Developmental Cell, Volume *31*

Supplemental Information

An Essential Role for Senescent Cells in Optimal Wound Healing through Secretion of PDGF-AA

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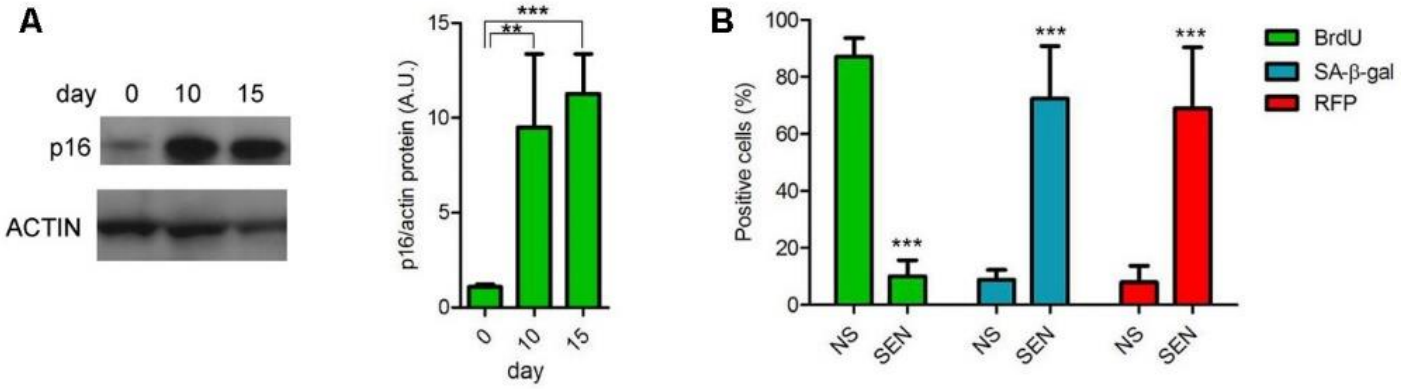


Figure S1. Related to Figure 1. Characterization of p16-3MR cells. (A) p16-3MR MEFs were induced to senescence by X-irradiation (IR; 10 Gy). $p16^{INK4a}$ (p16) protein levels were measured by immunoblotting on the indicated days after IR. Actin served as a loading control. Values were quantified using Image J (right panel). (B) MEFs were induced to senescence by IR (SEN) or mock irradiated (NS). 10 d after irradiation, the cells were assayed for BrdU incorporation (after a 24 hour pulse), SA-β-gal activity and RFP fluorescence, and the percentage of positive cells was determined. N=3. Data shown are the mean ± SD. **p<0.01; ***p<0.001.

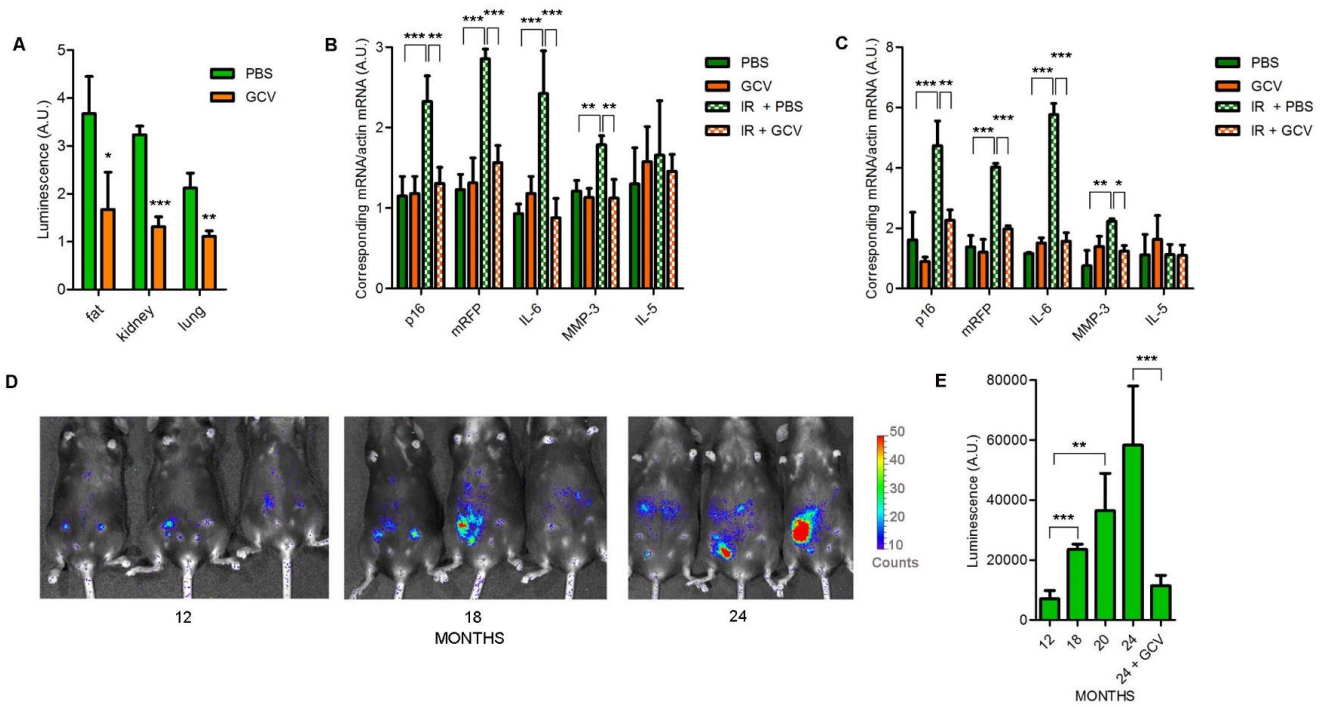


Figure S2. Related to Figure 2. Characterization of p16-3MR mice. (A-C) p16-3MR mice were X-irradiated (7 Gy whole body). 90 d later, they were treated with GCV or PBS for 5 d (25 mg/kg; daily i.p. injections). N=3. Mice were i.p. injected with coelentarazine and visceral fat, kidneys and lungs were excised and analyzed for luminescence using a Xenogen imager. (B,C) qRT-PCR was used to quantify levels of mRNAs encoding $p16^{INK4a}$, mRFP, the SASP factors IL-6 and MMP-3, and the non SASP factor IL-5 in kidneys (B) and lungss (C). Actin was used to control for RNA quantity. (D-E) p16-3MR mice were injected i.p. with coelentarazine at the indicated ages and luminescence was measured. (D) shows a representative image, (E) shows quantification of the luminescence. A cohort of 24 mo old mice (N=5) was treated with GCV (5 daily i.p. injections; 25 mg/kg), after which luminescence was measured. A.U.=arbitrary units. Data shown are the mean ± SD. *p<0.05; **p<0.01; ***p<0.001.

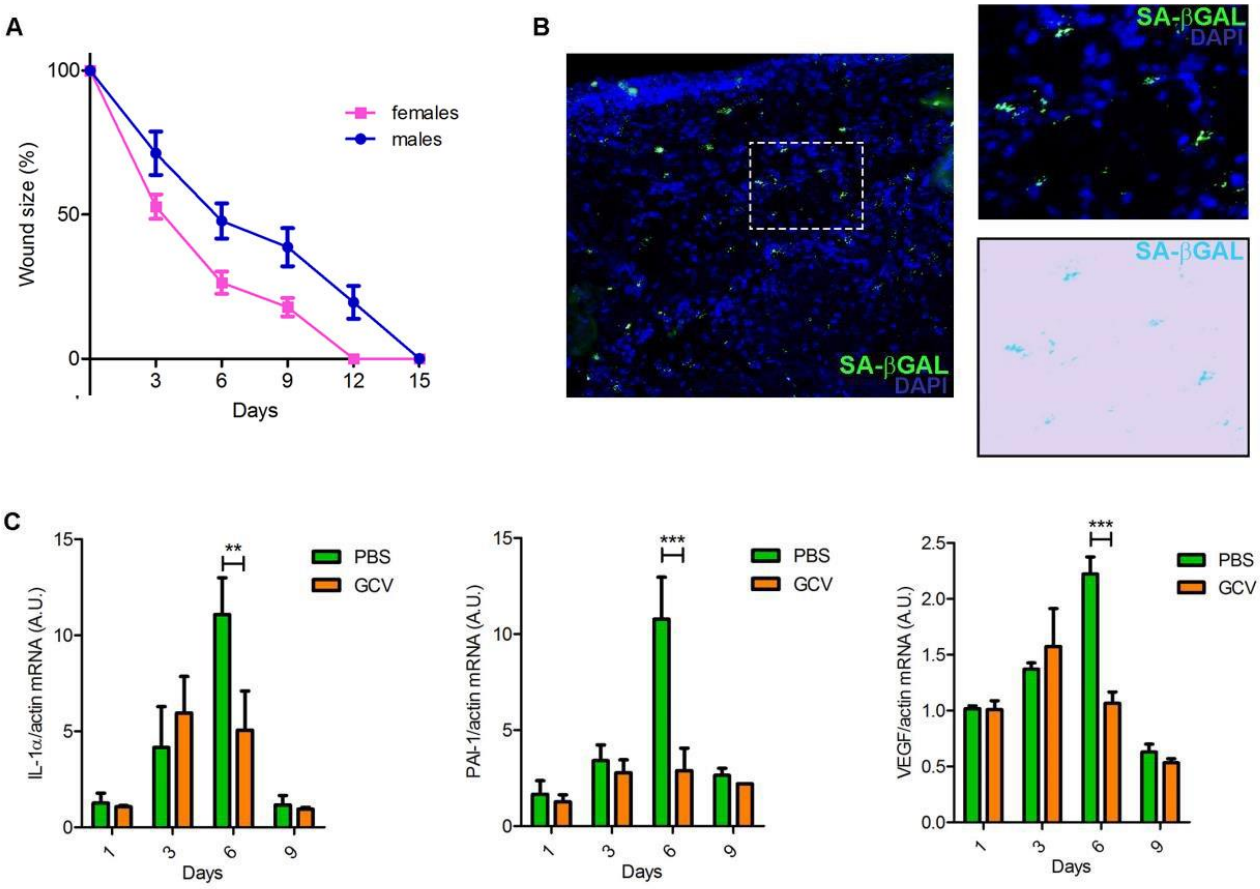
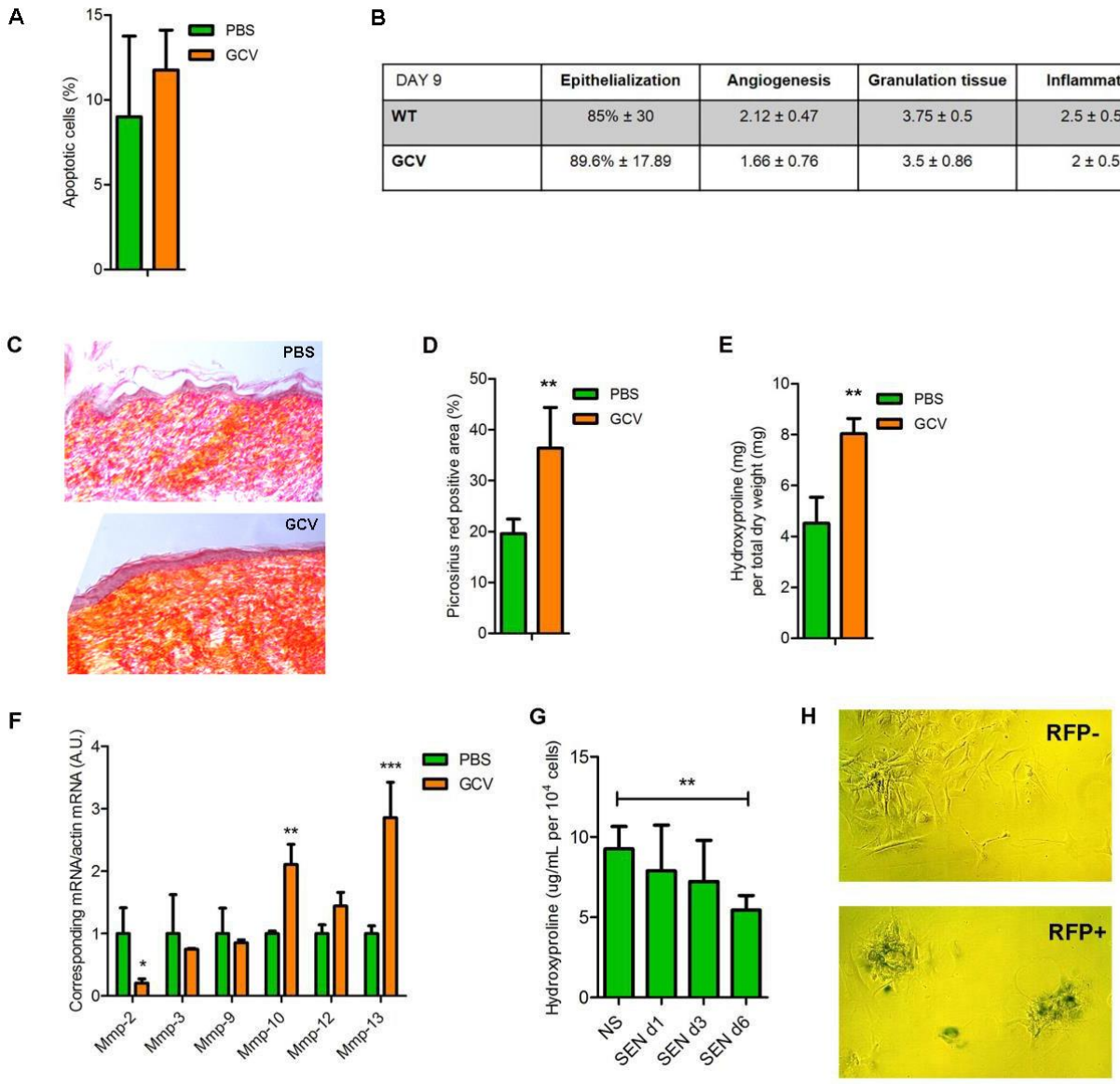


Figure S3. Related to Figure 3. Senescent cells are induced and necessary for optimal cutaneous wound healing. (A) 3-4 mos old female or male mice were wounded using a 6 mm punch to dorsal skin, and the size of the wound measured at the indicated day post-injury using a caliper. N=6. (B) Skin sections were collected 6 d after wounding female p16-3MR mice, fixed and stained for SA-β-gal (green, false-color image reflecting the negative of the original staining) and counterstained with DAPI (blue). Inset shows higher magnification (400x) of both the negative and the original (bright field) staining. (C) mRNA levels of the indicated genes were quantified by qRT-PCR from skin biopsies excised from PBS or GCV-treated wounds to p16-3MR mice at the indicated intervals after injury. Actin was used to control for cDNA quantity. N=4. Data shown are the mean ± SD. **p<0.01; ***p<0.001.



DAY 9	Epithelialization	Angiogenesis	Granulation tissue	Inflammation
WT	85% ± 30	2.12 ± 0.47	3.75 ± 0.5	2.5 ± 0.57
GCV	89.6% ± 17.89	1.66 ± 0.76	3.5 ± 0.86	2 ± 0.5

Figure S4. Related to Figure 4. Characterization of senescent cells induced during wound healing. (A) Longitudinal sections of wounds collected 6 d after injury were assayed for apoptosis by TUNEL staining. (B) Longitudinal sections of wounds collected 9 d after injury were evaluated as in Figure 4A-C. (C) Skin biopsies were collected 15 d after wounding, paraffin embedded and stained with picrosirius red (orange color) to visualize collagen deposition. Micrographs were taken at 100X magnification. (D) The area of positive picrosirius red staining was calculated using ImageJ software, and is expressed as a percentage of the entire section. (E) Skin biopsies were collected 15 d after wounding were lysed and assayed for hydroxyproline content as an indicator of collagen production. (F) Skin biopsies were excised from PBS- and GCV-treated wounds to p16-3MR mice 6 d after injury, and analyzed for mRNA levels of the indicated genes encoding proteases by qRT-PCR. Actin was

used to control for cDNA quantity. (G) Dermal fibroblasts from p16-3MR mice were induced to senescence by x- irradiation and hydroxyproline content of the conditioned media was measured at varying intervals after irradiation (NS = non-senescent; d1 = day 1; d3 = day 3; d6 = day 6). (H) Cells isolated from wounds of p16-3MR mice 6 d after injury were sorted for RFP, fixed and stained for SA-β-gal. N=4. Data shown are the mean ± SD. *p<0.05; **p<0.01; ***p<0.001.

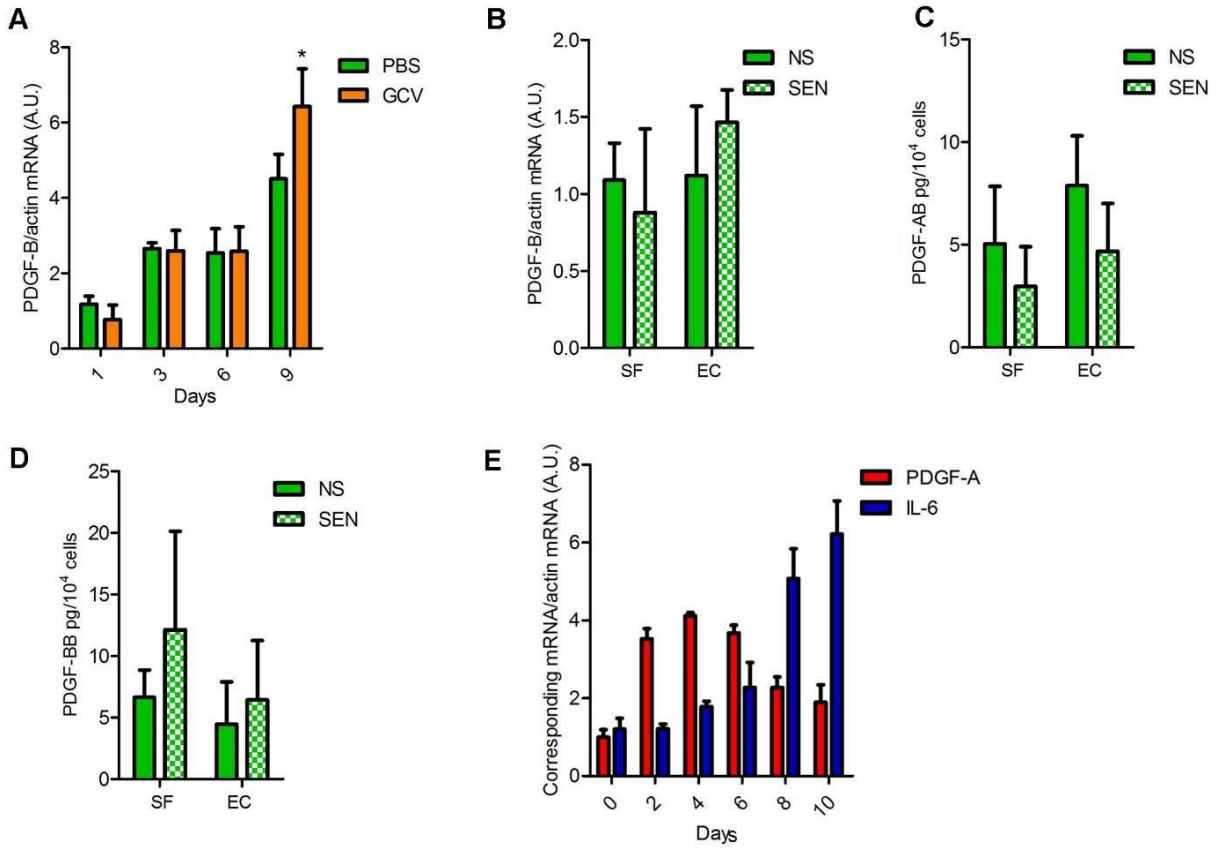


Figure S5. Related to Figure 5. PDGF-A is expressed and secreted by senescent cells.

(A) Skin biopsies excised from PBS- or GCV-treated wounds to p16-3MR mice were analyzed at the indicated intervals after injury for levels of mRNA encoding PDGF-B by qRT- PCR. Actin was used to control for cDNA quantity. (B-D) Murine skin fibroblasts (SF) and endothelial cells (EC) were mock irradiated (NS) or made senescent by X-irradiation (SEN; 10 Gy X-ray). N=4. (B) qRT-PCR was used to quantify PDGFB mRNA relative to actin mRNA levels. A.U.=arbitrary units. (C-D) PDGF-AB and PDGF-BB levels in conditioned media were quantified by ELISA and normalized to cell number. (E) MEFs were irradiated (IR; 7Gy) and samples collected at the indicated times after irradiation. qRT-PCR was used to quantify PDGFA and IL-6, relative to actin, mRNA levels. A.U.=arbitrary units. N=3. Data shown are the mean ± SD. *p<0.05.

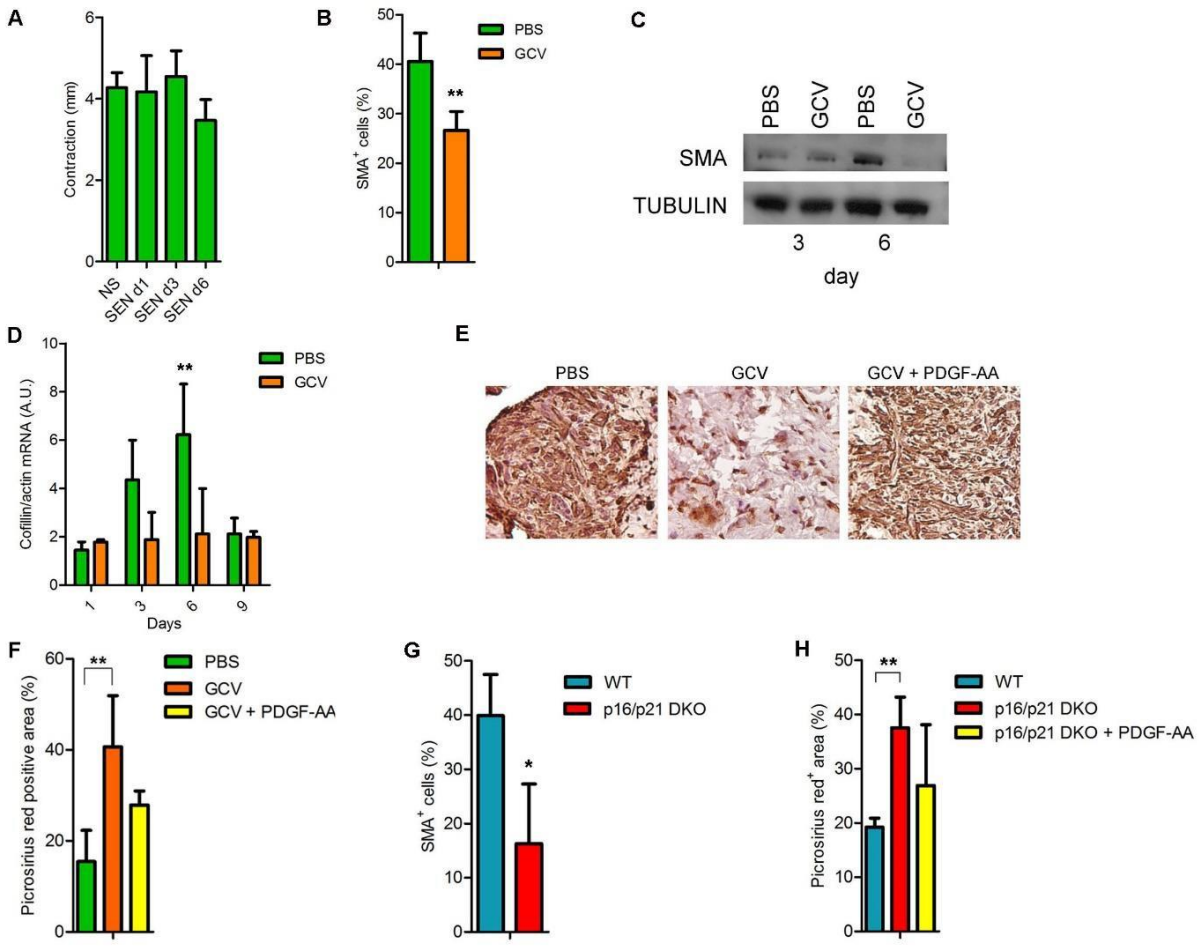


Figure S6. Related to Figure 6. Senescence-associated PDGF-A drives myofibroblast differentiation. (A) Dermal fibroblasts from p16-3MR mice were induced to senescence by X-irradiation and assessed for their ability to contract a collagen gel at the indicated intervals after irradiation (NS = non-senescent; d1 = day 1; d3 = day 3; d6 = day 6 after irradiation). N=4. (B) Paraffin-embedded longitudinal sections of wounds collected 6 d after wounding were immunostained for smooth muscle actin (SMA). The graph shows the percentage of SMA- positive cells compared to the total number of (FastRED-positive) cells in sections collected 6 d after wounding. N=5. (C) Levels of SMA were measured by immunoblotting at the indicated days after wounding. Tubulin served as a loading control. (D) mRNA levels of cofillin were quantified by qRT-PCR from skin biopsies excised from PBS or GCV-treated wounds to p16-3MR mice at the indicated intervals after injury. Actin was used to control for cDNA quantity. (E-F) Mice were injected with PBS, GCV or GCV followed by topical application of recombinant PDGF-AA. N=6. (E) Wounds were collected 6 d after injury and immunostained for SMA. (F) Skin biopsies collected 14 d after wounding were paraffin-embedded and stained with picrosirius red to detect collagen deposition. (G) Skin biopsies collected 6 d after injury to

WT or p16/p21 DKO mice were immunostained for SMA and nuclei were stained with FastRED. The graph shows the percentage of SMA-positive cells compared to total number of (FastRED-positive) cells. N=4. (H) Collagen deposition was measured as described in Fig S4D. Positive areas were quantified using ImageJ and expressed as percentage of the entire section. N=3. Data shown are the mean ± SD. *$p<0.05$; **$p<0.01$.

Supplemental Experimental procedures

Generation of p16-3MR mouse model**.** The 3MR cDNA (PMID: 14973078) was inserted into a bacterial artificial chromosome (BAC) containing the $p16^{INK4a}$ locus (BAC clone RP24-322D20 from the RPCI-24 C57BL/6J male *mus musculus* BAC library http://bacpac.chori.org/library.php?id=12). In addition to the $p16^{INK4a}$ locus, this clone contains the 3' end of the Mtap gene (lacking the promotor and 5' exons) but no other annotated full- length genes. The 3MR cDNA was inserted in frame with p16 into exon 2, creating a fusion protein containing the first 62 amino acids of p16, but no full-length WT p16 protein. Insertion of the 3MR cDNA also resulted in a stop codon in the $p19^{ARF}$ reading frame in exon 2, thereby preventing full-length $p19^{ARF}$ expression from the BAC. In a subsequent step, a neomycin resistance marker was inserted into the BAC backbone in the bacterial SacB gene using γ Red- mediated recombination (PMID: 10037821). The resulting p16-3MR-Neo BAC was linearized by PI-SceI digestion and electroporated into Bl6/129 ES cells. The Neo cassette was not removed after electroporation. Clones surviving G418 selection were screened for $p16^{INK4a}$ copy number by qPCR, karyotyped, and one clone (F7) was injected into blastocysts to generate chimeric animals. A single integration event of likely one functional copy of the BAC occurred in the central region of chromosome 13 in this clone. A transgenic founder was used to backcross the transgene onto the C57Bl6/J background using the speed congenic approach.

Detailed construction of p16-3MR BAC

1.Insertion of 3MR cDNA into p16 BAC

A 4 kb NheI fragment (restriction sites in red) with an internal BstB1 site (bold red, located in p16 exon 2) for insertion of 3MR cDNA was subcloned from RPCI-24-322D20 into pcDNA3.1 to provide 5' and 3' homology arms for RecA-mediated recombination into the parental BAC:

gctagccttcacaatttaaacttgagacttgttttgtgttcgttcgttctttttctttctttctttctttctctcttcttcttttttctttttttctttttttctttttattttttga
gacagggtttctctgtgtagccctggctgtcctggaactcactctgtagacttattactgttgtaacaaatgataagttttccttttatgtaaaata
gtttttgtcatataacacatcctgattacagtttccctcctagtccttcccctcctcccctcttttagtttggaaggggtgggggtctcatgcagct
cacattggcttccaggacaatatctgtacctgaaggtgactttgaaattctaaacctcctgcctctacaccctgggtgttgaggatacaggta
tgcagggttcctgcagtgttgcagtttgaacccagagtctctttcatatgctaggtcttataggtggttttacatgacaaactagagtaatttgga
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gttttcagtgagtaggtttgcataacggagctgaggaggcgtggccaggataaagaagtggttagagaagctgctaaggagagctgga
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gcctcagcccgccttttttcttcttagcttcacttctagcgatgctagc

The original exonic sequence of WT p16 locus is shown below, with 5' and 3'UTRs in purple, coding sequence in black, and the BstB1 restriction site used for integration of the 3MR cDNA in Exon 2 in red:

Exon 1

ACTGGTCACACGACTGGGCGATTGGGCGGGCACTGAATCTCCGCGAGGAAAGCGAACTC
GAGGAGAGCCATCTGGAGCAGCATGGAGTCCGCTGCAGACAGACTGGCCAGGGCGGCG
GCCCAGGGCCGTGTGCATGACGTGCGGGCACTGCTGGAAGCCGGGGTTTCGCCCAACGC
CCCGAACTCTTTCGGTCGTACCCCGATTCAG

Exon 2

GTGATGATGATGGGCAACGTTCACGTAGCAGCTCTTCTGCTCAACTACGGTGCAGATTCG
AACTGCGAGGACCCCACTACCTTCTCCCGCCCGGTGCACGACGCAGCGCGGGAAGGCTT
CCTGGACACGCTGGTGGTGCTGCACGGGTCAGGGGCTCGGCTGGATGTGCGCGATGCCT
GGGGTCGCCTGCCGCTCGACTTGGCCCAAGAGCGGGGACATCAAGACATCGTGCGATAT
TTGCGTTCCGCTGGGTGCTCTTTGTGTTCCGCTGGGTGGTCTTTGTGTACCGCTGGGAAC
GTC

Exon 3

GGCCCTGGAACTTCGCGGCCAATCCCAAGAGCAGAGCTAAATCCGGCCTCAGCCCGCCTT
TTTCTTCTTAGCTTCACTTCTAGCGATGCTAGCGTGTCTAGCATGTGGCTTTAAAAAATACA
TAATAATGCTTTTTTTGCAATCACGGGAGGGAGCAGAGGGAGGGAGCAGAAGGAGGGA

GGGAGGGAGGGAGGGACCTGGACAGGAAAGGAATGGCATGAGAAACTGAGCGAAGGCG
GCCGCGAAGGGAATAATGGCTGGATTGTTTAAAAAAATAAAATAAAGATACTTTTTAAAATG
TC

RecA-mediated integration of the 3MR cDNA into the parental BAC via homology arms and excision of vector sequences in E coli (PMCID:102750) resulted in the modified BAC exonic sequence shown below. Remains of the BstBI site is in red, linker sequence surrounding the 3MR cDNA is in orange, stop codon in the p19ARF reading frame is in blue, and the 3MR coding sequence is in green:

atgtctagcggtaccgccaccatggcttccaaggtgtacgaccccgagcaacgcaaacgcatgatcactgggcctcagtggtgggct
cgctgcaagcaaatgaacgtgctggactccttcatcaactactatgattccgagaagcacgccgagaacgccgtgatttttctgcatgg
taacgctgcctccagctacctgtggaggcacgtcgtgcctcacatcgagcccgtggctagatgcatcatccctgatctgatcggaatgg
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gccgacatccccgactacttgaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtg
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ccgttccatgcacgtctttatcctggattacgaccaatcgcccgccggctgccgggacgccctgctgcaacttacctccgggatggtcc

agacccacgtcaccacccccggctccataccgacgatatgcgacctggcgcgcacgtttgcccgggagatgggggaggctaactg

acccggggaattctgcagatatccagcacagtggcggccgcat

2.Insertion of Neo resistance cassette into modified BAC

A 774 bp fragment of the SacB gene (below) in the BAC backbone was amplified by PCR using the BAC as a template with SacB forward (SacB F, gtccggttctgcaacc) and reverse (SacB R, cctttggcttgcggcac) primers. A kan/neo resistance cassette was cloned into the HindIII site (in bold red below) and integrated into the modified BAC using γ Red-mediated recombination (PMID:10037821):

gtccggttctgcaacctttacatctgacggaaaaatccgtttattctacactgactattccggtaaacattacggcaaacaaagcctgac
aacagcgcaggtaaatgtgtcaaaatctgatgacacactcaaaatcaacggagtggaagatcacaaaacgatttttgacggagacg
gaaaaacatatcagaacgttcagcagtttatcgatgaaggcaattatacatccggcgacaaccatacgctgagagaccctcactacg
ttgaagacaaaggccataaataccttgtattcgaagccaacacgggaacagaaaacggataccaaggcgaagaatctttatttaac
aaagcgtactacggcggcggcacgaacttcttccgtaaagaaagccag**aagctt**cagcagagcgctaaaaaacgcgatgctgag
ttagcgaacggcgccctcggtatcatagagttaaataatgattacacattgaaaaaagtaatgaagccgctgatcacttcaaacacgg
taactgatgaaatcgagcgcgcgaatgttttcaaaatgaacggcaaatggtacttgttcactgattcacgcggttcaaaaatgacgatc
gatggtattaactcaaacgatatttacatgcttggttatgtatcaaactctttaaccggcccttacaagccgctgaacaaaacagggcttgt
gctgcaaatgggtcttgatccaaacgatgtgacattcacttactctcacttcgcagtgccgcaagccaaagg

Real Time-PCR. The primers and probes were as follows:

ACTIN: #64; FW 5'-ctaaggccaaccgtgaaaag-3'; RV 5'-accagaggcatacagggaca-3'

TUBULIN: #88; FW 5'-ctggaacccacggtcatc-3'; RV 5'-gtggccacgagcatagttatt-3'

p16^{INK4a}: #91; FW 5'-aatctccgcgaggaaagc-3';RV 5'-gtctgcagcggactccat-3'

mRFP: #161; FW 5'–gacctcggcgtcgtagtg–3';RV 5'–aagggcgagatcaagatgag–3'

CFL1 (cofilin): #6; FW 5'-tctgtctccctttcgtttcc-3';RV 5'-ttgaacaccttgatgacaccat-3'

PDGFA: #52; FW 5'-gtgcgacctccaacctga-3';RV 5'-ggctcatctcacctcacatct-3'

IL-6: #6; FW 5'-gctaccaaactggatataatcagga-3';RV 5'-ccaggtagctatggtactccagaa-3'

LMNB1: #15; FW 5'-gggaagtttattcgcttgaaga-3';RV 5'-atctcccagcctcccatt-3'

IL-5: #91; FW 5'-acattgaccgccaaaaagag-3';RV 5'-atccaggaactgcctcgtc-3'

p21: #9; FW 5'-ttgccagcagaataaaaggtg-3';RV 5'-tttgctcctgtgcggaac-3'

PDGF-B: #32; FW 5'-cggcctgtgactagaagtcc-3';RV 5'-gagcttgaggcgtcttgg-3'

IL-1α: #29; FW 5'-tccataacccatgatctggaa-3';RV 5'-ttggttgagggaatcattcat-3'

PAI-1: #69; FW 5'-aggatcgaggtaaacgagagc-3'; RV 5'-ttggttgagggaatcattcat-3'

VEGF: #1; FW 5'-aaaaacgaaagcgcaagaaa-3';RV 5'-tttctccgctctgaacaagg-3'

CCL5: #110; FW 5'-tgcagaggactctgagacagc-3';RV 5'-gagtggtgtccgagccata-3'

FGF: #53; FW 5'-aacgcatagcgcttctgaat-3';RV 5'-aaaacagctcccacagagga-3'

CCL2: #62; FW 5'-catccacgtgttggctca-3';RV 5'-gatcatcttgctggtgaatgagt-3'

TGF-β1: #72; FW 5'-tggagcaacatgtggaactc-3';RV 5'-cagcagccggttaccaag-3'

MMP2: #77; FW 5'-taacctggatgccgtcgt-3';RV 5'-ttcaggtaataagcacccttgaa-3'

MMP3: #36; FW 5'-caaaacatatttctttgtagaggacaa-3';RV 5'-ttcagctatttgcttgggaaa-3'

MMP9: #19; FW 5'-acgacatagacggcatcca-3';RV 5'-gctgtggttcagttgtggtg-3'

MMP10: #81; FW 5'-gagtctggctcatgcctacc-3';RV 5'-caggaataagttggtccctga-3'

MMP12: #15; FW 5'-tgaatttgcatttcctgtacatagt-3';RV 5'-tgctgtaagtccatgggtga-3'

MMP13: #10; FW 5'-aaggggataacagccactacaa-3';RV 5'-accaacataaaaattaagccaaatg-3'

VIMENTIN: #1; FW 5'-gtaccggagacaggtgcagt-3';RV 5'-ttctcttccatctcacgcatc-3'

SMA: #58; FW 5’-ctctcttccagccatctttcat-3’;RV 5’- tataggtggtttcgtggatgc-3’

ENDOTHELIN-1: #50; FW 5’-ctgctgttcgtgactttcca-3’;RV 5’-tctgcactccattctcagctc-3’

KERATIN-1: #62; FW 5’-tttgcctccttcatcgaca-3’;RV 5’-gttttgggtccgggttgt-3’

KERATIN-10: #95; FW 5’-cgtactgttcagggtctggag-3’;RV 5’-gcttccagcgattgtttca-3’