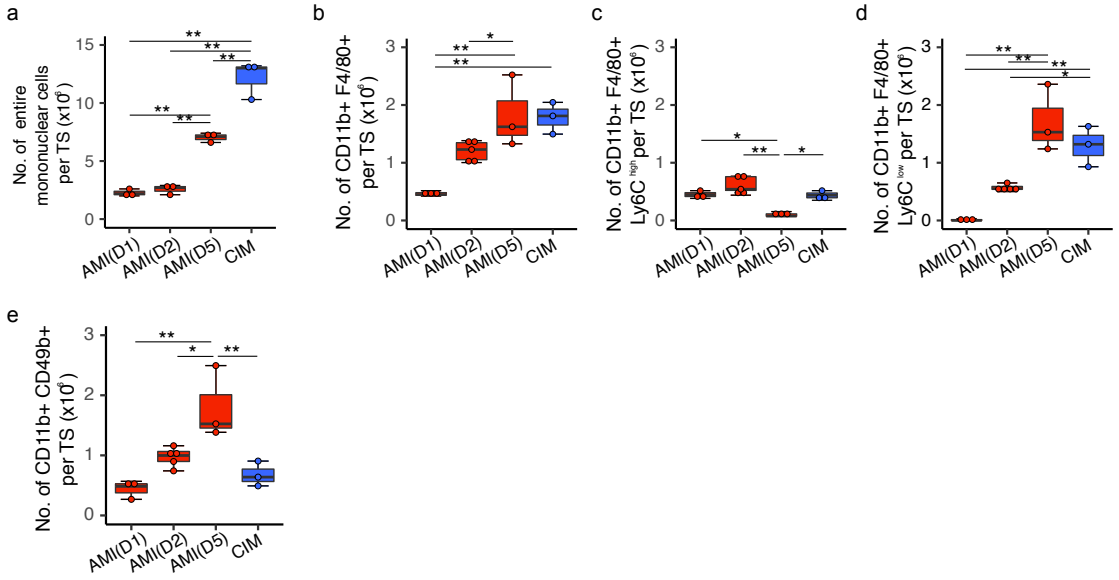
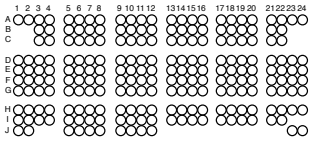


Fig. S4. Immune cell number in AMI and CIM mice, as determined by flow cytometry. (a) Total number of mononuclear cells in triceps surae; (b) CD11b+ F4/80+ macrophages; (c) CD11b+ F4/80+ Ly6C^{high} pro-inflammatory macrophages; (d) CD11b+ F4/80 Ly6C^{low} anti-inflammatory macrophages; and (e) CD11b+ CD49b NK cells in CIM and AMI (1, 2, and 5 days after injury). Quantitative data are shown as box-and-whisker plots with medians, interquartile range (IQR), and 1.5× IQR. P-values were determined by one-way ANOVA adjusted by Tukey's method. (*P < 0.05, **P < 0.001).

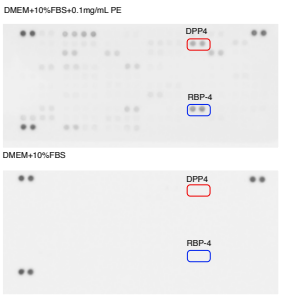
a



b

Coordinate	Analyte	Coordinate	Analyte	Coodinate	Analyte
A1, A2	Reference Spots	D1, D2	GROα	H1, H2	Myeloperoxidase
A3, A4	Adiponectin	D3, D4	Growth Hormone	H3, H4	Osteopontin
A5, A6	Apolipoprotein A-I	D5, D6	HGF	H5, H6	PDGF-AA
A7, A8	Angiogenin	D7, D8	ICAM-1	H7, H8	PDGF-AB/BB
A9, A10	Angiopoietin-1	D9, D10	IFN-γ	H9, H10	Pentraxin 3
A11, A12	Angiopoietin-2	D11, D12	IGFBP-2	H11, H12	PF4
A13, A14	BAFF	D13, D14	IGFBP-3	H13, H14	RAGE
A15, A16	BDNF	D15, D16	IL-1α	H15, H16	RANTES
A17, A18	Complement Component C5/C5a	D17, D18	IL-1β	H17, H18	RBP-4
		D19, D20	IL-1ra	H19, H20	Relaxin-2
A19, A20	CD14	D21, D22	IL-2	H21, H22	Resistin
A21, A22	CD30	D23, D24	IL-3	H23, H24	SDF-1α
A23, A24	Reference Spots	E1, E2	IL-4	I1, I2	Serpin E1
B3, B4	CD40 ligand	E3, E4	IL-5	I3, I4	SHBG
B5, B6	Chitinase 3-like 1	E5, E6	IL-6	I5, I6	ST2
B7, B8	Complement Factor D	E7, E8	IL-8	I7, I8	TARC
B9, B10	C-Reactive Protein	E9, E10	IL-10	I9, I10	TFF3
B11, B12	Cripto-1	E11, E12	IL-11	I11, I12	TfR
B13, B14	Cystatin C	E13, E14	IL-12 p70	I13, I14	TGF-α
B15, B16	Dkk-1	E15, E16	IL-13	I15, I16	Thrombospondin-1
B17, B18	DPPIV	E17, E18	IL-15	I17, I18	TNF-α
B19, B20	EGF	E19, E20	IL-16	I19, I20	uPAR
B21, B22	Emmprin	E21, E22	IL-17A	I21, I22	VEGF
C3, C4	ENA-78	E23, E24	IL-18 Bpa	J1, J2	Reference Spots
C5, C6	Endoglin	F1, F2	IL-19	J5, J6	Vitamin D BP
C7, C8	Fas Ligand	F3, F4	IL-22	J7, J8	CD31
C9, C10	FGF basic	F5, F6	IL-23	J9, J10	TIM-3
C11, C12	FGF-7	F7, F8	IL-24	J11, J12	VCAM-1
C13, C14	FGF-19	F9, F10	IL-27	J23, J24	Negative Controls
C15, C16	Flt-3 Ligand	F11, F12	IL-31		
C17, C18	G-CSF	F13, F14	IL-32		
C19, C20	GDF-15	F15, F16	IL-33		
C21, C22	GM-CSF	F17, F18	IL-34		
		F19, F20	IP-10		
		F21, F22	I-TAC		
		F23, F24	Kallikrein 3		
		G1, G2	Leptin		
		G3, G4	LIF		
		G5, G6	Lipocalin-2		
		G7, G8	MCP-1		
		G9, G10	MCP-3		
		G11, G12	M-CSF		
		G13, G14	MIF		
		G15, G16	MIG		
		G17, G18	MIP-1α/MIP-1β		
		G19, G20	MIP-3α		
		G21, G22	MIP-3β		
		G23, G24	MMP-9		

c



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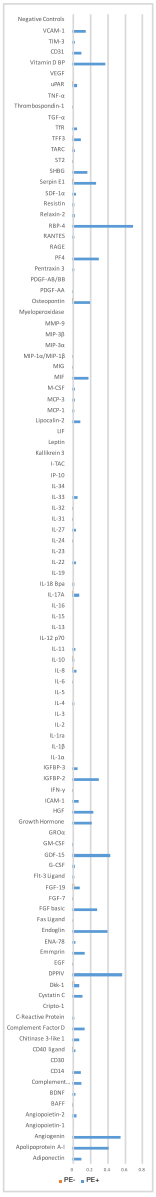


Fig. S5. PE contains various cytokines. (a) Human XL Cytokine Array coordinates and (b) names of cytokines represented on the array. (c–d) Proteome array analysis of DMEM + 10% FBS + PE and DMEM + 10% FBS containing protein, and relative expression levels of cytokines in DMEM + 10% FBS (PE-) and DMEM + 10% FBS + PE (PE+).

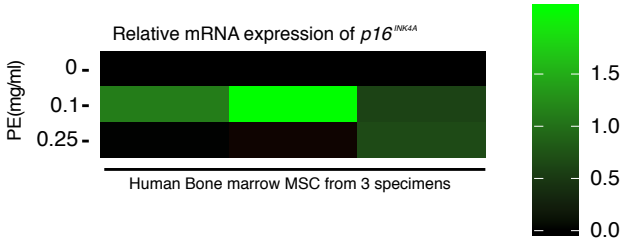


Fig. S6. Heat map of p16^{INK4A} gene expression when MSCs were cultured with 0, 0.1, or 0.25 mg/ml PE in the culture media. p16^{INK4A} gene expression increased in MSCs exposed to 0.1 mg/ml PE.

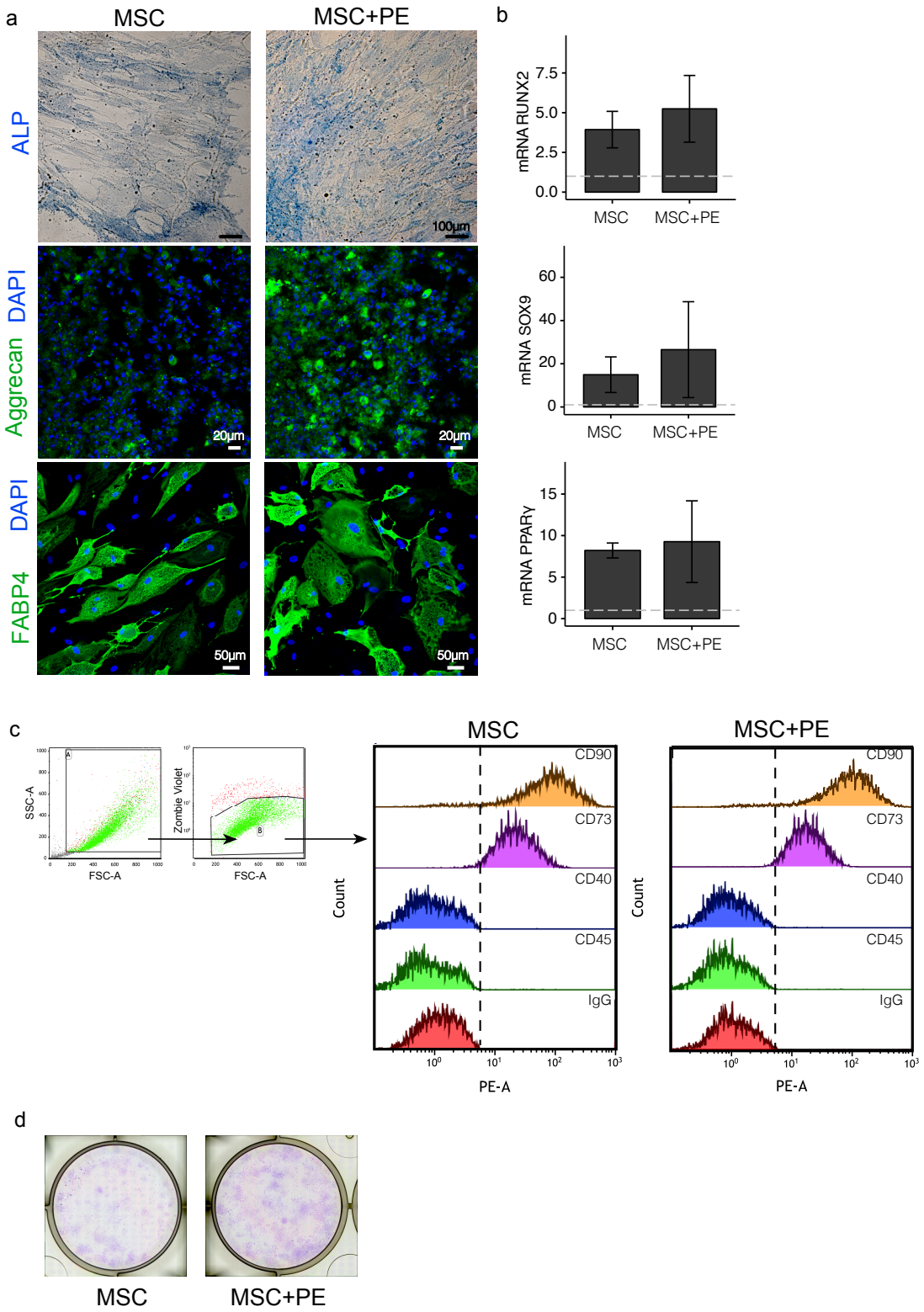


Fig. S7. Characterisation of MSC with or without PE treatment. (a) Multi-lineage

differentiation of MSCs with or without PE treatment. Osteocytes, chondrocytes, and adipocytes were detected by alkaline phosphatase (ALP) staining, immunofluorescence staining with anti-aggrecan antibody, and immunofluorescence staining with anti-FABP4 antibody, respectively. Representative images of three independent experiments are shown. (b) mRNA expression of RUNX2, SOX9, and PPARγ after induction of differentiation of MSCs with or without PE treatment. (n = 3) (c) Flow cytometry analysis of CD90, CD73, CD40, and CD45 expression in MSCs, with or without PE treatment. (d) Colony-forming unit assay of MSCs with or without PE treatment. Representative images of three independent experiments are shown.

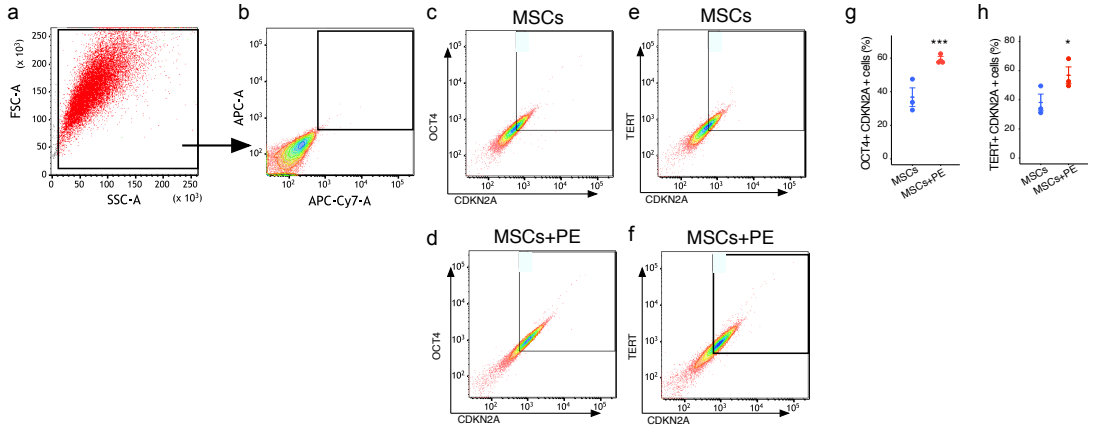


Fig. S8. Expression of OCT4, CDKN2A, and TERT increased in MSCs+PE, as determined by *in situ* hybridisation analysis using the PrimeFlow™ RNA Assay. (a and b) Positive gates were set by analysing the negative control sample. (c-f) Flow cytometry analysis of OCT4 and CDKN2A (c and d), and TERT and CDKN2A (e and f). (g and h) Percentage of the $OCT4^+$ $CDKN2A^+$ cells in MSCs and MSCs+PE (n = 3 per group) (g), and percentage of $TERT^+$ $CDKN2A^+$ cells in MSCs and MSCs+PE (n = 3 per group) (h).

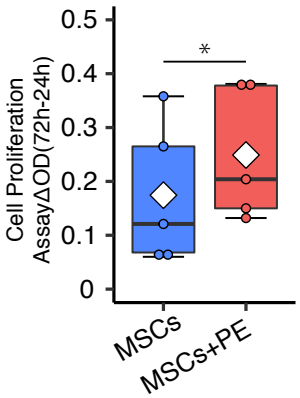
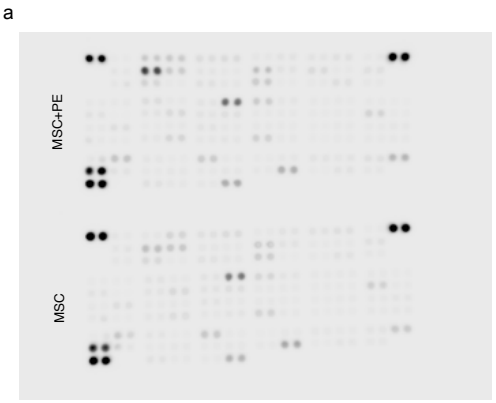
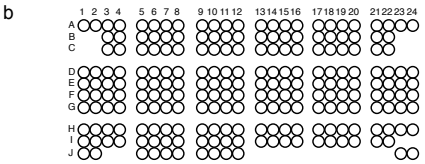


Fig. S9. Cell proliferation assay in MSCs and MSCs+PE. (a) Cell proliferation assay in MSCs, with or without PE treatment. Quantitative data are shown as box-and-whisker plots with medians, interquartile range (IQR), and 1.5× IQR. P-values were determined by two-tailed Student's t-test (*P < 0.05).





c

Coordinate	Analyte	Coordinate	Analyte	Coodinate	Analyte
A1, A2	Reference Spots	D1, D2	GROα	H1, H2	Myeloperoxidase
A3, A4	Adiponectin	D3, D4	Growth Hormone	H3, H4	Osteopontin
A5, A6	Apolipoprotein A-I	D5, D6	HGF	H5, H6	PDGF-AA
A7, A8	Angiogenin	D7, D8	ICAM-1	H7, H8	PDGF-AB/BB
A9, A10	Angiopoietin-1	D9, D10	IFN-γ	H9, H10	Pentraxin 3
A11, A12	Angiopoietin-2	D11, D12	IGFBP-2	H11, H12	PF4
A13, A14	BAFF	D13, D14	IGFBP-3	H13, H14	RAGE
A15, A16	BDNF	D15, D16	IL-1α	H15, H16	RANTES
A17, A18	Complement Component C5/C5a	D17, D18	IL-1β	H17, H18	RBP-4
		D19, D20	IL-1ra	H19, H20	Relaxin-2
A19, A20	CD14	D21, D22	IL-2	H21, H22	Resistin
A21, A22	CD30	D23, D24	IL-3	H23, H24	SDF-1α
A23, A24	Reference Spots	E1, E2	IL-4	I1, I2	Serpin E1
B3, B4	CD40 ligand	E3, E4	IL-5	I3, I4	SHBG
B5, B6	Chitinase 3-like 1	E5, E6	IL-6	I5, I6	ST2
B7, B8	Complement Factor D	E7, E8	IL-8	I7, I8	TARC
B9, B10	C-Reactive Protein	E9, E10	IL-10	I9, I10	TFF3
B11, B12	Cripto-1	E11, E12	IL-11	I11, I12	TfR
B13, B14	Cystatin C	E13, E14	IL-12 p70	I13, I14	TGF-α
B15, B16	Dkk-1	E15, E16	IL-13	I15, I16	Thrombospondin-1
B17, B18	DPPIV	E17, E18	IL-15	I17, I18	TNF-α
B19, B20	EGF	E19, E20	IL-16	I19, I20	uPAR
B21, B22	Emmprin	E21, E22	IL-17A	I21, I22	VEGF
C3, C4	ENA-78	E23, E24	IL-18 Bpa	J1, J2	Reference Spots
C5, C6	Endoglin	F1, F2	IL-19	J5, J6	Vitamin D BP
C7, C8	Fas Ligand	F3, F4	IL-22	J7, J8	CD31
C9, C10	FGF basic	F5, F6	IL-23	J9, J10	TIM-3
C11, C12	FGF-7	F7, F8	IL-24	J11, J12	VCAM-1
C13, C14	FGF-19	F9, F10	IL-27	J23, J24	Negative Controls
C15, C16	Flt-3 Ligand	F11, F12	IL-31		
C17, C18	G-CSF	F13, F14	IL-32		
C19, C20	GDF-15	F15, F16	IL-33		
C21, C22	GM-CSF	F17, F18	IL-34		
		F19, F20	IP-10		
		F21, F22	I-TAC		
		F23, F24	Kallikrein 3		
		G1, G2	Leptin		
		G3, G4	LIF		
		G5, G6	Lipocalin-2		
		G7, G8	MCP-1		
		G9, G10	MCP-3		
		G11, G12	M-CSF		
		G13, G14	MIF		
		G15, G16	MIG		
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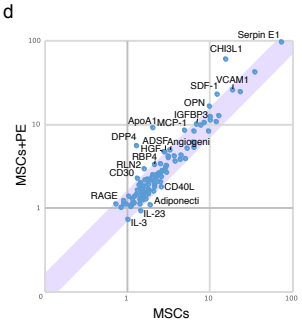

Fig. S10. Proteome array analysis of supernatant cytokines of MSC cultures. (a) Proteome array analysis of cytokines in culture supernatants of MSCs with or without PE. (b) Human XL Cytokine Array coordinates and (c) names of cytokines represented on the array. (d) Scatter plots of changes in protein expression in MSC cultures with or without PE supplementation.

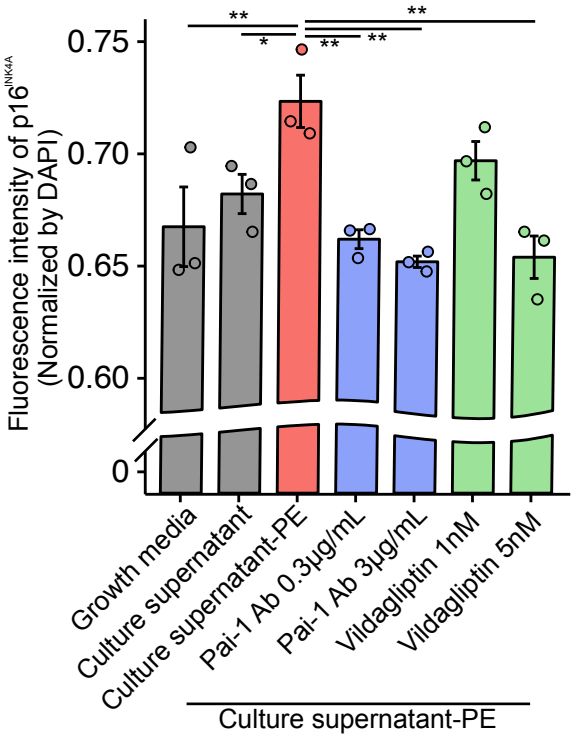

Fig. S11. Induction of FAP senescence by culture supernatant of MSCs or MSCs+PE.

(a) Fluorescence intensity of p16INK4A in FAPs treated with culture supernatant of MSCs or MSCs+PE in combination with vildagliptin, a DPP-4 inhibitor or neutralising antibodies of PAI-1. Quantitative data are shown as means ± SEM (dot plot). P-values were determined by one-way ANOVA, adjusted by Tukey's method. (*P < 0.05, **P < 0.001).

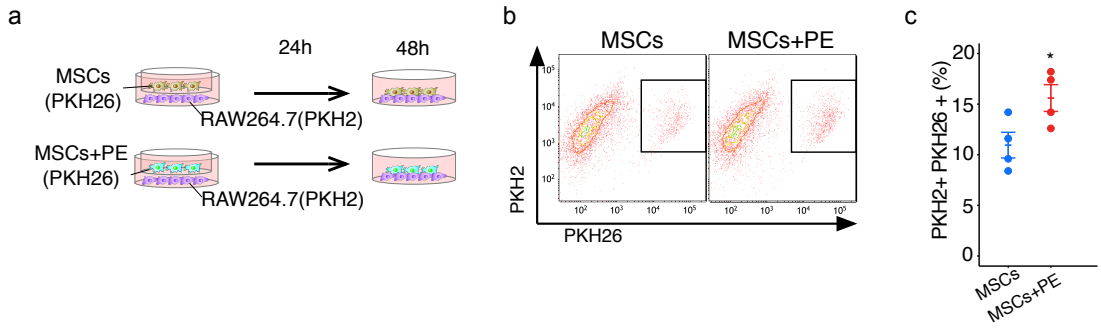

Fig. S12. Phagocytosis assay (a) Procedure for co-culture of MSCs or MSCs+PE with RAW264.7 macrophages. (b) Flow cytometry analysis of PKH2-labelled RAW264.7 and PKH26-labelled MSCs or MSCs+PE. (c) Cells engulfing PKH26-positive cells were identified as PKH2-labelled RAW264.7 macrophages, and the percentage of $PKH2^{+}$ $PKH26^{+}$ cells was determined (n = 3 per group). Quantitative data are shown as means ± SEM (dot plot). P-values were determined by two-tailed Student's t-test (*$P < 0.05$).

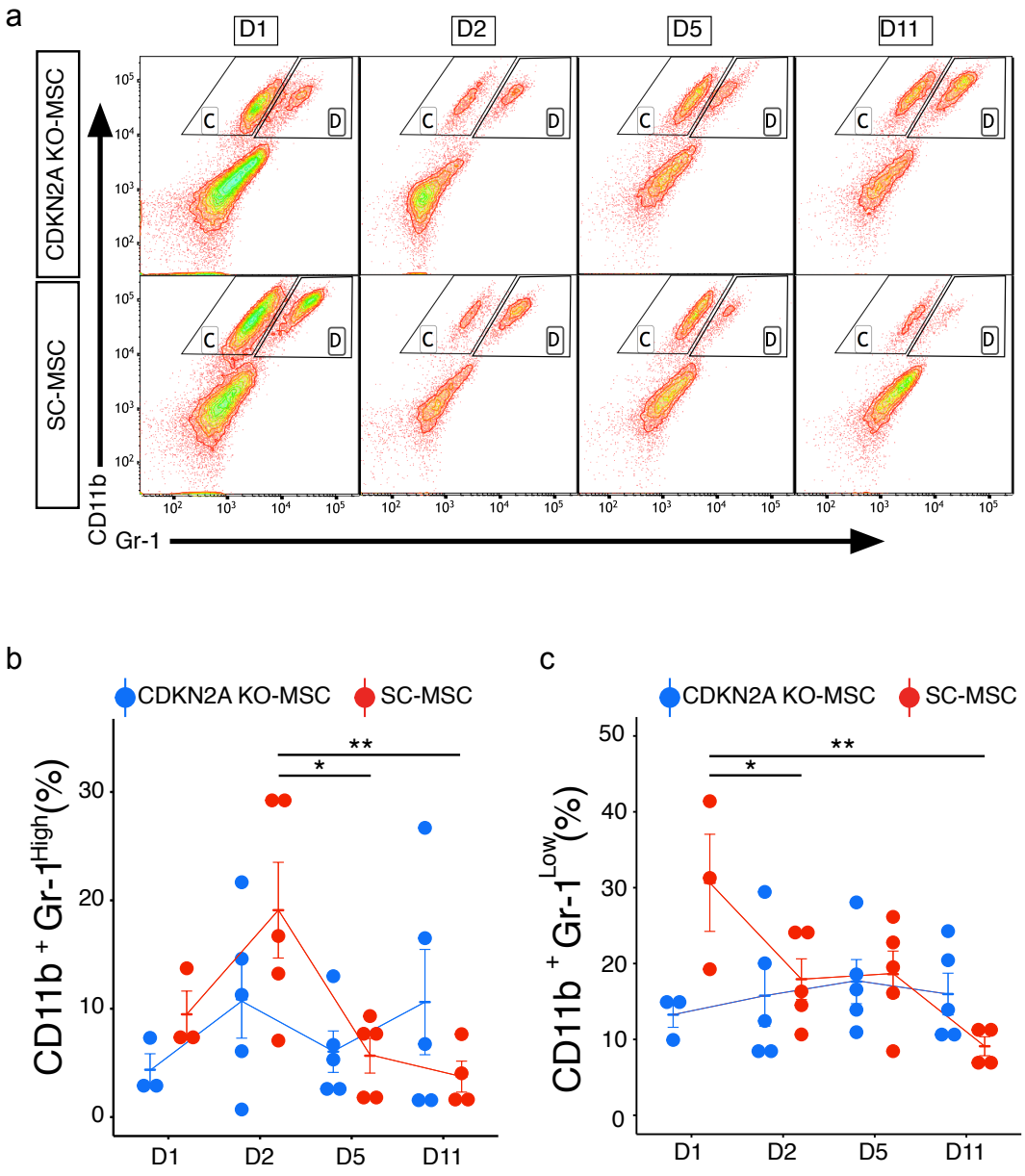

Fig. S13. CD11b^{+}Gr1^{+} cells after CDKN2A KO-MSC transplantation. (a) Representative CD11b and Gr-1 counter plot in CIM (1, 2, 5, and 11 days after CDKN2A KO-MSC or scramble control (SC)-MSC injection). (b) Percentage of CD11b+ Gr-1^{high} neutrophils and CD11b^{+} Gr-1^{low} immature myeloid cells. Quantitative data with dot plots are shown as means ± SEM. P-values were determined by one-way ANOVA, adjusted by

Tukey's method. (*P < 0.05, **P < 0.001).

Supplementary tables

Table 1

Primary antibodies Antibody	Clone	Conjugate(s)	Dilution	Source
Flow cytometry				
rat anti-CD45	30-F11	FITC	1:200	Biolegend
rat anti-CD11b	M1/70	PE/Cy7	1:80	Biolegend
rat anti-Gr-1	RB6-8C5	PerCP/Cy5.5	1:80	Biolegend
rat anti-F4/80	BM8	Alexa Fluor APC	1:80	Biolegend
rat anti-CD31	390	FITC	1:80	Biolegend
rat anti-CD49b	DX5	PE	1:200	Biolegend
rat anti-Ly-6C	HK1.4	FITC	1:200	Biolegend
rat anti-integrin α7	3C13	PE	1:20	Miltenyi Biotec
rabbit anti-p16INK4A	Polyclonal	unconjugated	1:200	ProteinTech
Immunohistochemistry and Immunofluorescence				
rabbit anti-YAP	63.7	unconjugated	1:100	Santa Cruz Biotechnology
rabbit anti-p16INK4A	Polyclonal	unconjugated	1:200	ProteinTech
rabbit anti-OCT4	Polyclonal	unconjugated	1:100	Abcam
rabbit anti-phospho-LATS1/2	Polyclonal	unconjugated	1:50	Sigma Aldrich
rabbit anti-YAP1(phosho-Ser127)	EP1675Y	unconjugated	1:250	Abcam
F-actin	-	Phalloidin-iFluor 488 reagent	1:1000	Abcam
rabbit anti-laminin	Polyclonal	unconjugated	1:100	Abcam
rat anti-CD8a	53-6.7	unconjugated	1:80	Biolegend

mouse anti-H-2Kd	SF1-1.1	biotin	1:80	Biolegend
rat anti-CD140a	APA5	unconjugated	1:50-200	Biolegend
rat anti-CD49b	DX5	Alexa Fluor 488	1:200	Biolegend
rat anti-F4/80	BM8	Alexa Fluor 488	1:80	Biolegend

Secondary reagents

Antibody or reagent	Conjugate(s)	Source
goat polyclonal anti-rabbit IgG	Alexa Fluor 488, Alexa Fluor 647, Cy3	Jackson ImmunoResearch
goat polyclonal anti-rat IgG	Alexa Fluor 488, Alexa Fluor 647, Cy3	Jackson ImmunoResearch
Streptavidin	Alexa Fluor 647	Jackson ImmunoResearch
Donkey polyclonal anti-rabbit IgG	PE	Biolegend

Table 2

Specific primer sequences used for real-time PCR

Gene	Forward Sequence	Reverse Sequence	Size (bp)	Accession Number
POU5F1 (OCT4)	5' GACAGGGGAGGGGAGGAG 3'	5' CTTCCCTCCAACCAGTTGCCC 3'	143	NM_001159542
SOX2	5' GGGAAATGGGAGGGGTGCAA 3'	5' TTGCGTGAGTGTGGATGGGA 3'	151	NM_003106
NANOG	5' TGGACACTGGCTGAATCCTTC 3'	5' CGTTGATTAGGCTCCAACCAT 3'	142	NM_024865
DNMT1	5' CGTAAAGAAGAATTATCCGAGG 3'	5' GTTTTCTAGACGTCCATTCAC 3'	123	NM_001130823
p16INK4A (CDKN2A)	5' AGCATGGAGCCTTCGGCTGA 3'	5' CCATCATCATGACCTGGATCG 3'	142	NM_000077
p14ARF (CDKN2A)	5' TACTGAGGAGCCAGCGTCTA 3'	5' TGCACGGGTCGGGTGAGAGT 3'	146	NM_058195
p21 (CDKN1A)	5' GAGACTCTCAGGGTCGAAAA 3'	5' TTAGGGCTTCCTCTTGGAGA 3'	92	NM_000389
TP53	5' TGACTGTACCACCATCCACTA 3'	5' AAACACGCACCTCAAAGC 3'	143	NM_000546
RB1	5' GCTAGCCTATCTCCGGCTAAA 3'	5' CTGGAAAAGGGTCCAGATGA 3'	88	NM_000321
TERT	5' CGGAAGAGTGTCTGGAGC 3'	5' GGATGAAGCGGAGTCTGGA 3'	145	NM_198253
TSG-6	5' CCCATTGTGAAGCCAGGGCCCAACTG 3'	5' GGAAGCTCATCTCCACAGTATCTTCCC 3'	362	NM_007115
IDO1	5' GCATTTTTCAGTGTTCTTCGCATA 3'	5' TCATACACCAGACCGTCTGATAGC 3'	77	NM_002164
IL-6	5' GATGAGTACAAAAGTCCTGATCCA 3'	5' CTGCAGCCACTGGTTCTGT 3'	130	NM_000600
α-SMA (ACTA2)	5' GCAGCCCAGCCAAGCACTGT 3'	5' TGGGAGCATCGTCCCCAGCA 3'	135	NM_001613
CD47	5' AGAAGGTGAAACGATCATCGAGC 3'	5' CTCATCCATACCACCGGATCT 3'	160	NM_001777
PPARγ (PPARG)	5' GACTTCTCCAGCATTTCTAC 3'	5' TCCACTTTGATTGCACTTTG 3'	122	XM_024453606
RUNX2	5' AAGCTTGATGACTCTAAACC 3'	5' TCTGTAATCTGACTCTGTCC 3'	164	NM_001024630
SOX9	5' CATGAGCGAGGTGCACTCC 3'	5' TCGCTTCAGGTCAGCCTTG 3'	112	NM_000346
18S rRNA	5' ATCGGGGATTGCAATTATTC 3'	5' CTCACTAAACCATCCAATCG 3'	130	X03205
GAPDH	5' ATTGCCCTCAACGACCACTT 3'	5' TGCTGTAGCCAAATTCGTTGTC 3'	64	NM_002046