Supplemental Material

Patient Population. Patients undergoing aortic valve surgery were enrolled in the study following Institutional Review Board (IRB) approved guidelines of University of Pennsylvania Perelman School of Medicine. Informed consent was obtained for the subject enrollment and clinical information was obtained by patient interview and chart review. Exclusion criteria for the study included: presence of bicuspid aortic valve, premature menopause and/or osteoporosis, prior aortic valve surgery, rheumatic heart disease, endocarditis, active malignancy, chronic liver failure, calcium regulation disorders (hyperparathyroidism, hyperthyroidism, and hypothyroidism), serum creatinine ≥ 1.5mg/dl, chronic or acute inflammatory states (sepsis, autoimmune disease, and inflammatory bowel disease, etc), and other pulmonary diseases. Control tissues were obtained through collaboration with the heart transplant research program of the University of Pennsylvania Perelman School of Medicine and The Gift of Life Program.
Echocardiographic and Doppler data. All patients underwent a comprehensive echocardiographic assessment including, M-mode, two-dimensional and Color Doppler, conducted by a certified echo cardiographer using commercially available ultrasound systems. All measurements were performed according to the American Society of Echocardiography recommendations. The presence of aortic stenosis was defined as an Aortic Valve Area (AVA) <2.0 cm^2. Aortic valve calcification was assessed, and a calcium score of 1 to 4 was assigned by a single cardiologist based on the method described as: 1 - no calcification; 2 - mildly calcified (small isolated spots); 3 - moderately calcified (multiple larger spots); 4 - severely calcified (extensive thickening and calcification of all cusps).

Supplemental Table I

A – Demographics

Demographics	Controls N =13		Aortic Sclerosis N =13		Aortic Stenosis N =13		p
Age	45.2	± 15.3	57.8	± 10.3	77.2	± 8.2	< 0.001
Male subjects	9	(69.2%)	10	(76.9%)	3	(23.1%)	0.013
Smokers	5	(38.5%)	5	(38.5%)	5	(38.5%)	0.983
Diabetes	2	(15.4%	1	(7.7%)	4	(30.8%)	0.359
Hypertension	4	(30.8%)	6	(46.2%)	11	(84.6%)	0.033
Cerebral vascular accident	1	(7.7%)	NA		NA		0.353
Coronary artery disease	1	(7.7%)	2	(15.4%)	5	(38.5%)	0.174
End Stage Congestive Heart Failure	NA		8	(61.5%)	1	(7.7%)	<0.001
Heart Failure	NA		5	(38.5%)	2	(15.4%)	0.193
Hyperlipidemia	3	(23.0%)	2	(15.4%)	7	(53.9%)	0.119

B- Echocardiographic Measurements

Demographics	Controls N =13	Aortic Sclerosis N =13	Aortic Stenosis N =13	p
Aortic Valve Area (AVA) (cm^2)	>2	2.3 (±0.5)	0.7(±0.2)	< 0.01
Doppler Velocity (m/s)	<2	0.5 (±0.9)	3.7 (±1.01)	< 0.01
Calcium Score (1-4)	1	1.6 (±0.5)	3.5 (±0.5)	< 0.01

Histological analysis. Histological analysis of the aortic valve tissues; Hematoxylin and Eosin (H&E), Modified Movat Pentachrome staining (for proteoglycans, elastin and collagen) and

Alizarin Red staining (for calcium) was performed according to the protocol of the Histology Laboratory of the University Of Pennsylvania Perelman School of Medicine.

TUNEL Assay. Paraffin-embedded tissue sections were dewaxed at 60 °C for 15 min, washed in xylene, and then rehydrated through a graded series of ethanol and distilled water. After incubation with proteinase K for 20 min, sections were kept in a permeability solution (0.1% Triton X-100 in PBS) at room temperature for 5 minutes and subsequently incubated with TUNEL reaction mixture (Promega) for 60 min at 37 °C in a humidified chamber. Blocking was performed in 0.3% H_2O_2 in methanol for 5 min, followed by incubation with streptavidin–HRP solution for 30 min at room temperature, 3,3′-diaminobenzidine solution for 10 min and HRP-coupled anti-mouse IgG staining. A negative control using all reagents except terminal transferase was performed in parallel. Images were taken using Olympus Fluoview 1000 Confocal microscope. For cultured VICs, cells were grown in 8 well chamber slides and treated with 1mM H_2O_2 for 1hr in PBS and allowed to recover for 24 hours. Cells were washed with PBS and fixed with 4% paraformaldehyde followed by incubation with equilibration buffer for 10 minutes. Slides were subsequently incubated with 50 µl TUNEL reaction mixture for 60 min at 37 °C in a humidified chamber, 30 min with 50 µl Streptavidin–HRP solution, and then incubated with 60 µl 3,3′-diaminobenzidine (DAB) solution for 10 min. Coverslips were mounted and images were captured by Leica 4000B microscope (Leica Microsystems, IL). A negative control using all reagents except terminal transferase was performed in parallel. The nucleus of positive cells was stained brown as detected under light microscopy.

RNA isolation. RNA extraction was performed using the RNeasy Fibrous tissue kit (QIAGEN, Valencia, CA) and homogenizing the entire AV cusps obtained from Controls (n=3, Calcium Score=1) AVSc (n=3, Calcium Score=1-2) and AVS (n=3, Calcium score=3-4). RNA concentrations were measured spectrophotometrically at 260 nm (Nanodrop). RNA quality and integrity were determined utilizing an Agilent 2100Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and absorbance at A260/A280. Only high quality RNA, having a RIN of >7.0, and an A260/280 absorbance ratio of >1.8, was utilized for further analysis.

Isolation of Aortic Valvular Interstitial Cells (VIC). Isolation of aortic VICs was performed using a modified method described before. Aortic cusps were placed in 2 mg/ml type II collagenase (Worthington Biochemical Corp., Worthington, VA) in Dulbecco's modified Eagle's medium containing 1% Penicillin/Streptomycin solution and incubated in a shaker for 20 min at 37°C. Loosened endothelial layer was removed by wiping the cusp surfaces with sterile cotton swabs. Tissues were then finely minced and dissociated in type II collagenase (1mg/ml) and hyaluronidase (100U/ml) for 4 h at 37°C. The resulting VICs were seeded in tissue culture plates in DMEM media and maintained at 37°C and 5% CO_2. VICs growth medium contained Advanced DMEM supplemented with 10% Fetal Bovine Serum (Thermo Scientific, Hudson, NH), and 1% Penicillin/Streptomycin solution (Life Technologies, Carlsbad, CA). All the experiments were performed on cultured cells between their second and fifth passages. Isolated cells were banked in liquid nitrogen for further studies.

H_2O_2 treatment of human-derived VICs. To test cellular transdifferentiation, VICs were exposed to 0.1 mM H_20_2 in osteogenic media for 15 days with media change every three days. The effect of oxidative stress on the expression of bone-related (Runx-2, MSX-2 and OPN) was determined by Real time PCR. Total RNA was isolated from VICs using the RNA extraction kit (QIAgen) and reverse transcribed into cDNA. SYBR Green chemistry-based Real Time PCR was performed using the 7500 Fast PCR protocol from Applied Biosystems. Briefly, 1µg total RNA was converted to cDNA using the RT reagents (Applied Biosystems, CA) and 20ng of total cDNA was used for the subsequent PCR amplification. Gene expression was normalized to Beta Actin and the relative quantification of the transcripts was determined using the ddCT method. The analysis was performed using VICs Control untreated as basal.

Immunofluoresence analysis. VICs were cultured on glass coverslips and treated with H_20_2. Cells were fixed in an ice-cold 1:1 methanol:acetone mixture for 10 minutes. Cells were subsequently permeabilized with 0.5% Triton for 5 minutes at 4°C and then incubated with the appropriate primary antibody for 20 minutes at 37°C. Cells were then washed with PBS–Tween 20 and incubated with secondary antibody for 30 minutes at 37°C. Following washing, coverslips were mounted onto glass slides using Vectashield mounting media with DAPI (Vector Labs) and visualized with an Olympus Fluoview 1000 Confocal microscope. The following antibodies were used: 8-Oxo-dG (ab64548, Abcam), γH2AX (JBW301, Millipore), MRE11 (ab33125, Abcam), XRCC1 (2735, Cell Signaling)

Western blotting and Immunohistochemistry. Protein expression was demonstrated by western blot and immunofluorescence techniques using specific antibodies against Nitrotyrosine (MAB5404 clone 2A82), γH2AX (JBW301), 8-Oxo-dG (ab64548, Abcam), GAPDH (Ab9485, AbCam), Cyclin A (06-138, Millipore), RunX-2 (Abnova), αSMA (AbCam), pAKT (Cell Signalling).

Adenoviral transduction. Replication defective, type 5 Ad-SOD3, Ad-CAT and Ad-eGFP constructs under the CMV promoter for cell transduction with human extracellular superoxide dismutase, mouse catalase, and enhanced green fluorescent proteinwere purchased from Vector BioLabs and Penn Vector Core of the University of Pennsylvania, respectively. Transduction was performed using MOI:100. VICs were seeded and cultured up to 80% confluency in Advanced DMEM and transduced overnight. Twenty-four or forty-eight hours after transduction cells were treated with H_2O_2 to induce DNA damage, cellular transdifferentiation and calcification. To test the effect of Ad-SOD3 and Ad-CAT on the activation of osteogenic genes, VICs were transduced and kept in osteogenic media with or without H_2O_2 for 15 days. Transduction was repeated every four days. At the end of the experiment VIC were either fixed for immunofluorescence or harvested for Western blotting or RealTime analysis.

Comet Assay. The Comet assay was performed using a Comet Assay kit (Trevigen Inc, Gaithersburg, MD) according to the manufacturer's instructions. VICs were treated with 1mM H_2O_2 in PBS for 1 hour at 37C. The cells were collected in PBS followed by mixing the cells suspension with liquefied agarose at a 1:10 (vol/vol) ratio. 50ul of this mixture was immediately transferred onto the slide provided. After cell-lysis at 4°C, slides were treat ed with alkali solution (0.3MNaOH, 1mM EDTA) for 30 min to unwind the double stranded DNA. Slides were electrophoresed at 1 V/cm for 20 min. After staining with SYBR green dye, samples were visualized and photographed by fluorescent microscope. Tail length was defined as the distance between the leading edge of the nucleus and the end of the tail. Image analysis was carried out using Comet Score software (Tritek Corporation, VA).

***In Vitro* Calcification.** VICs were treated with 1mM H_2O_2 in PBS for 1 hour and allowed to recover for 7 days in the presence or absence of Ad-SOD3 or Ad-CAT. At day 7, VICs were incubated overnight at 4°C in 0.6N HCL. Calcium was estimated using the calcium assay kit (Biovision, Mountain View, CA). After washing the cells twice with 1× PBS, cells were harvested using 1N NaOH+0.3% SDS, and total protein was estimated. Amount of total calcium was expressed as μg of calcium/μgof proteins.

Supplemental Figure I

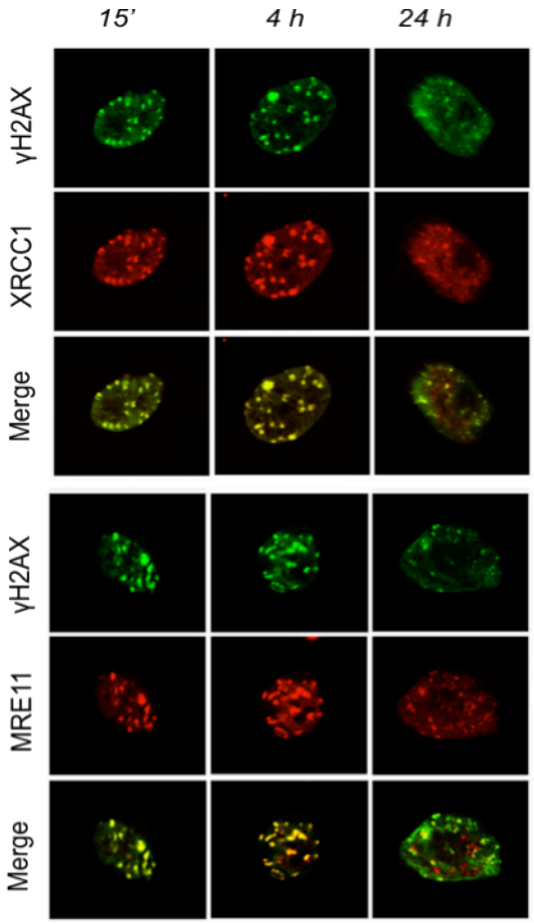

Fig SI. XRCC1 and Mre11 colocalize with γH2AX in control VICs exposed to H_2O_2 at different recovery time. (A) Immunofluorescence images showing colocalization of γH2AX (green) and XRCC1 (red) 24 hours after exposure to H_2O_2 in VICs isolated from AVC patients. (B) γH2AX (green) and Mre11 (red) expression under the conditions described above. Magnification 100X.

Supplemental Table II

Gene		Primer sequence
OPN	Fw	5'- TTG CAG CCT TCT CAG CCA A -3'
	Rv	5'- GGA GGC AAA AGC AAA TCA CTG -3'
RUNX2	Fw	5'- CCA ACC CAC GAA TGC ACT ATC -3'
	Rv	5'- TAG TGA GTG GTG GCG GAC ATA C -3'
MSX2	Fw	5'- CCT ACC CGT TCC ATA GAC CT-3'
	Rv	5'- TAC TGG CTG GTA CTG CCT TC-3'
SOD1	Fw	5'- TCA GGA GAC CAT TGC ATC AT-3'
	Rv	5'- GAA TGT TTA TTG GGC GAT CC-3'
SOD2	Fw	5'- TGT CAC CCA GTG GTT TTT GT-3'
	Rv	5'- GCC CTG CAA ATA AAC ATC CT-3'
SOD3	Fw	5'- TCC TCT GCT CCA ACA GAC AC-3'
	Rv	5'- GGA GGC CTT CAG ACC TAC TG-3'
CAT	Fw	5'- ACT TCT GGA GCC TAC GTC CT-3'
	Rv	5'- CGC ATC TTC AAC AGA AAG GT-3'
ActinB	Fw	5'- AGG GGC CGG ACT CGT CAT ACT -3'
	Rv	5'- GGC GGC ACC ACC ATG TAC CCT -3'