

Supplementary Figures and Tables

The senescence-like activity of BMS-470539 is associated with anti-fibrotic actions in models of dermal fibrosis

Camilla S.A. Davan-Wetton, Natalya Khodeneva, Christopher P Denton, David J Abraham, Mauro Perretti, Trinidad Montero-Melendez

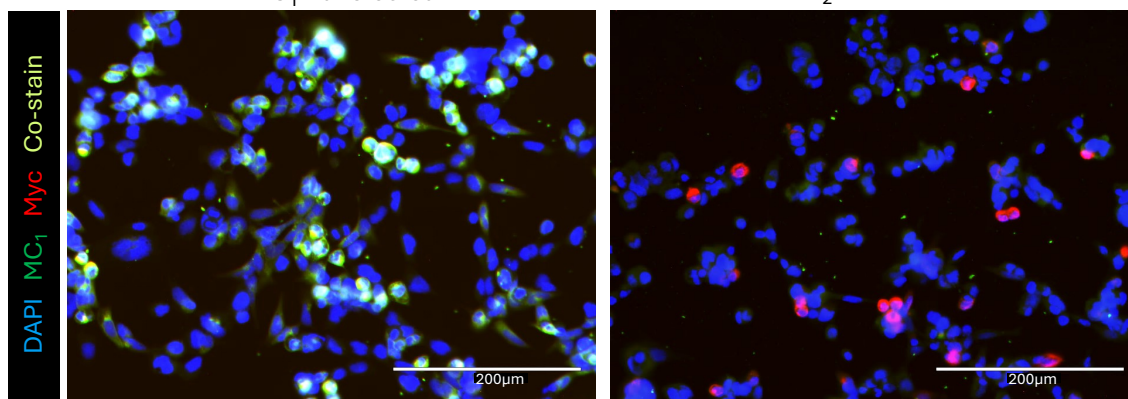
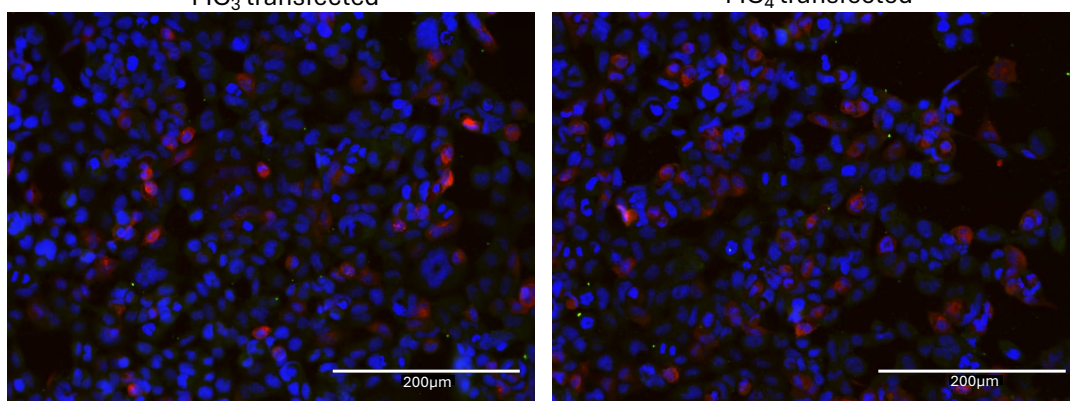
Supplementary Figure 1

A

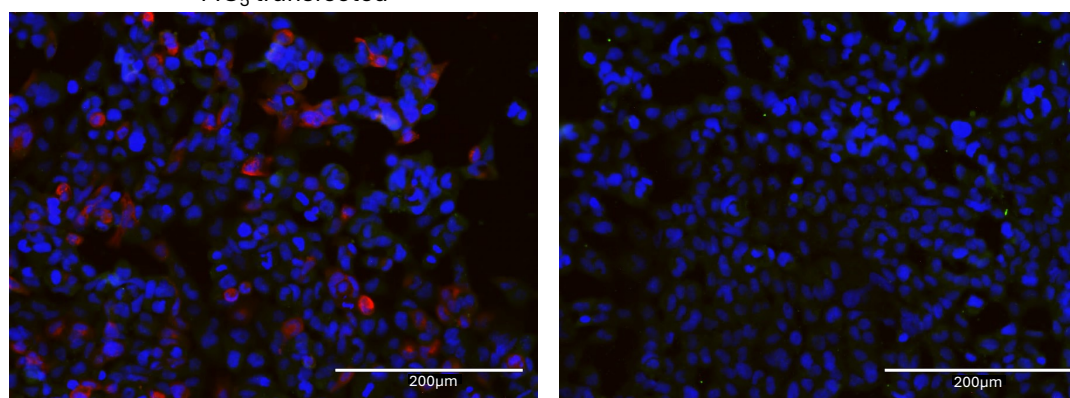
IC3 (217-232)

MC₁ VVFFFLAMLVLMAVLVYVHMLARACQHAQGIARLHKRQR-PVHQGFGFLKGAVTLTILLGIFFLCWGPFFFLH
MC₂ TSLFPLMLVFILCLYVHMFLLARSHTRKISTLPR-----ANMKGAITLTILLGVFIFCWAPFVLH
MC₃ ITMFFAMMLMGSLYVHMFLLARLHVKRIALALPADGVAPQQHSCMKGAVTITILLGVFIFCWAPFFLLH
MC₄ ITMFFTMLALMASLTVHMFLLARLHKRIALVPGTG-----AIRQANMKGAITLTILLGVFIFCWAPFFLLH
MC₅ ISMFFAMLFLVLSLYIHMFLLARLTHVKRIALALPGAS-----SARQRTSMQGAIVTMTLLGVFTVCWAPFFLLH

B

MC₁ transfectedMC₂ transfectedMC₃ transfectedMC₄ transfectedMC₅ transfected

Untransfected control

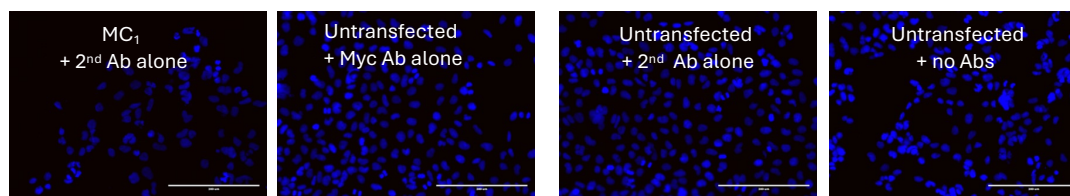


MC₁
+ 2nd Ab alone

Untransfected
+ Myc Ab alone

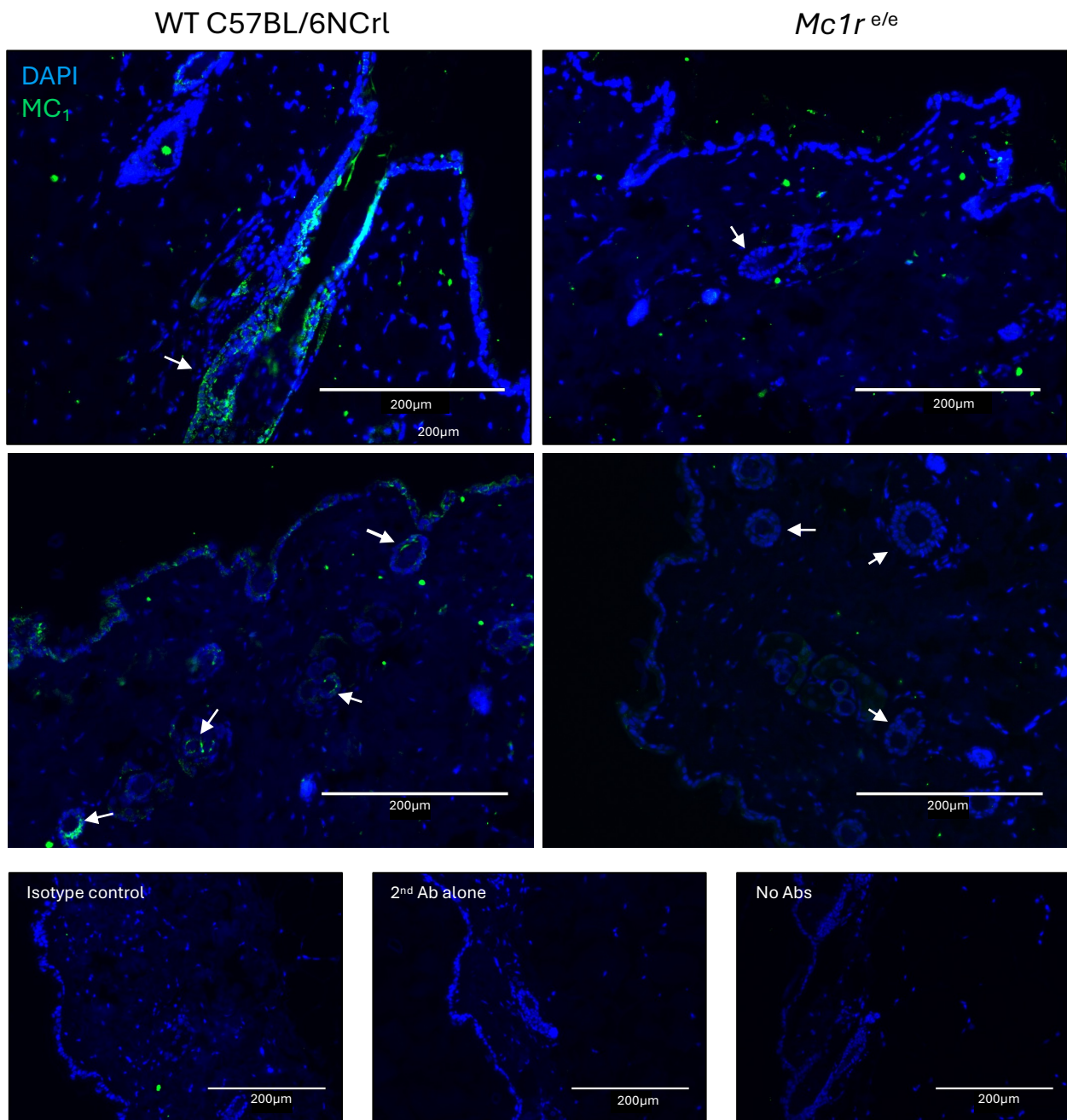
Untransfected
+ 2nd Ab alone

Untransfected
+ no Abs



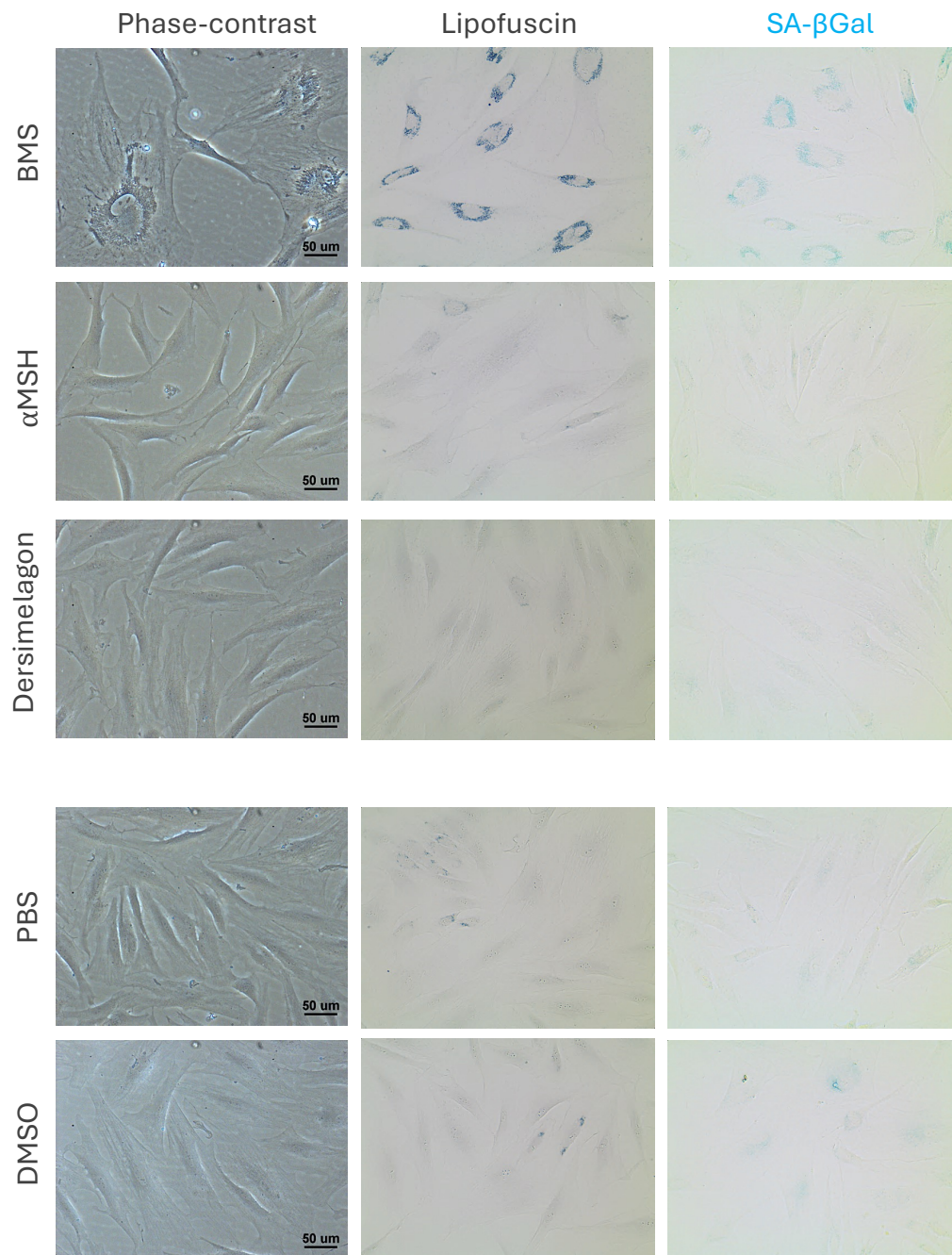
Supplementary Figure 1. *In vitro* validation of an anti-MC₁ receptor antibody on transfected HEK293T cells. **A)** Amino acid sequence alignment of the five human melanocortin receptors. The peptide used as immunogen to generate the anti-MC₁ receptor rabbit polyclonal antibody (Alomone, Cat. No. AMR-020) corresponds to the intracellular domain 3, a region of high variability. **B)** HEK293T cells were transiently transfected with each of the melanocortin receptors, using Myc-tagged expression vectors, and stained 24 h later with an anti-Myc antibody (Cell Signalling Technology) and the anti-MC₁ receptor rabbit polyclonal antibody (Alomone). Yellow colour indicates co-staining of MC₁ with the Myc tag. Images were captured at 20X and scale bars represent 200 μm .

Supplementary Figure 2



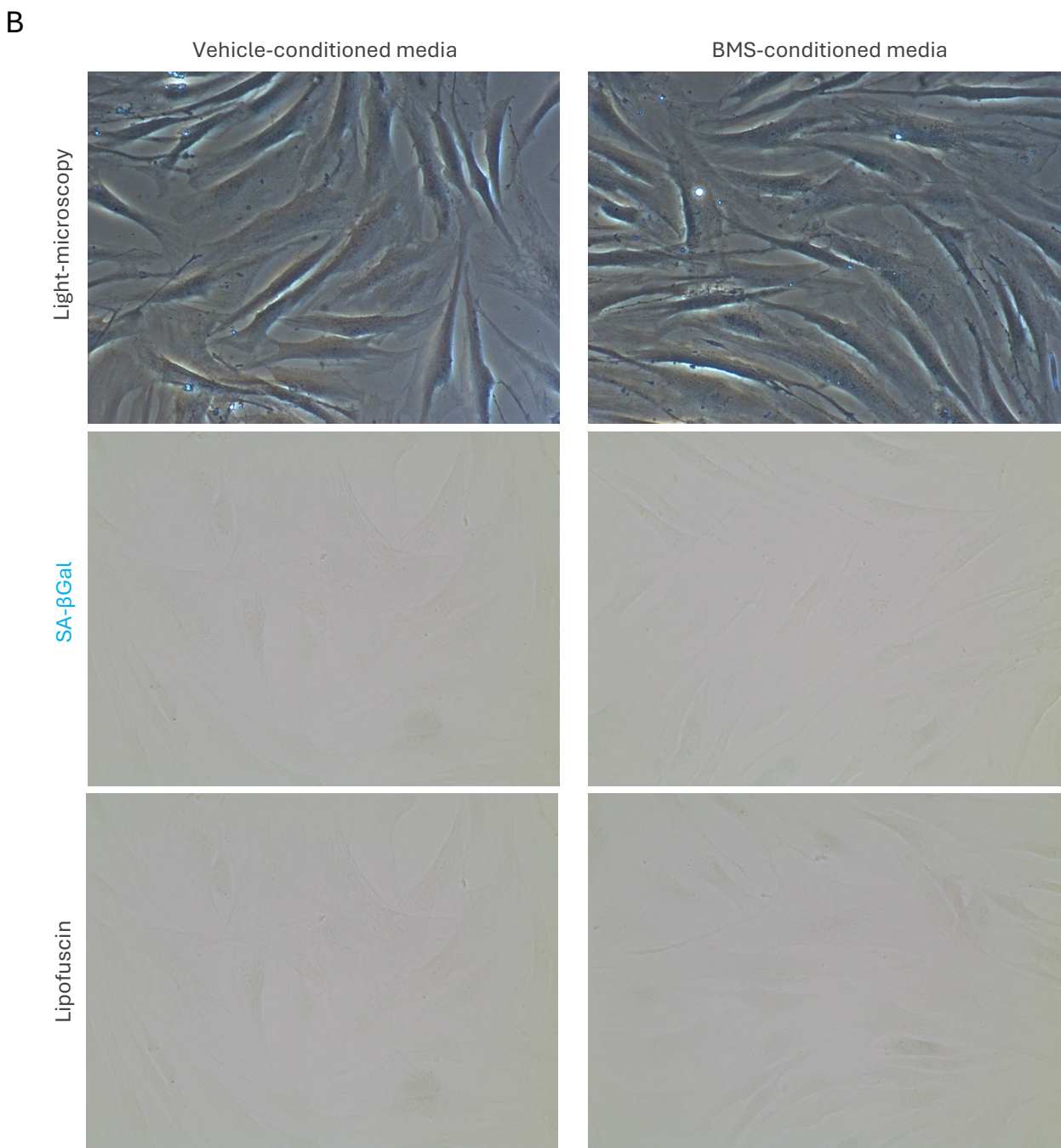
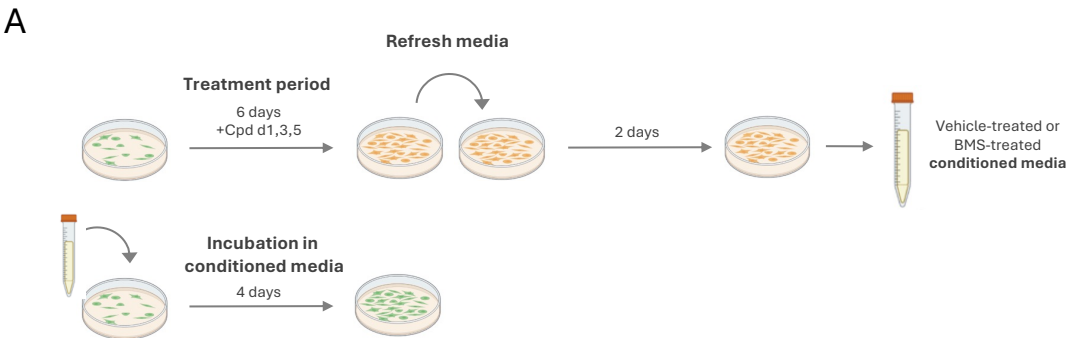
Supplementary Figure 2. *In vivo* validation of an anti-MC₁ receptor antibody using *Mc1r*^{e/e} mouse tissues. Formalin-fixed paraffin-embedded skin tissues from wild type (WT) C57BL/6NCrl and *Mc1r*^{e/e} mice (lacking a functional MC₁ receptor) were subjected to immunofluorescence staining using the anti-MC₁ receptor rabbit polyclonal antibody (Alomone, Cat. No. AMR-202). Images were captured at 20X and scale bars represent 200 μm. While arrows indicate hair follicles where MC₁ expression is expected.

Supplementary Figure 3



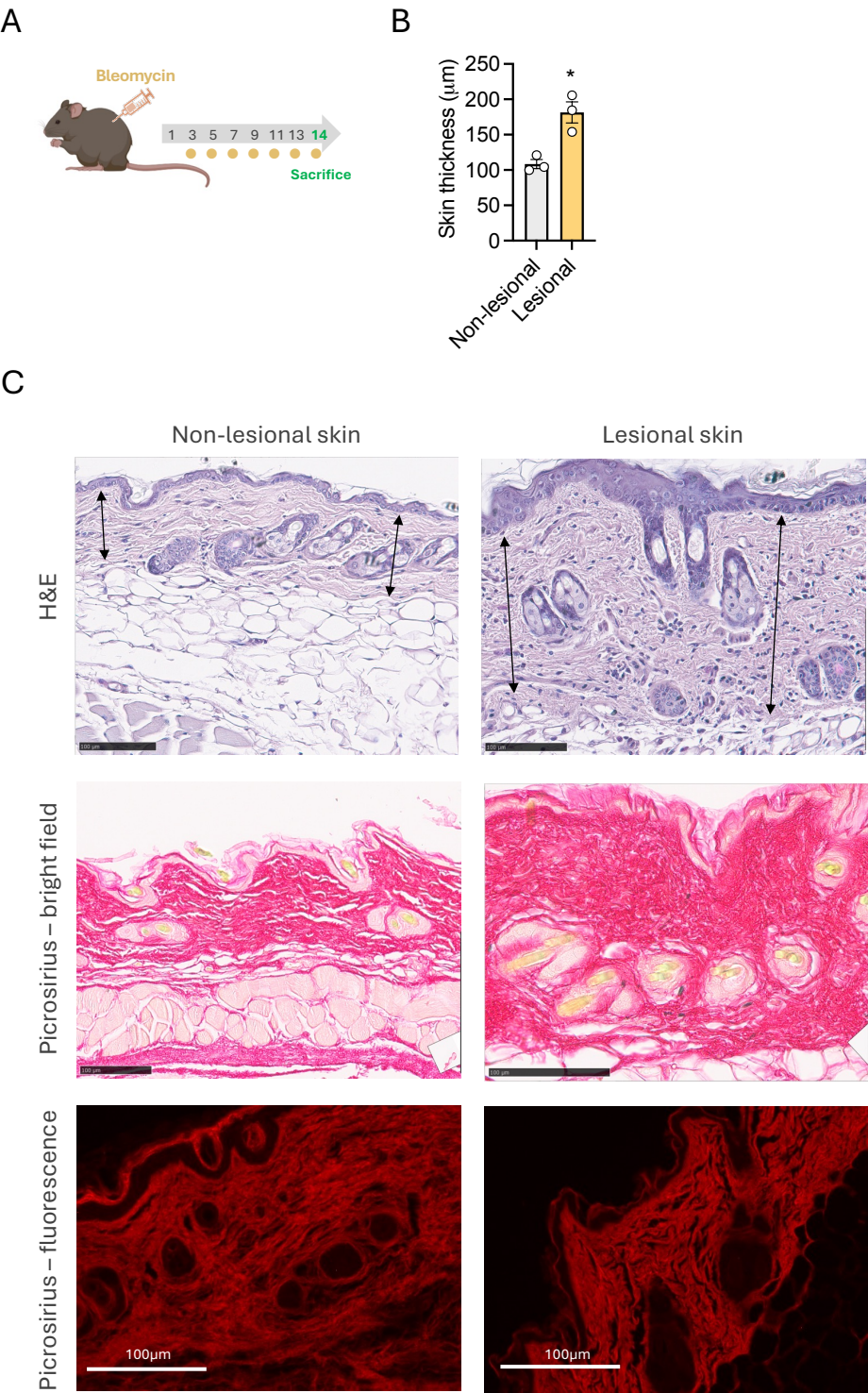
Supplementary Figure 3. Induction of senescence markers by BMS-470539, dersimelagon and α MSH on *in vitro* cultured dermal fibroblasts. Systemic sclerosis (SSc) fibroblasts were treated with vehicle (PBS or DMSO), 10 μ M BMS-470539, 10 μ M dersimelagon or 10 μ M α MSH every other day for 6 days. Cells were stained for senescence associated β -galactosidase (SA- β Gal) activity or lipofuscin using Sudan black B. Images were captured at 40X with a light microscope. Representative images are shown.

Supplementary Figure 4



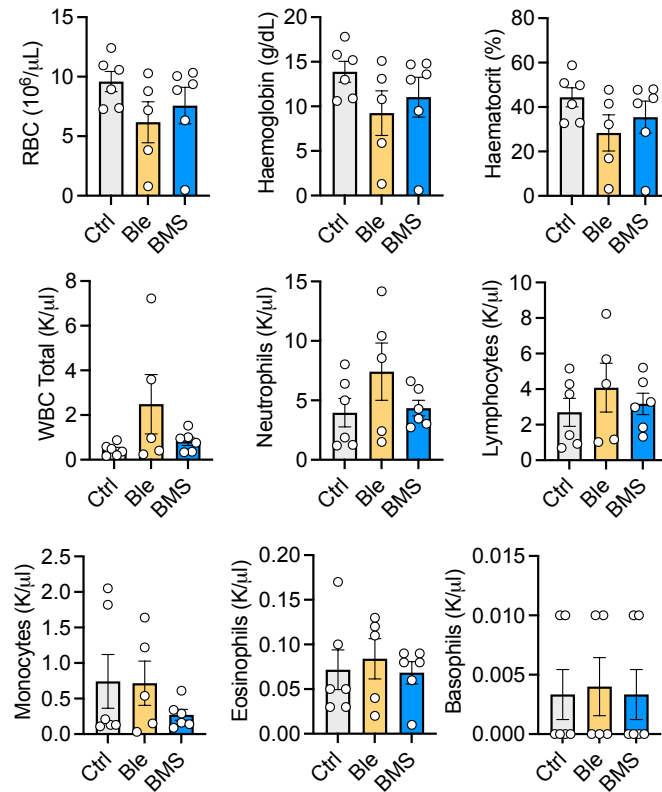
Supplementary Figure 4. Absence of secondary senescence on *in vitro* cultured human dermal fibroblasts treated with BMS-conditioned media. **A)** Diagram representing the procedure to obtain conditioned media. Systemic sclerosis (SSc) fibroblasts were treated with vehicle (Ctrl) or 10 μ M BMS-470539, every other day for 6 days. Cells were washed to remove any remaining BMS-470539 compound and fresh media added. Cells were incubated for 2 days to allow the release of mediators. Media was then collected, filtered and used on newly plated cells for 4 days. **B)** Cells were analysed by visualising the presence of a perinuclear lysosomal ring (light microscopy images), senescence associated β -galactosidase (SA- β Gal) activity and by lipofuscin staining using Sudan black B. Images were captured at 40X. Representative images of n=3.

Supplementary Figure 5



Supplementary Figure 5. Establishment of fibrosis on the bleomycin-induced skin fibrosis murine model at day 14. A) Diagram representing the experimental design used to induce dermal fibrosis. 1 mg/ml bleomycin was administered intradermally (i.d.) every other day from day 1 until day 14. **B)** The dermal thickness of the H&E-stained sections was quantified in NDP.view2. **C)** Representative images of H&E and picrosirius red stained tissue sections of skin from saline and bleomycin treated mice as a visualisation of skin thickness and collagen deposition Picrosirius red sections were visualised by bright field and fluorescence microscopy. Data represent the mean $\pm$ SEM (n=3; unpaired t-test, *p<0.05). Images were captured at using a Nanozoomer slide scanner and scale bars represent 100 μ m.

Supplementary Figure 6



Supplementary Figure 6. Systemic changes in mice treated intradermally with bleomycin or BMS-470539. 50 μL of saline, 1 mg/ml bleomycin or 3.6 mg/ml BMS-470539 were administered intradermally (i.d.) from day 1 until day 27. Mice were sacrificed at day 28 and blood collected by cardiac puncture. Blood was analysed using a ProCyte Dx Haematology Analyzer (IDEXX). Data represent mean $\pm$ SEM of $n=4$ and was analysed by paired one-way ANOVA vs. control followed by Dunnett's multiple comparisons test.

Supplementary Table 1

Donor	Sex	Age (years)	Diffuse or limited	Auto-antibodies	Co-morbidities	Medication	<i>MC1R</i> genotype
HV101	F	54	n/a	n/a	n/a	n/a	R151C
HV102	M	73	n/a	n/a	n/a	n/a	WT
HV103	F	54	n/a	n/a	n/a	n/a	R151C
HV104	F	73	n/a	n/a	n/a	n/a	WT
SSc1	F	53	Diffuse	ARA	ILD, OA, aortic valve replacement	Mycophenolate	V60L , R160W
SSc5	F	42	Diffuse	-	Lung fibrosis, severe midgut disease	Lansoprazole, Proventil	R151C
SSc7	F	47	Diffuse	Anti-Scl-70	Hypothyroidism, thalassaemia trait	Iloprost, Mycophenolate mofetil	WT
SSc8	F	41	Diffuse	Anti-Scl-70	ILD, myocardial involvement, inflammatory arthritis	No active medications	V60L , R151C
SSc10	F	53	Diffuse	ANA, ENA, anti-Ro, anti-Scl-70	Myositis overlap, cardiac involvement, ILD, RP, hypothyroidism, AF	Hydroxychloroquine, Iloprost, Prednisolone, Rituximab	V60L , R151C
SSc11	F	52	Diffuse	-	None	Lansoprazole	WT
SSc12	F	45	Limited	ARA	Carcinoid of appendix, hypothyroidism, RP	Folic acid, Levothyroxine, Methotrexate, Sildenafil	WT
SSc13	M	51	Diffuse	Anti-RNA pol III	Inflammatory arthritis, GAVE, anaemia, ischaemic heart disease, depression	Tocilizumab	V60L , R151C
SSc14	F	51	Diffuse	-	Hypomagnesaemia, hypothyroidism, ILD, anaemia, osteoporosis	Iloprost, Prednisolone, Myfortic, Adizem-SR	V92M , R163Q , T314T
SSc15	F	44	Limited	Anti-Scl-70	Inflammatory arthropathy, digital ulcer, myocardial involvement	Iloprost, Hydroxychloroquine, Sildenafil	V60L , R151C

Supplementary Table 1. Characteristics of healthy volunteers (HV), and systemic sclerosis patients (SSc) which donated skin samples for the obtention of dermal fibroblasts. AF: atrial fibrillation; ANA: anti-nuclear antibody; Anti-RNA poly III: anti-RNA polymerase III antibody; Anti-Ro: anti-Sjogren's-syndrome-related antigen A antibody; Anti-Scl-70: anti-topoisomerase 1 antibody; ARA: anti-reticulin antibody; ENA; anti-extractable nuclear antigen antibody; GAVE: gastric antral vascular ectasia; ILD: interstitial lung disease; OA: osteoarthritis; RP: Raynaud's phenomenon. *MC1R* gene variants with strong association with red hair are highlighted in red.

Supplementary Table 2

Haplotypes	No.	%	RHC phenotype
WT	3	30	n/a
V60L / R151C	4	40	r / R
V60L / R160W	1	10	r / R
V92M / R163Q	1	10	r / r
R151C	1	10	R

Supplementary Table 2. Summary of *MC1R* genotyping results for the systemic sclerosis (SSc) patients fibroblasts (n=10) used in this study indicating the number and proportion of donors carrying the specified haplotype and the red hair colour (RHC) phenotype as described in literature (R= high association with red hair (in red), r= weak association with red hair).

Supplementary Table 3

Variant	Substitution	RHC	AA	Aa	aa	p	q	%
F45L	133 T>C	r	96	1	0	0.995	0.005	1.0
V60L	178 G>T	r	64	32	1	0.825	0.175	34.0
D84E	252 C>A	R	96	1	0	0.995	0.005	1.0
V92M	274 G>A	r	80	14	3	0.897	0.103	17.5
T95M	284 C > T	R	96	1	0	0.995	0.005	1.0
R151C	451 C>T	R	85	12	0	0.938	0.062	12.4
R160W	478 C>T	R	80	17	0	0.912	0.088	17.5
R163Q	488 G>A	r	88	8	1	0.948	0.052	9.3
V193L	577 G>A	R	96	1	0	0.995	0.005	1.0
F196L	586 T>C	R	96	1	0	0.995	0.005	1.0
D294H	880 G>C	R	94	2	1	0.979	0.021	3.1

Supplementary Table 3. Summary of *MC1R* genotyping results for the systemic sclerosis (SSc) patients DNA samples (n=97) used in this study indicating the individual variants identified, nucleotide position and substitution, red hair colour (RHC) phenotype (highly associated shown in red), number of patients homozygotes for mayor allele (AA), number heterozygotes (Aa), number of homozygotes for minor allele (aa), mayor allele frequency (p), minor allele frequency (q) and proportion of patients carrying that variant (%).

Supplementary Table 4

Haplotypes	No.	%	RHC
WT	18	18.6	n/a
F45L	1	1.0	r
V60L	21	21.6	r
V60L / V92M	3	3.1	r / r
V60L / R151C	4	4.1	r / R
V60L / R160W	1	1.0	r / R
V60L / R163Q	3	3.1	r / r
V60L / V193L	1	1.0	r / R
V60L / D294H	1	1.0	r / R
D84E	1	1.0	R
V92M	11	11.3	r
V92M / R160W	3	3.1	r / R
T95M	1	1.0	R
R151C	8	8.2	R
R160W	11	11.3	R
R160W / R163Q	1	1.0	R / r
R160W / D294H	1	1.0	R / R
R163Q	5	5.2	r
F196L	1	1.0	R
D294H	1	1.0	R

Supplementary Table 4. Summary of *MC1R* genotyping results for the systemic sclerosis (SSc) patients DNA samples (n=97) used in this study indicating the number and proportion of donors carrying the specified haplotype and the red hair colour (RHC) phenotype as described in literature (R= high association with red hair, in red, r= weak association with red hair).

Supplementary Table 5

Gene Symbol	Transcript ID	Gene Name	Fold change	p value
ACTA2	NM_001613	Actin alpha 2, smooth muscle	0.25	0.038
BAG2	NM_004282	BAG cochaperone 2	0.80	0.015
BCL2L2	NM_004050	BCL2 like 2	1.23	0.003
BIRC5	NM_001168	Baculoviral IAP repeat containing 5	0.53	0.007
BOK	NM_032515	BCL2 family apoptosis regulator BOK	0.79	0.027
CALD1	NM_033138	Caldesmon 1	0.56	0.010
CAVIN2	NM_004657	Caveolae associated protein 2	0.40	0.040
CDC27	NM_001256	Cell division cycle 27	0.78	0.022
CDCA3	NM_031299	Cell division cycle associated 3	0.54	0.039
CDK1	NM_001786	Cyclin dependent kinase 1	0.59	0.024
CDKN1A	NM_078467	Cyclin dependent kinase inhibitor 1A	1.91	0.043
CDKN3	NM_005192	Cyclin dependent kinase inhibitor 3	0.53	0.003
CEMP	NM_018689	Cell migration inducing hyaluronidase 1	0.80	0.013
CENPF	NM_016343	Centromere protein F	0.59	0.033
CENPH	NM_022909	Centromere protein H	0.67	0.026
CNN1	NM_001299	Calponin 1	0.35	0.004
CNN2	NM_004368	Calponin 2	0.66	0.014
COL5A2	NM_000393	Collagen type V alpha 2 chain	0.82	0.049
EIF2S2	NM_001316364	Eukaryotic translation initiation factor 2 subunit beta	0.75	0.024
FHL1	NM_001159699	Four and a half LIM domains 1	0.35	0.041
FSTL1	NM_007085	Follistatin like 1	0.82	0.023
GDF6	NM_001001557	Growth differentiation factor 6	0.38	0.034
GLB1	NM_000404	Galactosidase beta 1	1.39	0.032
GTSE1	NM_016426	G2 and S-phase expressed 1	0.55	0.026
IFI6	NM_002038	Interferon alpha inducible protein 6	3.31	0.006
IL7R	NR_120485	Interleukin 7 receptor	0.54	0.037
INSIG1	NM_001346592	Insulin induced gene 1	4.37	0.008
ITGA1	NM_181501	Integrin subunit alpha 1	0.82	0.026
ITGA3	NM_002204	Integrin subunit alpha 3	0.83	0.042
LAMP1	NM_005561	Lysosomal associated membrane protein 1	1.49	0.001
LDLR	NM_001195799	Low density lipoprotein receptor	2.71	0.021
LMOD1	NM_012134	Leiomodin 1	0.38	0.012
LOXL1	NM_005576	Lysyl oxidase like 1	0.71	0.020
MIR503HG	NR_024607	MIR503 host gene	0.29	0.033
MKI67	NM_001145966	Marker of proliferation Ki-67	0.53	0.019
MMP14	NM_004995	Matrix metalloproteinase 14	1.48	0.006
MMP2	NM_001127891	Matrix metalloproteinase 2	1.75	0.010
MVK	NM_000431	Mevalonate kinase	2.26	0.002
MYL6	NM_079423	Myosin light chain 6	0.66	0.039
MYO1B	NM_001161819	Myosin IB	0.60	0.030
NGF	NM_002506	Nerve growth factor	0.52	0.032
P4HA1	NM_001142595	Prolyl 4-hydroxylase subunit alpha 1	0.83	0.017
S100A16	NM_001317007	S100 calcium binding protein A16	0.52	0.042
SERPINE1	NM_000602	Serpin family E member 1	0.47	0.018
TAGLN	NM_003186	Transgelin	0.55	0.003
TGFB111	NM_001042454	Transforming growth factor beta 1 induced transcript 1	0.71	0.005
TPM1	NM_001018007	Tropomyosin 1	0.78	0.035
TPM2	NM_213674	Tropomyosin 2	0.59	0.009
TRIAP1	NM_016399	TP53 regulated inhibitor of apoptosis 1	1.20	0.032
VEGFA	NM_001287044	Vascular endothelial growth factor A	0.79	0.022

Supplementary Table 5. Details of selected genes provided in Figure 5E, related to RNA sequencing analysis (n=3). Fold change is calculated as the ratio of BMS-470539 treated cells vs. controls.