Antioxidant Enzymes Reduce DNA Damage and Early Activation of Valvular Interstitial Cells in Aortic Valve Sclerosis

Emanuela Branchetti, Rachana Sainger, Paolo Poggio, Juan B. Grau, Jeffrey Patterson-Fortin, Joseph E. Bavaria, Michael Chorny, Eric Lai, Robert C. Gorman, Robert J. Levy, Giovanni Ferrari

Objective—Accumulation of reactive oxygen species (ROS) and remodeling of the microstructure of the cusp characterize aortic valve sclerosis, the early phase of calcific aortic valve disease. These events are associated with activation of valvular interstitial cells (VICs) toward an osteogenic-like phenotype. Because ROS cause DNA damage and transcriptional activation we investigated the relationship between ROS, DNA damage response, and transdifferentiation of VICs.

Methods and Results—Human aortic valve cusps and patient-matched VICs were collected from 39 patients both with and without calcific aortic valve disease. VICs were exposed to hydrogen peroxide (0.1–1 mmol/L) after cell transduction with extracellular superoxide dismutase/catalase adenoviruses and characterized for DNA-damage response, osteogenic transdifferentiation, and calcification. ROS induce relocalization of phosphorylated γH2AX, MRE11, and XRCC1 proteins with expression of osteogenic signaling molecule RUNX2 via AKT. We report a sustained activation of γH2AX in aortic valve sclerosis-derived VICs suggesting their impaired ability to repair DNA damage. Adenovirus superoxide dismutase/catalase transduction decreases ROS-induced DNA damage and VIC transdifferentiation in aortic valve sclerosis-derived cells. Finally, adenoviral transduction with catalase reverts ROS-mediated calcification and cellular transdifferentiation.

Conclusion—we conclude that the ROS-induced DNA damage response is dysfunctional in early asymptomatic stages of calcific aortic valve disease. We unveiled an association among ROS, DNA damage response, and cellular transdifferentiation, reversible by antioxidant enzymes delivery. (Artroscler Thromb Vasc Biol. 2013;33:XXX-XXX.)

Key Words: aortic valve sclerosis ■ DNA-damage response ■ reactive oxygen species ■ valvular interstitial cells

Calcific aortic valve disease (CAVD) is an active multifactorial process more common with increasing age, although not an inevitable consequence of aging. Over the last decade, several clinical trials have been performed to halt the progression of CAVD. Early enthusiastic findings, documenting a reduction in the progression of the disorder, have been questioned by later randomized studies, which show substantial equivalence between treatments and placebo. It has been proposed that the therapy may have been initiated too late in the course of the disease to be effective. Initial asymptomatic phases of CAVD include mild thickening of the cusps, without affecting the mechanical proprieties of the valve, a condition called aortic valve sclerosis (AVSc). Advanced stages are associated with impaired leaflets motion, resistance to blood flow, and biomineralization (aortic valve stenosis–AVS). The prevalence of AVSc has been estimated at 25% to 30% in patients >65 years of age and up to 40% in those >75 years of age. These patients are largely asymptomatic, and challenging to identify, because of the variable and qualitative nature of AVSc description by echocardiographic evaluation. Once AVSc is detectable, there is an increased risk of cardiovascular events from the expected event-free survival. At the onset of early symptoms (stenosis), survival curve deviates even more from expected, with a dramatic decline in case of severe symptomatic AVS. Despite its high prevalence, little is known about the early stages of development of aortic valve disease and the initiating pathogenetic mechanisms determining the thickening of the cusps, the activation of valvular interstitial cells (VICs), and their transdifferentiation into osteoblastic-like cells. In addition, asymptomatic AVSc tissues are generally not available to investigators because these valves are not surgically replaced until moderate to severe stenosis occurs. At a microstructural level CAVD is characterized by extensive remodeling of the cusps with biomineralization of the fibrosa layer and by VICs adopting an osteogenic-like...
phenotype with expression of markers, such as osteopontin (OPN), osteonectin, and transcription factors, runt-related transcription factor 2 (Runx2) and muscle segment homeobox 2 (MSX2). Our previous work suggested that matricellular proteins, such as OPN, could be used for the identification of asymptomatic patients even before calcium deposition is detectable by echocardiography. However, the cellular mechanisms responsible for the early transdifferentiation of VICs remain unidentified. Recent studies support the concept that reactive oxygen species (ROS), likely produced by inflammatory infiltrates, play an important role in the development of the early cellular and extracellular changes associated with AVSc. Evidence of increased oxidative stress has been shown in experimental mouse models of valve stenosis, suggesting that the oxidant environment is not merely the consequence of increased cusp stress associated with calcification. ROS have also been implicated in pro-osteogenic and profibrotic signaling cascades, exogenous ROS accelerates calcification of vascular smooth muscle cells in vitro. In this regard, transcription factors, such as Runx2 and MSX2, have been shown to directly contribute to vascular calcification. Furthermore, antioxidant enzymes, such as superoxide dismutases (SOD) and catalase (CAT), are downregulated in calcified region of human aortic valves.

Several studies indicate that ROS can cause DNA strand breaks and base modifications. Elaborate cellular repair and genome surveillance mechanisms counteract genomic damage induced by ROS. The DNA damage response (DDR) process is manifested cytologically by the formation of DNA-repair foci. These subnuclear structures are formed by the recruitment and accumulation of DNA-repair factors at sites of DNA damage. DNA damage results in rapid phosphorylation of γH2AX by PI3K-like kinases, including ATM, ATR, and DNA-PK, regulatory kinases of DDR. γH2AX is required for checkpoint-mediated cell cycle arrest and DNA repair after single and double-stranded DNA breaks. This very early event in the DDR is required for recruitment of a subsequent multitude of proteins, including MDC1, XRCC1, RAD50, MRE11, 53BP1, and BRCA1. In a recent study, it has been proposed that increased transcript level of poly(ADP-ribose) polymerase-1 in human tricuspid compared with bicuspid aortic valves correlates with stenosis severity. The study showed transcriptional activation of the DNA damage nick sensor protein poly(ADP-ribose) polymerase-1 in stenotic valves and in VICs. In addition, DNA strand breaks and poly(ADP-ribose) polymerase-1 promote recruiting of homologous recombination factors, such as ATM, ATR, and other DDR kinases.

Here, we explore the intriguing hypothesis that ROS act on the valve cusp microstructure through the activation of the DDR mechanism, inducing VICs to adopt an osteogenic phenotype typical of advanced disease phases. Oxidative stress was analyzed in explanted aortic valve tissues by immunostaining for nitrotyrosine, a product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide. We provide evidence that ROS promote extensive oxidative DNA damage on VICs isolated from patients at different stages of CAVD. We analyzed the subnuclear relocalization of γH2AX, MRE11, and XRCC1 proteins in repair foci. Furthermore, for the first time using AVSc-derived VICs, we tested the ability of ROS to induce the expression of early osteogenic markers such as RUNX2 and MSX2 and in vitro calcification. Finally, using adenoviral transduction of SOD3 and CAT, we tested the ability of these enzymes to protect VIC from DNA damage and early phenotypic alteration.

Patients and Methods

Patient Population

Subject enrollment for the present study was performed at the Perelman School of Medicine of the University of Pennsylvania based on comprehensive echocardiographic assessment, and aortic valves were assigned calcium scores of 1 to 4. (Complete patient enrollment and demographics details are provided in Table I and Methods in the online-only Data Supplement).

Antioxidant Enzymes Expression and Activity

RNA isolation was performed on frozen tissue using the RNA Extraction Kit for Fibrotic Tissue (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Real-time polymerase chain reaction was performed by SYBR green chemistry using the 7500 Fast PCR protocol (Applied Biosystems). The relative quantification of the transcripts was determined using the ddCT method calculated using the SDS software version 1.4.0. Gene expression level was standardized to actinB, and fold changes were calculated using aortic valve calcification (AVC) tissues as basal. The complete list of primer used for real-time analysis is provided in Table II in the online-only Data Supplement. SOD and CAT activity assay kits (Biovision, CA) were used to test the activity of these enzymes in the tissue of AVC, AVSc, and AVS patients.

Histological Dot Blot and Western Blotting Analysis

Protein expression was analyzed using whole tissue extract from explanted aortic valve or whole cells extract from isolated VICs using standard protocol as described in the online-only Data Supplement.

Isolation of Human Aortic VICs

Isolation of aortic VICs was performed using a modification of the method previously described. All the experiments were performed with cultured cells between the second and fifth passages. Cells were grown in Advanced DMEM supplemented with 10% FBS, L-glutamine, Pen/Strep and Fungicid at 37°C, and 5% CO2.

Hydrogen Peroxide Treatment of Human-derived VICs

To induce DNA damage, human VICs isolated from AVC, AVSc, and AVS patients were treated with 1 mmol/L hydrogen peroxide (H2O2) in PBS for 1 hour at 37°C. Cells were either fixed or harvested 15 minutes, 4 hours, or 24 hours after treatment. To test cellular transdifferentiation, VICs were collected at 1, 4, and 7 days after being exposed to 1 mmol/L H2O2 in PBS for 1 hour or after 15 days of 0.1 mmol/L H2O2 exposure in osteogenic media with media changed every 3 days. The effect of oxidative stress on the expression of bone-related markers was determined by real time polymerase chain reaction and Western blotting.

Immunofluorescence Analysis

VICs were cultured on glass coverslips and treated with H2O2. After fixation, immunofluorescence was performed using confocal microscopy as described in the online-only Data Supplement.

Adenoviral Transduction

Replication-defective, type 5 Ad-SOD3, Ad-CAT, and Ad-eGFP constructs under the CMV promoter for cell transduction with human...
extracellular SOD, mouse CAT, and enhanced green fluorescent protein were purchased from Vector BioLabs and Penn Vector Core of the University of Pennsylvania, respectively. Transduction was performed as described in Materials in the online-only Data Supplement.

DNA Damage and Apoptosis
Comet assay and TUNEL assay were performed following manufacturer’s instruction using VICs exposed with 1 mmol/L H₂O₂ in PBS for 1 hour, as described in Materials in the online-only Data Supplement.

Statistical Analysis
The data were analyzed using SPSS software (version 15; SPSS). Continuous variables were expressed as mean±SE of mean. Comparisons of continuous variables between groups were performed with the Student t test or nonparametric (Mann–Whitney U test) tests as appropriate, depending on normal distribution. A value of P<0.05 was considered to be statistically significant. Comparisons between >2 groups were performed using Kruskal–Wallis test, with post hoc pair wise Mann–Whitney tests using the Bonferroni correction to determine significance of difference between individual groups.

Results
Oxidative stress accumulation is associated with reduced antioxidant enzymes expression and increased DNA damage in the early stage of CAVD. In control (transplant derived) aortic valve tissue (AVC), nitrotyrosine staining is barely detectable and evenly distributed throughout the tissue (Figure 1A and 1B). In contrast, aortic valve tissue from patients with pathological dysfunctions of the valve (both aortic sclerosis and severe aortic stenosis) showed accumulation of nitrotyrosine, with side-specific accumulation in the fibrosa layers and accumulation of oxidative injuries around the calcified regions in AVS patients. Dot blot analysis, performed using whole cell extracts, from AVC-, AVSc- and AVS-derived tissues (n=6/group) confirmed that peroxynitrite levels were higher in calcified-stenotic (13.2±0.3 fold) and noncalcified sclerotic aortic valve tissues (14.9±0.5 fold) when compared with controls (Figure 1A and 1B; P<0.05). We then analyzed the expression of copper zinc SOD (SOD1), manganese SOD (SOD2), extracellular SOD (SOD3), and CAT (Figure 1C and 1D). mRNA analysis showed a differential downregulation of SODs expression linked to different phase of the disease with SOD1 significantly downregulated in the AVSc specimens, SOD2 downregulated in the severe stages of the disease, and SOD3 generally downregulated in both pathological stages of CAVD (Figure 1C). In accordance, SOD total activity was reduced in sclerotic and stenotic patients, whereas CAT activity was mostly reduced in AVS patients (P<0.05; Figure 1D).

Aortic valve tissues from controls also showed weaker immunoreactivity for oxidative DNA-damage marker 8-oxo-dG, whereas increased immunoreactivity of 8-oxo-dG was found in sclerotic tissues. Stenotic tissue showed strong 8-oxo-dG staining throughout all the 3 layers with highest immunoreactivity around calcifying foci (black arrows; Figure 1E and 1F).

Figure 1. Oxidative stress accumulation is associated with reduced antioxidant enzymes expression and increased DNA damage in the early stage of calcific aortic valve disease. A, Representative images of Alizarin red stained calcifications (calcium) and nitrotyrosine staining in aortic valve tissue from AVC (control), AVSc, and AVS patients. Magnification, 10×. B, Dot blot for nitrotyrosine and relative densitometry using whole tissue extract from AVC, AVSc, and AVS tissues. Images are representative of dot blot performed in n=6 tissue/group. C, Bar graphs show fold change gene expression for superoxide dismutase 1, 2, 3 (SOD1, 2, 3) and catalase (CAT) in aortic valve tissues. Real Time polymerase chain reaction data were normalized against actinB gene expression and AVC tissue was used as basal. D, SOD and CAT activity measured in aortic valve tissue (n=3/group). *represents P<0.05 and refers to values toward AVC. E, Representative images of 8-oxo-guanine staining in aortic valve tissue from AVC, AVSc, and AVS patients. Magnification, 10× to 60×. F, Quantification of 8-oxo-guanine staining. Positive nuclei were counted in 4 different field/tissue section and averaged (n=3 tissues/group analyzed). G, Quantification of apoptotic cells detected by TUNEL assay performed on tissues section. *represents P<0.05 and refers to values toward AVC. **represents P<0.05 and refers to values toward AVSc and AVC. AVC indicates aortic valve calcification; AVSc, aortic valve sclerosis; AVS, aortic valve stenosis; Ao, aortic; Ve, ventricularis; and Pt1, 2, 3, patient number 1, 2, 3.

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Severe DNA damage is often associated with increased rate of apoptosis in vitro. Accordingly, the analysis of native tissues demonstrated an increase in apoptosis between ex vivo control and sclerotic tissues with a significant increase in explanted, severely calcified, stenotic valves (Figure 1G).

Impaired DDR was found in noncalcified, asymptomatic, patients with AVS(Figure 2A, left panels). To examine how human VICs responded to oxidative DNA damage, cells were exposed to H\textsubscript{2}O\textsubscript{2} (from 0.1–1 mmol/L) and early (15 minutes–4 hours) and late (24 hours) DDR was tested. As shown in Figure 2A (left panels), 15 minutes after H\textsubscript{2}O\textsubscript{2} treatment phosphorylated H\textsubscript{2}AX (\(\gamma\)H2AX) begin to accumulate in the nuclei of control, sclerotic, and stenotic VICs. After 4 hours of recovery all 3 cellular population showed \(\gamma\)H2AX immunoreactivity with no differences in the nuclear localization (middle panel). Twenty-four hours after treatment, control cells showed the presence of very few repair foci, whereas sclerotic and stenotic VICs showed higher number of \(\gamma\)H2AX foci. Western blotting analysis on total protein extracts confirm an impaired DDR in AVSc and AVS interstitial cells (Figure 2B and 2C) with sustained activation of phosphorylated H\textsubscript{2}AX (Figure 2A–2C). H\textsubscript{2}AX phosphorylation and subnuclear relocalization is a very early event of the DNA-damage response system but could also be the result of alternative events of chromatin remodeling.44,45 To show that the presence and subnuclear relocalization of \(\gamma\)H2AX foci is a direct representation of oxidative-induced DDR mechanisms, and not because of chromatin remodeling, we tested the subnuclear colocalization of additional DDR enzymes, such as MRE11 and XRCC1. Figure 2D shows a colocalization of these proteins with \(\gamma\)H2AX foci. Consistently, VICs derived from controls show a similar response to that of \(\gamma\)H2AX (Figure 1 in the online-only Data Supplement).

Oxidative stress results in unresolved DNA damage in AVSc VICs. To test whether the sustained activation of \(\gamma\)H2AX foci is the result of a greater accumulation of DNA damage in AVS-derived VIC or a delay in the repair mechanism, we tested the level of DNA fragmentation and apoptosis after 1 mmol/L H\textsubscript{2}O\textsubscript{2} treatment. Single cell electrophoresis (Comet Assay) on isolated VICs from controls and AVSc patients suggest a similar degree of DNA damage after oxidative stress exposure and 4 hours of recovery. At a later time point (24 hours of recovery), the damage in controls VICs is significantly lower than in sclerotic cells (Figure 3A–3C). To confirm this observation, we noticed an increase in TUNEL-positive staining in 1 mmol/L H\textsubscript{2}O\textsubscript{2}-treated VICs from sclerotic patients when compared with controls (Figure 3D and 3E). Finally, cell cycle arrest and recovery of H\textsubscript{2}O\textsubscript{2}-treated cells were confirmed by the reduction of cyclin A expression, 15 minutes after H\textsubscript{2}O\textsubscript{2} treatments and recovery at later time-points (Figure 3F).

Oxidative stress modulates AVSc-derived VIC transdifferentiation via AKT signaling pathway. To directly test the impact of ROS on VIC transdifferentiation, AVSc-derived cells were tested for markers of osteogenic-like transdifferentiation and DNA damage up to 7 days after 1 mmol/L H\textsubscript{2}O\textsubscript{2}.
exposure. Figure 4A shows accumulation of markers of early osteogenic transdifferentiation, such as RUNX2, with concurrent reduction of myofibroblast marker α-smooth muscle actin. Accordingly with previous results, we also noticed a sustained activation of phosphorylated H2AX in AVSc VICs. Because RUNX2 has been reported to be modulated by AKT signaling, we tested the effect of AKT inhibitor IV on AVSc-derived cells induced to osteogenic transdifferentiation via H2O2 treatment. AVSc-derived VICs were pretreated with 0.1 μmol/L of AKT inhibitor IV, followed by H2O2 (1 mmol/L for 1 hour) and recovery. Figure 4B shows that H2O2-mediated RUNX2 accumulation at 24 hours of recovery is mediated by the activation of phospho-AKT signaling. Interestingly, H2O2-induced α-smooth muscle actin reduction at 24 hours is concomitant with the decrease in phospho-AKT activation.

Adenoviral transduction of antioxidant enzymes (SOD3 and CAT) rescues VICs from an impaired DDR and reduces the expression of early markers of VICs activation. Because AVSc and AVS VICs showed an impaired oxidative DDR, we tested whether adenoviral transduction of antioxidant enzymes (SOD3 and CAT) could rescue VICs. VICs from 8 control, 8 AVSc, and 8 AVS patients were isolated and exposed to 1 mmol/L H2O2 in the presence or absence of adenovirally transduced SOD3 and CAT. DDR was assayed by visualizing H2AX phosphorylation at 24 hours of recovery. GFP-adenoviral delivery was used to determine transduction efficiency (Figure 5A, left panel). To confirm the efficiency of the adenoviral transduction, SOD3 and CAT activities were tested with results showing increased enzymatic activity up to 7 days after transduction (Figure 5B). Immunofluorescence (Figure 5A) and Western blotting on total protein extracts (Figure 5C) revealed that Ad-SOD3 and Ad-CAT are able to rescue VICs from an impaired DDR (decrease in the expression of γH2AX). We then tested the relationship among

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**Figure 3.** Oxidative stress results in unresolved DNA damage in aortic valve sclerosis valvular interstitial cells (VICs). A, Representative pattern for comet assay to show DNA damage induced by 1 mmol/L H2O2 in AVC and AVSc-derived VICs. B, Single cell representing head and tail length. C, DNA in comet tail (mean±SE) calculated using CometScore software. *P<0.05. D, Percentage of apoptotic nuclei detected by TUNEL assay. Positive nuclei were counted in 4 different field/slide and averaged. E, TUNEL assay representative images. Magnification, 20x. F, Cyclin A expression detected by Western blotting and relative densitometry in VICs isolated from AVSc patients. Whole cell extract was made at different recovery time (15 minutes, 4 and 24 hours) after treatment with H2O2. *P<0.05. GAPDH was used as loading control. AVSc indicates aortic valve sclerosis; AVC, aortic valve calcification; and U, untreated VICs.

**Figure 4.** Oxidative stress modulates aortic valve sclerosis (AVSc)-derived valvular interstitial cell (VIC) transdifferentiation via AKT signaling pathway. A, Western blotting showing the expression of runt-related transcription factor 2 (RUNX2), α-smooth muscle actin (αSMA), and γH2AX using whole cell lysate from AVSc-derived VICs treated with 1 mmol/L H2O2 and recovered at different time (1d, 1 day; 4d, 4 days; 7d, 7 days). GAPDH was used as loading control. B, pAkt, RUNX2, αSMA expression 15′, 4 hours, 24 hours, after treatment with 1 mmol/L H2O2. GAPDH was used as loading control.  pAkt, RUNX2, αSMA expression 15’, 4 hours, 24 hours, after treatment with 1 mmol/L H2O2. **Figure 5.** Adenoviral transduction of antioxidant enzymes (SOD3 and CAT) rescues VICs from an impaired DDR and reduces the expression of early markers of VICs activation.
oxidative stress, DDR, and VICs transdifferentiation toward an osteogenic phenotype. AVSc-derived VICs were treated with 100 μmol/L H2O2 in osteogenic media for 15 days to determine the effect of oxidative stress on RUNX2, MSX2, and OPN expression. As shown in Figure 5D, ROS induce RUNX2, and MSX2 upregulation by 3.2- and 3.1-fold, respectively (P<0.05). These results are consistent with an early stage of osteogenic activation because both these markers have been reported to be initiators of osteogenic activation.28,31 Adenoviral delivery of SOD3 and CAT partially reduced RUNX2, MSX2, and OPN, suggesting a functional correlation between the DDR and early events of VICs transdifferentiation toward an osteogenic phenotype (Figure 5D).

Adenoviral delivery of CAT reduces H2O2-mediated in vitro calcification and osteogenic-like transdifferentiation of VICs. Finally, we investigated the impact of oxidative stress on in vitro calcification of AVSc-derived VIC (Figure 6). Our experiments show that although control-derived VIC are mostly unresponsive to in vitro calcification 7 days after being exposed to 1 mmol/L H2O2, the same treatment resulted in a significant calcium accumulation in AVSc VICs (Figure 6A and 6B). In vitro calcification was reduced to basal level when cells were transduced with Ad-CAT (Figure 6B), whereas Ad-SOD3 is not able to revert H2O2-induced in vitro calcification. Finally, accordingly with the in vitro calcification results, Ad-CAT, but not Ad-SOD3, reduces the expression of osteogenic markers RUNX2 and reverts H2O2-induced α-smooth muscle actin downregulation (Figure 6C).

Discussion

Elevated levels of oxidative DNA damage have been reported in a number of diseases, but their presence may simply be
epiphenomenal rather than pathogenic. A direct mechanism has been postulated to describe the role of oxidative DNA damage in the development of cardiovascular diseases, with increasing evidence of a direct role of the cascade oxidative stress-DNA damage-diseases in experimental model of atherosclerosis.34,49 It is now understood that aortic valve stenosis is the end-stage of a disease that progresses from microscopic early changes to aortic sclerosis and then, in a subset of patients, to severe biominalization.50 To impact the progression from sclerosis to stenosis, we need to understand the earliest stages of disease so that we can measure the effects of targeted therapy on the microscopic processes in the valve cusps.12,51–53 Despite our best efforts, progress to understand, diagnose, and treat CAVD have been hindered by our inability to quantify in vivo the dynamic molecular events associated with early calcific changes in the valves.3,45 Despite its high prevalence, little is known about the developmental stages and pathogenetic mechanisms of aortic sclerosis. Our study provides several new insights into the early pathogenesis and the progression of AVSc.

First, the lack of oxidant defense in disease may lead to increased ROS and subsequent DNA damage (Figure 1). The oxidative stress typical of the very early events of valvular and vascular dysfunctions is mainly created by the production of ROS.20–24 Several studies indicate that ROS can cause DNA strand breaks and base modifications.32–34 Oxidative DNA damage resulting from free radical attack remains, however, a poorly examined field in CAVD. Our analysis shows, for the first time, accumulation of oxidative stress along with early calcific changes in the valves.3,45,50 Despite its high prevalence, little is known about the developmental stages and pathogenetic mechanisms of aortic sclerosis. Our study provides several new insights into the early pathogenesis and the progression of AVSc.

Because DNA damage cannot be tolerated by the cell if left unrepaired, elaborate cellular repair and genome surveillance mechanisms counteract genomic damage induced by ROS. The DDR is manifested cytologically by the formation of DNA-repair foci.35–38 These structures can be seen as an affinity platform for a substantial number of proteins, allowing for the local subnuclear concentration of these factors.35,54

We therefore investigated the cellular response to DNA damage and its link to osteogenic phenotype in VICs. DNA damage results in rapid phosphorylation of H2AX by PI3K-like kinases, including ATM, ATR, and DNA-PK, regulatory kinases of DDR,35,37 and cell cycle arrest. γH2AX is required for checkpoint-mediated cell cycle arrest and DNA repair after double-stranded DNA breaks. We show that oxidative stress induces a rapid subnuclear accumulation of γH2AX, MRE11, and XRCC1 proteins in human isolated VICs. AVSc-isolated VICs show a sustained phosphorylation of H2AX, suggesting an altered organization of the DDR after ROS treatments. Single gel electrophoresis and TUNEL assays suggest that the sustained activation of γH2AX, MRE11, and XRCC1 proteins could be the results of an impaired mechanisms of repair rather than a greater accumulation of DNA damage in AVSc-isolated VICs (Figures 2 and 3). As a note, the incomplete colocalization between MRE11 and γH2AX foci at 15 minutes could be a result of the formation of the MNR (MRE11, Rad 50, and NSB1) complex and it is consistent with previous reports.35,37,49

The molecular cascade generated by the DDR proteins may have a number of effects on the valvular cellular populations. Here we show that ROS induces the expression of early markers of osteogenic transdifferentiation, such as RUNX2, via AKT signaling. AKT signaling has been implicated in the in vitro differentiation of skeletal cells, such as chondrocytes, osteoblast, myoblast, and adipocytes. Mice lacking of Akt1 and Akt2 show delayed bone development, suggesting an important role of AKT signaling in the differentiation of bone cells. Furthermore it has been shown that H2O2-induced activation of AKT signaling regulates RUNX2 expression and calcification in vascular smooth muscle cells.36 ROS-mediated VICs activation can be reversed by adenoviral transduction of SOD3 and CAT. In our experiments, in accordance with previous results,35 oxidative mediated-DNA damage results in VIC activation toward an osteogenic-like
phenotype via AKT-RUNX2 signaling pathway reverted by antioxidant enzymes delivery (Figures 4 and 5). Finally, in vitro calcification assays show a direct link among ROS, VIC activation, and biomineralization of AVSc-derived VIC when compared with controls (Figure 6). In AVSc-derived VIC exposure to 1 mmol/L H2O2 resulted in parallel accumulation of RUNX2 and calcium, with this effect reverted by adenoviral transduction of antioxidant enzyme CAT. The ineffectiveness of SOD3 in contrasting high dose of H2O2, in this experiment, is in agreement with the specificities of the enzymes56: CAT acting specifically on H2O2 is capable of neutralizing it effectively within a broad range of H2O2 concentrations. On the contrary, SOD3 may be able to prevent toxic effects developing at low H2O2 concentrations by rapidly neutralizing superoxide anion formed presumably from the ROS, DNA-damage response, and cellular transdifferentiation reversible by antioxidant enzymes delivery. A better understanding of mechanisms that lead to increases in oxidative stress in CAVD, and indeed in many other cardiovascular diseases, may lead to more effective antioxidant prevention or treatments of this largely understudied patient population.

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Disclosures
None.

References
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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 cm². 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

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<td>1 (7.7%)</td>
<td>2 (15.4%)</td>
<td>5 (38.5%)</td>
<td>0.174</td>
</tr>
<tr>
<td>End Stage Congestive Heart Failure (%)</td>
<td>NA</td>
<td>8 (61.5%)</td>
<td>1 (7.7%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart Failure (%)</td>
<td>NA</td>
<td>5 (38.5%)</td>
<td>2 (15.4%)</td>
<td>0.193</td>
</tr>
<tr>
<td>Hyperlipidemia (%)</td>
<td>3 (23.0%)</td>
<td>2 (15.4%)</td>
<td>7 (53.9%)</td>
<td>0.119</td>
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</tbody>
</table>

B- Echocardiographic Measurements

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Controls N =13</th>
<th>Aortic Sclerosis N =13</th>
<th>Aortic Stenosis N =13</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic Valve Area (AV/A) (cm²)</td>
<td>&gt;2</td>
<td>2.3 (±0.5)</td>
<td>0.7(±0.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Doppler Velocity (m/s)</td>
<td>&lt;2</td>
<td>0.5 (±0.9)</td>
<td>3.7 (±1.01)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Calcium Score (1-4)</td>
<td>1</td>
<td>1.6 (±0.5)</td>
<td>3.5 (±0.5)</td>
<td>&lt;0.01</td>
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</table>

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$_2$O$_2$ 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$_2$O$_2$ 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 2100 Bioanalyzer (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$_2$O$_2$ treatment of human-derived VICs.** To test cellular transdifferentiation, VICs were exposed to 0.1 mM H$_2$O$_2$ 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 H2O2. 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 H2O2 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 H2O2 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 H2O2 in PBS for 1 hour at 37°C. 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 H2O2 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/µg of proteins.
Supplemental Figure I

15'  4 h  24 h

Fig S1. XRCC1 and Mre11 colocalize with γH2AX in control VICs exposed to H₂O₂ at different recovery time. (A) Immunofluorescence images showing colocalization of γH2AX (green) and XRCC1 (red) 24 hours after exposure to H₂O₂ in VICs isolated from AVC patients. (B) γH2AX (green) and Mre11 (red) expression under the conditions described above. Magnification 100X.
## Supplemental Table II

<table>
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