SUPPLEMENTAL MATERIAL

Methods

Cell culture, viral transductions and antibiotic selection

c-kit+ CPCs isolated from 3 and 13 month old male FVB mice hearts (1,2), human patients receiving left ventricular assist device (3,4), aborted human fetuses (4) were cultured as described previously. Mouse CPCs (mCPCs) and human CPCs (hCPCs) were cultured for at least 8 and 3 passages respectively to obtain sufficient cells for experiments. Experiments on mCPCs and hCPCs were conducted between passages 8-20 and 4-10 respectively. mCPCs demonstrate no significant change in molecular or morphological characteristics between passages. However hCPCs demonstrate differences consistent of replicative senescence and therefore passages 4-5 were classified as early passages, 6-8 as mid passages, 9-10 as late passages (4).

Lentiviral constructs harboring sh-RNA against p53 (clone ID: TRCN0000003753, TRCN0000012359), c-Myc (TRCN0000234923, TRCN0000039641), Pim-1 (TRCN0000010116) were purchased from Sigma Aldrich. Lentiviral constructs for Tetracycline – inducible c-Myc and pLVX-Tet-on were purchased from Addgene (Plasmid 20723, FUW-tetO-hMYC) and Clontech respectively. Lentiviruses were prepared and concentrated as described previously (5). Lt-sh-NS (5), Lt-sh-control (5), lentivirus overexpressing Pim-1 and GFP (2), GFP (2), NS-GFP (5), AzG-Geminin and mKO-Cdt1 (6) have been previously described. mCPCs were transduced with lentiviruses and after 48 hours were treated with puromycin (1-10 μg/ml, overnight) or neomycin/G418 (for pLVX-Tet-on, 400 μg/ml, overnight), with the exception of CPCs that were transduced with viruses harboring a fluorescent protein like GFP/AzG/mKO, in which case the cells were FACS sorted based on expression of fluorescence marker. hCPCs were transduced as described previously (3). Following Lt-sh-NS transduction, CPCs were observed for 3-4 days and samples were collected after detecting morphological changes. For dual knockdown experiments, CPCs were transduced with Lt-sh-p53 prior to NS knockdown.

Pharmacological inhibitors

Pharmacological inhibition of c-Myc was achieved by treatment with 10058-F4 (c-Myc in, 50μM, overnight; Sigma Aldrich, #F3680) as has been described before (1,7).

Human Myocardium

Non-failing human myocardium was obtained from donors whose hearts were declined for transplantation. Institutional Review Board-approved prospective informed consent for research use of non-failing hearts through Temple University

School of Medicine and the local organ procurement organization (Gift of Life, Inc) ensured ethical use of all tissue. Myocardial perfusion with cold, 4:1 blood cardioplegia and prompt transport to the laboratory was performed as previously described (8). Tissue pieces were snap-frozen in liquid nitrogen and stored at -80C until time of analysis. The cardiac region from non-failing human hearts included the mid left ventricle free wall with full thickness from epicardium to endocardium. Human fetal and adult failing myocardium were obtained as previously described (4). Adult failing heart samples were small explants of the left ventricle isolated from patients with end stage heart failure receiving a left ventricular assist device and the tissue was used either for isolation of adult human CPCs (AhCPCs) or for biochemical analyses. Fetal whole heart tissue from spontaneously aborted fetuses at 16-17 weeks of age was used for isolation of fetal human CPCs (FhCPCs) or for biochemical analyses. Each sample was isolated from a different patient and tissue from individual patients was not combined. Experimental values obtained from multiple patients of a sample category (fetal, adult failing, adult non-failing) were averaged for statistical analyses. Human patient information and disease state of samples used have been listed in Supplemental Table 2.

Sample preparation and immunoblot analyses

Protein lysates from cultured cells were prepared using sample buffer (containing 150mM Tris (pH 6.8), 150mg/ml sucrose, 2mM ethylene diamine tetraaceticacid (EDTA) (pH 7.5-8), 480mg/ml urea, 8mg/ml dithiothreitol, 0.2% sodium dodecyl sulfate, 0.2 mg/ml bromophenol blue at a final pH of 6.8, supplemented with protease and phosphatase inhibitor cocktails, Sigma-Aldrich #P8340, #P5726, #P0044). For detection of some proteins (p53, c-Myc) samples were extracted on dry ice and ethanol instead of on ice. Samples from mice left ventricles and human myocardium were prepared by homogenizing tissue in isolation buffer (containing 70 mM sucrose, 190 mM mannitol, 20mM HEPES solution, and 0.2 mM EDTA in de-ionized water) using a Next Advance bullet blender. Protein lysates were prepared from homogenized tissue by adding sample buffer in a 1:1 ratio. Samples were boiled for 5 minutes at 100*C to denature proteins. Immunobloting was done as described before (5). Antibodies used are listed in Supplemental Table 3. Blots were quantified using ImageJ (NIH).

RNA extraction and quantitative real time PCR

Total RNA was extracted using TRIzol (Invitrogen). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Real time PCR was performed on all samples in triplicate using iQ SYBR Green (Bio-Rad) following the manufacturer's protocol. The fold change in gene expression was determined using the *ddCt* method (5). Primer sequences are listed in Supplemental Table 4. Telomere lengths were measured using quantitative real time PCR as described previously (4).

Flow Cytometry

To determine the cell cycle profiles, CPCs were fixed in 70% ethanol and stained with propidium iodide (PI)/ RNase buffer (BD Biosciences, #550825) for 3 hours at 37*C, as described before (9,10). To determine Bromodeoxyuridine (BrdU) rates, mCPCs were treated with 10μM BrdU over a 24 hour time course and fixed in 70% ethanol (overnight, 4*C), denatured (2N HCl/0.2% Triton-X100 for 30 minutes), neutralized (0.1 mol/L TrisHCl pH 8.5 for 10 minutes), blocked (PBS supplemented with 10% horse serum for 30 minutes), incubated in primary (overnight, 4*C) and secondary antibodies (1:50,1 hour at room temperature) and stained with PI/RNase buffer (3 hours, 37*C). For cell death assays, CPCs were stained with propidium iodide for 2 minutes (10). Live cells were sorted on FACSAria (BD Biosciences) to detect fluorescent protein expression. Data was processed using FlowJo software.

Proliferation and Differentiation Assays

CPCs were seeded in quadruplicates at a density of 1000 cells/well in a 96-well flat bottom culture plate. Proliferation rates were determined using Cyquant direct cell proliferation assay (Life Technologies, #C35011). To determine differentiation potential, mCPCs were cultured at a density of 10,000 cells/well in a 6 well plate over a 7 day period and were treated with either full media or α-minimal essential media with 10% FBS, 1% penicillin streptomycin glutamine, in the presence and absence of dexamethasone (Dex,10nM), following which protein lysates were extracted.

Microscopy

Senescent CPCs were detected using SA-β-gal assay (Abcam #ab65351) on Olympus IX70 microscope. Immunofluorescent images of paraffin sections or cultured cells were acquired as described before on Leica SP2 or SP8 confocal microscopes (3). For experiments with immunostaining and SA-β-gal staining of cultured cells, SA-β-gal assay was performed first followed by immunostaining (3). Phalloidin conjugate 633 and sytox blue (SB) were used to stain the actin filaments and nuclei respectively (5). Image processing of single channel scans to generate the overlaid image was done on Adobe Photoshop.

Electron microscopy

Cells were grown in hCPC media on glass cover slips placed in a 6-well plate. After 24 hours of culture, cells were transduced with lentiviruses following which coverslips were removed and rinsed before being fixed at room temperature with 1% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for one hour. After fixation, cover slips were rinsed and samples were dehydrated at room temperature through a graded series of ethanol solutions (25%, 35%, 50%, 70%, 75%, 85%, 95% and 100%). Samples were then dried using a Samdri-790 Critical Point Dryer and coated with 6-nm Platinum

(QT150 Sputter Coater). Scanning electron microscopy was performed on a FEI Quanta 450 FEG as described previously (11).

Evaluation of cell morphology

Images of cultured CPCs were obtained on a Leica DMIL microscope and outlines were traced using ImageJ software. Parameters such as area, “shape descriptors” and “Feret’s diameter” were measured, based on which the cross sectional area and roundness were determined (4). Feret/MinFeret was used to evaluate length/width ratio of each cell.

Experimental Animals

Transgenic mice harboring eGFP driven by the NS promoter (pNSeGFP) (12), cardiac specific overexpression of Pim-1 (Pim-1-WT) (13), global deletion of Pim-1 (Pim-1-KO) (13), and NS heterozygous knockout mice (NS+/-) (14) have been described before. Nontransgenic (NTg) mice were used as controls. Only males were used for adult NS+/- mice studies. Cardiac function determination using echocardiography analyses has been reported previously (3,6). Mice were euthanized by anoxia with 100% carbon-di-oxide, the heart was harvested and washed with cold PBS, both auricles and the right ventricle were separated from the left ventricle (LV) which was used for biochemical analyses. The base of the LV was used for protein, RNA and DNA analyses and the apex of the LV was fixed in formalin and processed for confocal microscopy analyses. All animal protocols and studies were approved by the review board of the Institutional Animal Care and Use Committee at San Diego State University.

Human studies

Study was approved by the institutional review committees at San Diego State University and Temple University School of Medicine. Written informed consent was received from human patients prior to inclusion in this study.

Statistics

All data are expressed as mean +/- SEM. Pearson correlation analyses and statistical analyses were done using paired or unpaired Student’s t-test, one way-ANOVA with Newman-Keuls multiple comparison test or using two way ANOVA with Bonferroni post-tests on Graph Pad Prism v5.0. A value of $p<0.05$ was considered statistically significant.

Figure Legends

Figure S1. Cellular flattening of CPCs. A-D. Confocal microscopic image of AhCPCs at late passage (Line H10-001, p13) stained with phalloidin (green) and a nuclear stain, sytox blue (red) indicating a morphologically altered (white arrow) and unaltered cell (yellow arrow, **A**.) **B-C.** Z-stack images of the cells indicated by arrows demonstrating that the

morphological alteration of cell flattening is evident three dimensionally as thinner cells. Z-stack images have been shown to the bottom and right of the overlaid images. The white lines indicate the sectioning axes to visualize the Z-stack. **D.** 3-dimensional topographical view of the Z-stack with the colors indicating their thickness.

Figure S2. Senescence characteristics of CPCs. A. OCPCs have reduced proliferation rate relative to YCPCs as determined by Cyquant assay (N=4). **B.** OCPCs have increased G1, decreased S and G2 phase populations determined by DNA content measurements using FACS (N=5). **C.** Bi- and polynucleate OCPCs are predominantly SA-β-gal+ as seen from percentage distribution of cells based on nuclei number and SA-β-gal activity (N=1200 cells). **D.** Confocal microscopy of myocardial sections in 12 month (12mo) and 3 month old (3mo) mice stained for c-kit (green), nucleus (sytox blue (SB), white), senescence marker γ-H2AX (red) and sarcomeric actin (s-actin, blue). Insets **(*a*)** and **(*b*)** indicate higher magnification of overlaid image and selected single channel scans. Arrows point to CPC. **E.** 12 month old FVB mice (12mo) have more CPCs positive for senescence marker γ-H2AX than 3 month (3mo) old mice *in vivo*. **F.** More binucleate CPCs are observed with increasing age *in vivo*. **G.** Percentage distribution of cells based on nuclei number and expression of γ-H2AX (12 mo: N= 17 cells from 4 mice, 3 mo: N=17 cells from 3 mice). **H.** Cell autonomous role for diminished NS expression and binucleation is established from confocal microscopic image of cultured OCPCs stained with Phalloidin (Phal, grey), NS (red) and SB (blue). White arrows indicate binucleate cells with decreased NS. Red arrowheads indicate cells expressing NS. **I.** Confocal microscopic images of FhCPCs (line FH05) and AhCPC (H13-068) stained with Phal (grey), NS (red), SB (blue). Yellow and white arrows respectively indicate morphologically altered and binucleate AhCPCs with decreased NS. Scale bar indicates 50μm (D), 100μm (H,I). Human CPCs at mid passages were used.

Figure S3. Loss of NS causes CPC senescence. A. Morphometric analyses of relative cross sectional area (Rel.CSA), roundness and length/width ratio reveals that NS knockdown (Lt-sh-NS) causes cell flattening in AhCPCs (N>30 cells, 3 experiments each). **B.** Morphological changes caused by NS knockdown are also evident by comparing forward scatter areas (FSC-A) from FACS analyses of YCPCs. **C-F.** YCPCs were transduced with transduced with indicated lentiviruses and stained with SA-β-gal. **C.** Phase contrast microscopic images with representative binucleate (binuc.) and polynucleate (polynuc.) SA-β-gal+ cells upon NS knockdown are shown in the lower panel **D.** Senescent cell counts. **E-F.** Percentage of multinucleate cells **(E)** and CPCs that are multinucleate and SA-β-gal+ **(F).** (**C-F**: N>1700 cells). Scale bar indicates 100μm. *p<0.05, **p<0.01, ***p<0.001 (relative to each corresponding group in Lt-sh-control in **A, E**). The black and red lines and * in **E** and **F**. indicate comparison between different mononucleate and binucleate groups respectively.

Figure S4. Loss of NS inhibits proliferation and differentiation without affecting basal cell death of CPCs. A. NS knockdown increases G1 and decreases S and G2 phases of the cell cycle, determined by DNA content measurements using FACS in YCPCs (N=6). **B.** NS is silenced in YCPCs expressing FUCCI reporter construct harboring Azami green-tagged Geminin (AzG). NS knockdown reduces the AzG+ cells indicative of decreased S-M phase as determined by FACS (N=4). **C.** NS knockdown upregulates senescence markers p53 and p16 in AhCPCs (Line H11-020) **D.** Loss of NS does not increase basal cell death as determined by representative FACS histograms (left) and mean percentage of dead cells (right) in YCPCs (N=7). **E.** NS silencing decreases the basal mRNA level of c-kit and lineage commitment markers SM-22, α-smooth muscle actin (α-SMA) and myocyte enhancement factor-2c (MEF2c) as seen by qRTPCR analyses in YCPCs. Dashed line indicates value of Lt-sh-control (N=3 for each gene). * indicates $p<0.05$, ** indicates $p<0.01$ vs Lt-sh-control. N.S not significant.

Figure S5. Knockdown of p53 restores CPC morphology lost upon NS silencing. A-B. Phase contrast micrographs of AhCPC (Line H10-005) and YCPCs with knockdown of p53 and/or NS. **C.** NS knockdown-induced loss of CPC morphology is prevented by p53 silencing as indicated by morphometric measurements of relative cross sectional area, length/width ratio and roundness in YCPCs (N>30 cells from 3 experiments). **D.** Silencing p53 and NS decreases SA-β-gal+ mononucleate cells without affecting senescent multinucleate cells relative to sh-NS, as seen by percentage distribution of YCPCs based on nuclei number and SA-β-gal+ activity (N=700 cells, 3 experiments). Scale bar indicates 100μm. ** indicates $p<0.01$.

Figure S6. Pim-1 regulates NS. A-B. Pim-1 overexpression in AhCPC (hCPCeP) increases NS expression relative to AhCPC expressing eGFP (hCPCe), as demonstrated by immunoblot and densitometric analyses. Fast growing line H10-014 at mid (p5-7) and late passages (p10) was used **(A).** Slow growing line H10-004 fast at late passage (p10) was used (N=3). *$p<0.05$, ** indicates $p<0.01$, *** $p<0.001$.

Figure S7. Senescence markers in NS+/- mice. Immunoblot and denistometric analyses show that NS heterozygous knockout mice (NS+/-) have increased cardiac p53 expression, but not p16, by 1 month of age relative to nontransgenic mice (NTg) (N=4 NTg, 4 NS+/-).

Supplemental Table 1. M-mode echocardiography based functional analyses of NS+/- mice at different ages. Key: NTg: Non-transgenic, NS+/-: mouse with heterozygous deletion of NS, (s): systole, (d): diastole, LVAW: left ventricular anterior wall, LVID: left ventricular inner diameter, LVPW: left ventricular posterior wall, EF: ejection fraction, FS: fractional shortening, LV vol: left ventricular volume. N indicates number of mice. Only male mice were used in the study. * $p<0.05$ vs age-matched NTg.

Supplemental Table 2. Patient information for human samples. Key: W: Weeks, Y: Years, M: Male, F: Female, NYHA: New York Heart Association, EF%: Ejection Fraction, HyperLipid: Hyperlipidemia, N/Ap: Not Applicable (criteria not applicable), N/Av: Not Available (Patient information not available). For fetal and adult failing heart samples - C: CPCs obtained, H: Heart tissue used for biochemical analyses, C/H: both CPCs and heart tissue used. Patient information for many AhCPC lines used in the study has been recently published (4) and this table represents an update with inclusion of more samples.

Supplemental Table 3. Antibody list.

Supplemental Table 4. Primer list.

Supplemental Table 1: M-mode echocardiography based functional analyses of NS+/- mice

		1 month		3 month		6 month	
Parameter	**Units**	**NTg**	**NS+/-**	**NTg**	**NS+/-**	**NTg**	**NS+/-**
LVAW (s)	mm	1.45	1.16	1.25	1.01 *	1.24	1.34
LVAW (d)	mm	1.05	0.76 *	0.86	0.83	0.81	0.95 *
LVID (s)	mm	2.21	2.09	2.93	3.33 *	2.83	3.28 *
LVID (d)	mm	3.27	3.32	3.82	3.99	3.86	4.11
LVPW (s)	mm	1.31	1.21	0.97	0.9	1.16	0.86 *
LVPW (d)	mm	0.97	0.67	0.69	0.78	0.91	0.71
EF	%	61.85	67.5	46.64	32.37 *	55.08	41.30 *
FS	%	32.93	37.59	23.21	18.66	28.38	20.01 *
LV Vol (s)	µl	17.39	16.01	33.31	45.15 *	31.22	43.7 *
LV Vol (d)	µl	44.43	45.98	62.75	70.15	64.65	74.91
N		4	3	6	5	6	5

Supplemental Table 2: Patient Information for Human Samples

Sample ID	Sample Type	Age	Sex	NYHA	EF%	Cardiac Index	Diabetes	Hyper Lipid.	Smoking	Infarct	Ischemia	Ace Inhibitor	β-Blocker	Anti-Coagulant
FH-01 (C)	Fetal	16 w	F	NOT APPLICABLE (N/Ap)										
FH-03 (H)		16 w	N/Av											
FH-04 (H)		16 w												
FH-05 (C/H)		17 w												
FH-08 (C/H)		16 w												
H10-001 (C)	Adult, Failing	68 Y	M	IV	11	1.6	Yes	No	No	No	No	Yes	No	Aspirin
H10-004 (C)		82 Y	M	IV	8	2	Yes	Yes	1 pack/day for 30 Years	Yes (Multiple)	Yes	No	No	Aspirin
H10-005 (C)		52 Y	M	IV	20	1.3	Yes	No	No	Yes	Yes	Yes	Yes	Aspirin
H10-014 (C)		73 Y	M	IV	17	1.6	No	No	Yes, stopped 25 Years ago	Yes (Multiple with recent cardio-genic shock)	Yes	No	No	Aspirin
H11-020 (C)		68 Y	M	IV	20	1.7	No	No	No	No	No	No	No	Aspirin
H11-039 (H)		65 Y	M	NOT AVAILABLE (N/Av)										
H12-041 (H)		59 Y	M											
H12-043 (C)		42 Y	M	IV	20	1.6	No	No	No	No	No	Yes	Yes	Aspirin
H12-044 (H)		81 Y	M	IV	14	1.47	Yes	Yes	Yes, stopped 29 Years ago	Yes (Multiple)	Yes	No	No	Aspirin
H12-045 (C/H)		75 Y	M	IV	19	2.4	No	Yes	No	Yes	Yes	No	No	Aspirin
H12-046 (H)		47 Y	M	IV	20	1.3	No	No	No	No	No	No	No	Aspirin
H12-047 (C/H)		72 Y	M	IV	8	1.1	Yes	No	No	No	No	No	No	Aspirin
H12-053 (C)		61 Y	M	IV	15	1.75	Yes	Yes	No	Yes	Yes	Yes	Yes	Aspirin
H13-061 (C)		65 Y	M	IV	20	1.62	No	Yes	No	No	No	No	Yes	Aspirin
H13-068 (C)		62 Y	M	III	20	1.6	Yes	Yes	Yes, stopped 3 Years ago	Yes	Yes	Yes	Yes	Aspirin
AD NF 322	Adult, Non-Failing	75 Y	M	N/Ap	Normal > 50%	NOT AVAILABLE (N/Av)								
AD NF 331		57 Y	F											
AD NF 365		58 Y	F											
AD NF 443		72 Y	F											
AD NF 446		40 Y	M											
AD NF 506		76 Y	M											

Supplemental Table 3: Antibody list

Antibody / Species raised	Company	Catalog	Dilution	Application
NS / goat	R&D Systems	AF1638	1:500, 1:100	IB, IHC
NS / rabbit	Santa Cruz	sc-67012	1:500	IB
p53 / mouse	Abcam	ab26	1:500	IB
p53 / rabbit	Santa Cruz	sc-6243	1:1000	IB
p16 / mouse	Santa Cruz	sc-55600 sc-1661	1:100	IB
c-kit / goat	R&D Systems	AF1356	1:1000, 1:50	IB, IHC
c-Myc / mouse	Santa Cruz	sc-42	1:500	IB
c-Myc / mouse	Zymed		1:500	IB
c-Myc/ rabbit	Cell Signaling	5605	1.500	IB
Pim-1 / mouse	Zymed	394600	1:1000	IB
α-SMA / mouse	Sigma	A2547	1:1000	IB
α-Tubulin / rabbit	Cell Signaling	2144	1:1000	IB
β-actin / mouse	Santa Cruz	sc-81178	1:1000	IB
BrdU / rat	Novus Biologicals	NB500-169	1:100	FACS
Cyclin D1 / rabbit	Biosource	AHF0102	1:500	IB
GAPDH / mouse	Millipore	MAB374	1:1000	IB
GFP / goat	Rockland	600-101-215	1:500	IB
γ-H2AX / rabbit	Millipore	MABE205	1:500	IB
α-Sarcomeric actin / mouse	Sigma	A2172	1:100	IHC
Aurora B / rabbit	Zymed	365200	1:200	IB
Calsequestrin	Thermo Scientific	PA1-913	1:1000	IB
Oct 4/rabbit	Abcam	Ab19857	1:500	IB
Nanog	Millipore	AB5731	1:500	IB
KLF4/rabbit	Cell Signaling	4038	1:500	IB

Supplemental Table 4: Primer List

Primer Name	Forward/Reverse	Sequence
c-kit	Forward Reverse	ATTGTGCTGGATGGATGGAT GATCTGCTCTGCGTCCTGTT
SM22	Forward Reverse	GACTGCACTTCTCGGCTCAT CCGAAGCTACTCTCCTTCCA
α-SMA	Forward Reverse	GTTCAGTGGTGCCTCTGTCA ACTGGGACGACATGGAAAAG
MEF2c	Forward Reverse	GCACAGCTCAGTTCCCAAAT TGGAGAGATGAAGTGAAGCG
NS (mouse) NS (human)	Forward Reverse Forward Reverse	GGGAGCTGTCACCTGAGCAA CATCCTCTTGACTCGCTCTATCC GGGAAGATAACCAAGCGTGTG CCTCCAAGAAGTTTCCAAAGG
18S	Forward Reverse	CGAGCCGCCTGGATACC CATGGCCTCAGTTCCGAAAA

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