

## COX2 Inhibition Reduces Aortic Valve Calcification In Vivo

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**Objective**—Calcific aortic valve disease (CAVD) is a significant cause of morbidity and mortality, which affects ≈1% of the US population and is characterized by calcific nodule formation and stenosis of the valve. *Klotho*-deficient mice were used to study the molecular mechanisms of CAVD as they develop robust aortic valve (AoV) calcification. Through microarray analysis of AoV tissues from *klotho*-deficient and wild-type mice, increased expression of the gene encoding cyclooxygenase 2 (COX2; *Ptgs2*) was found. COX2 activity contributes to bone differentiation and homeostasis, thus the contribution of COX2 activity to AoV calcification was assessed.

**Approach and Results**—In *klotho*-deficient mice, COX2 expression is increased throughout regions of valve calcification and is induced in the valvular interstitial cells before calcification formation. Similarly, COX2 expression is increased in human diseased AoVs. Treatment of cultured porcine aortic valvular interstitial cells with osteogenic media induces bone marker gene expression and calcification in vitro, which is blocked by inhibition of COX2 activity. In vivo, genetic loss of function of COX2 cyclooxygenase activity partially rescues AoV calcification in *klotho*-deficient mice. Moreover, pharmacological inhibition of COX2 activity in *klotho*-deficient mice via celecoxib-containing diet reduces AoV calcification and blocks osteogenic gene expression.

**Conclusions**—COX2 expression is upregulated in CAVD, and its activity contributes to osteogenic gene induction and valve calcification in vitro and in vivo. (*Arterioscler Thromb Vasc Biol.* 2015;35:938-947. DOI: 10.1161/ATVBAHA.114.305159.)

**Key Words:** aortic valve, calcification of ■ cyclooxygenase 2 ■ heart valves

Calcific aortic valve disease (CAVD) is characterized by mineralized nodules on the valve cusps and typically results in progressive aortic valve stenosis.<sup>1,2</sup> CAVD is a significant cause of morbidity and mortality, thus research into the prevention or delay of CAVD may ultimately lead to improved clinical outcomes.<sup>3,4</sup> In the United States, aortic valve stenosis affects 0.4% of the total population; however, the risk of developing aortic valve stenosis because of CAVD increases with age, and the prevalence is estimated at 2.8% in the elderly.<sup>5</sup> There are many factors that contribute to the development of CAVD, such as abnormal valve development, advanced age, end-stage kidney disease, and inflammation secondary to increased lipid deposition.<sup>3</sup> Valve replacement surgery is the current standard of care; however, mechanical valves are associated with the risk of thromboembolism, and bioprosthetic valves have limited durability.<sup>4,6,7</sup> Investigation into the cellular and molecular changes that occur at early stages of disease may lead to alternative therapies and alleviate the need for surgery.

During disease, valvular interstitial cells (VICs) from mineralized valves activate molecular pathways associated with valve development and bone differentiation.<sup>8,9</sup> Expression of the osteogenic gene markers osteocalcin (OCN), Runx2, osteopontin (OPN), alkaline phosphatase, and bone sialoprotein

(BSP) is increased in human CAVD in comparison with control valves.<sup>8</sup> Additional pathways involved in bone and cartilage formation, such as bone morphogenetic protein (BMP), Notch, and Wnt signaling, have also been shown to be involved in valve calcification.<sup>10–13</sup> Together, the evidence suggests that an osteogenic-like mechanism is active in CAVD although the contribution of such pathways may vary depending on the underlying cause of CAVD.

*Klotho*-deficient mice develop robust nodular aortic valve (AoV) calcification similar to that observed in human CAVD.<sup>14,15</sup> *Klotho*-deficient mice were originally described as a model of premature aging, because they have a shortened lifespan, but they also develop kidney disease and have increased serum phosphate levels, which are associated with an increased risk of CAVD in humans.<sup>16–19</sup> In addition to exhibiting AoV calcification, *klotho*-deficient mice have increased osteogenic gene expression in the AoVs, similar to human CAVD.<sup>9,14</sup> Thus, *klotho*-deficient mice are useful for studying the cellular and molecular changes that occur during valve calcification in the early stages of CAVD, and, as they have little immune cell infiltration in the AoV, study of the VIC-intrinsic molecular changes are possible in this model.<sup>14</sup>

Here, we show that cyclooxygenase 2 (COX2) expression is increased in the mineralized AoVs of *klotho*-deficient

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### Nonstandard Abbreviations and Acronyms

<b><math>\alpha</math>SMA</b>	$\alpha$ -smooth muscle actin
<b>AS</b>	aortic valve stenosis
<b>AoV</b>	aortic valve
<b>CAVD</b>	calcific aortic valve disease
<b>COX2</b>	cyclooxygenase 2
<b>Ptgs2</b>	prostaglandin-endoperoxide synthase 2
<b>VIC</b>	valvular interstitial cell

mice and in human CAVD. COX2 has a key role in prostaglandin synthesis and is active during inflammation, as well as in bone formation and repair.<sup>20–23</sup> COX2-specific inhibitors are commonly used to treat pain and inflammation but have an increased risk of cardiovascular side effects.<sup>24–27</sup> In vitro, COX2 activity is necessary for osteogenic gene expression and VIC calcification. In *klotho*-deficient mice, genetic and pharmacological manipulation of COX2 activity in vivo reduces AoV calcification. Thus, COX2 activity contributes to osteogenic gene induction and calcification in AoVs in mice.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

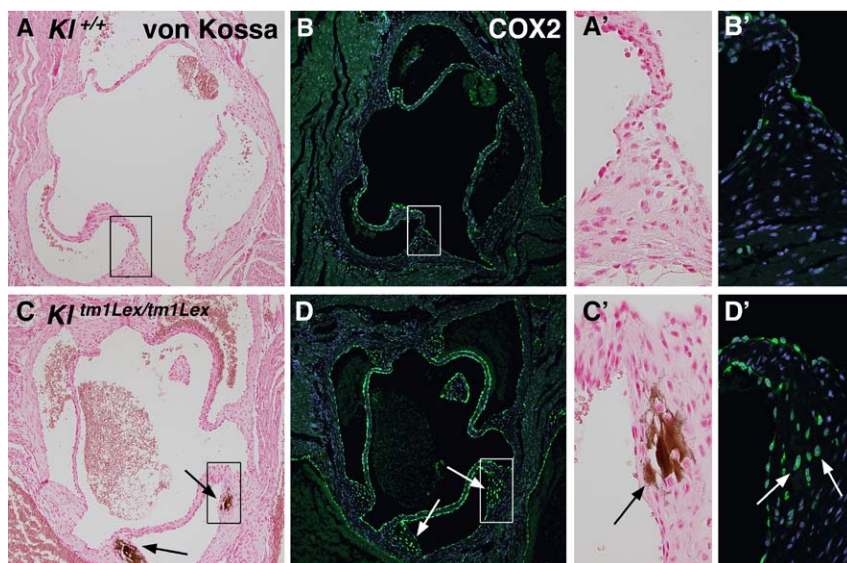
#### COX2 Expression Is Increased in Regions of AoV Calcification in *klotho*-Deficient Mice

To identify novel molecular mechanisms involved in AoV calcification, gene expression in 6.5-week-old *klotho*-deficient and wild-type mice was evaluated by microarray. Among the genes with the most increased expression in *klotho*-deficient animals were *Spp1* (osteopontin), previously reported to be increased in *klotho*-deficient valves,<sup>14</sup> and prostaglandin-endoperoxide synthase 2 (*Ptgs2*; Tables I and II in the online-only Data Supplement). Expression of *Ptgs2*, the gene encoding COX2 protein, was increased 4.3-fold in AoV tissues of *klotho*-deficient animals, and increased COX2 expression was

confirmed by immunohistochemical analysis. *Klotho*-deficient mice develop calcification in the hinge region of the AoV, as apparent in von Kossa stained sections of hearts from 6- to 6.5-week-old wild-type (*Kl<sup>+/+</sup>*) and *klotho*-deficient (*Kl<sup>tm1Lex/tm1Lex</sup>*) mice (Figure 1A, 1A', 1C, and 1C').<sup>14</sup> In wild-type mice, COX2 expression is limited to endothelial cells lining the AoV cusps and aortic wall (Figure 1B and 1B'). However, *klotho*-deficient mice have increased COX2 expression in the VICs of the AoV hinge region (Figure 1D and 1D'), in addition to the normal endothelial pattern. Importantly, COX2 expression in VICs spatially overlaps with the region of AoV calcification in *klotho*-deficient mice (Figure 1C–D'). These results demonstrate that increased COX2 protein expression is localized to regions of AoV calcification in *klotho*-deficient animals.

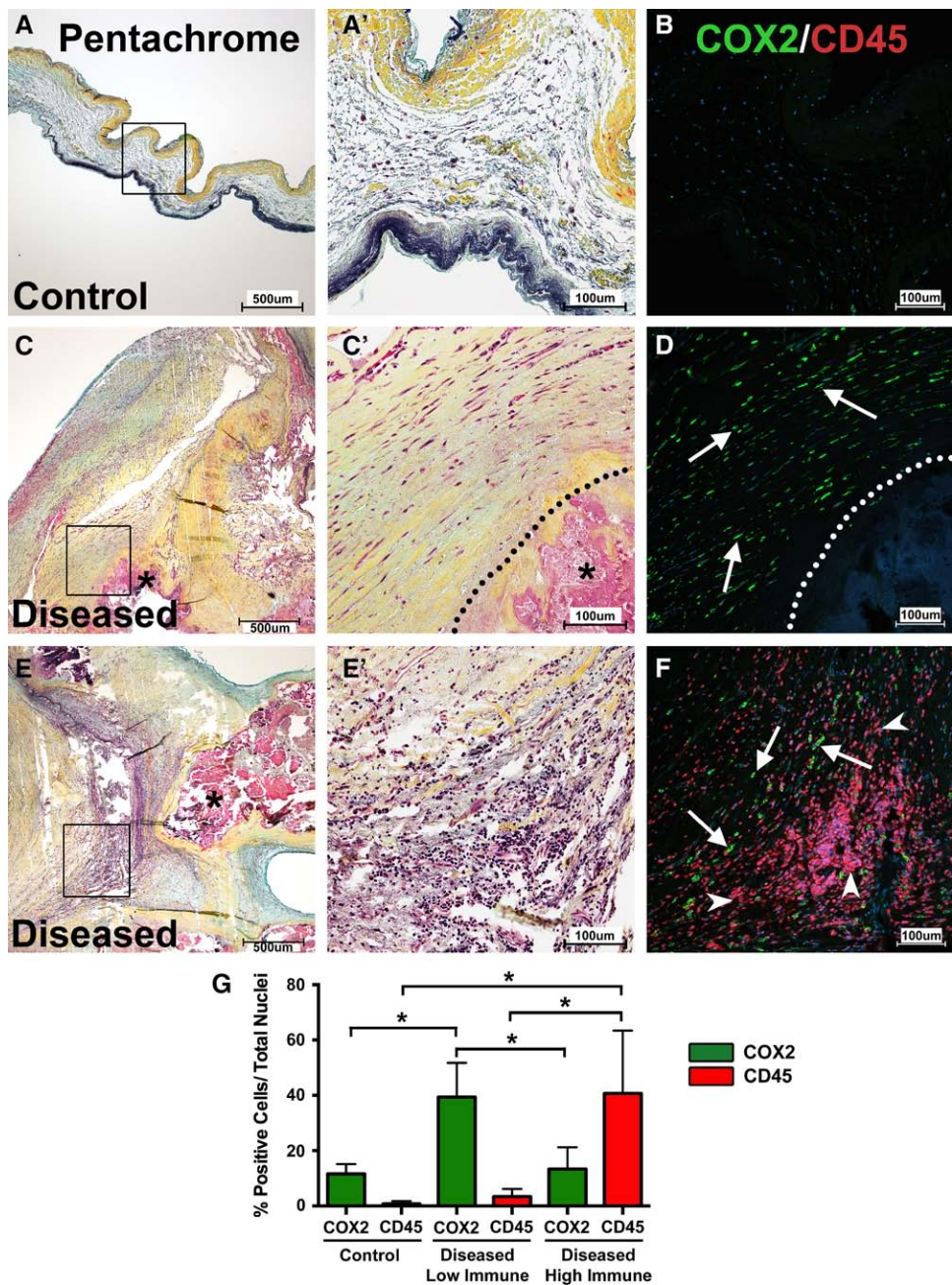
#### COX2 Expression Is Increased in Human CAVD

To examine whether COX2 expression is increased in human CAVD, explanted diseased and control AoV tissues were stained for COX2 by immunofluorescence. As COX2 is often associated with immune cell infiltration, COX2 protein expression was compared with the leukocyte/hematopoietic cell marker CD45. Pentachrome staining was used to examine the histology of control and diseased valves, particularly regions of immune cell infiltration and extracellular matrix thickening (Figure 2A, 2A', 2C, 2C', 2E, and 2E'). Areas of intense COX2 protein expression are apparent in regions of the diseased valves surrounding calcification and, in comparison, little COX2 protein is observed in control AoVs (Figure 2B, 2D, and 2F). COX2 is expressed in regions adjacent to calcific nodules where CD45 expression is absent (Figure 2D) but can also be readily observed in regions with intense CD45 expression (Figure 2F). Although COX2-positive and CD45-positive cells can be found in overlapping areas with immune cell infiltration, few cells coexpress these proteins (Figure 2F). Control AoV tissues contain few COX2- or CD45-positive cells (Figure 2B). Quantification of COX2-positive, CD45-positive, and double-positive cells shows that the percentage of COX2-expressing cells is significantly increased in diseased tissues in regions of low



**Figure 1.** Cyclooxygenase 2 (COX2) expression is increased and localized to the region of aortic valve calcification in *klotho*-deficient (*Kl<sup>tm1Lex/tm1Lex</sup>*) mice. Positive von Kossa staining (black arrows) demonstrates aortic valve calcification in the hinge region of *klotho*-deficient mice (C and C') compared with no observable calcification in wild-type controls (*Kl<sup>+/+</sup>*; A and A'). The boxed regions in A and C are magnified and are shown in A' and C'. Sections are counterstained with nuclear fast red (A, A', C, and C'). In wild-type animals, immunofluorescence demonstrates that COX2 is primarily expressed in endothelial cells (green staining in B and B'). In contrast, *Klotho*-deficient mice have increased COX2 expression in valvular interstitial cells surrounding and throughout regions of valve calcification (green staining in D and D' indicated by white arrows). Boxed regions in B and D are magnified and are shown in B' and D'. Nuclei are counterstained with ToPro3 (blue staining in B, B', D, and D').





**Figure 2.** Cyclooxygenase 2 (COX2) expression is increased in human calcific aortic valve disease. Movat pentachrome staining delineates the extracellular matrix of human control (A, boxed region magnified in A'; n = 5) and diseased (C and E, boxed regions magnified in C' and E'; n=7) aortic valve (AoV) tissues. Coimmunofluorescence of COX2 (green) and CD45 (red; B, D, and F) shows increased COX2 expression in diseased AoVs (D and F) compared with controls (B). COX2 expression is low in human control AoVs (green staining in B), and few cells express the leukocyte/hematopoietic cell marker CD45 (red staining in B). In diseased tissues, COX2 expression is high in regions void of CD45-positive cells (low immune regions; green staining in D indicated by white arrows). COX2 expression also is observed in regions of diseased valves with high levels of CD45 expression (high immune regions), but costaining of COX2 and CD45 is rarely observed in the same cell (CD45=red staining indicated by white arrowheads [F]; COX2=green staining indicated by white arrows [F]). Quantification of COX2-positive cells (green bars) versus total nuclei demonstrates that COX2 expression is significantly increased in regions of few infiltrating immune cells in diseased AoVs when compared with controls (G). As expected, CD45 expression (red bars) was increased in diseased valves in regions of high immune cell infiltration when compared with controls (G). Dotted lines delineate calcified regions (C and D), and calcification is also marked by an asterisk (C, C', and E). Nuclei are counterstained with ToPro3 (blue in B, D, and F). A 1-way ANOVA test with a post hoc multiple comparisons test was used to compare COX2 and CD45 expression between control and diseased valves with regions of low immune cell infiltration or regions of high immune cell infiltration; significant differences are indicated by asterisks ( $P<0.05$ ), and error bars represent the SD (G).

immune cell infiltration when compared with control tissues (Figure 2G). As expected, regions of high immune cell infiltration in diseased AoV tissues have significantly higher

numbers of CD45-positive cells when compared with control valves (Figure 2G). Furthermore, expression analysis of COX2 and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) in human

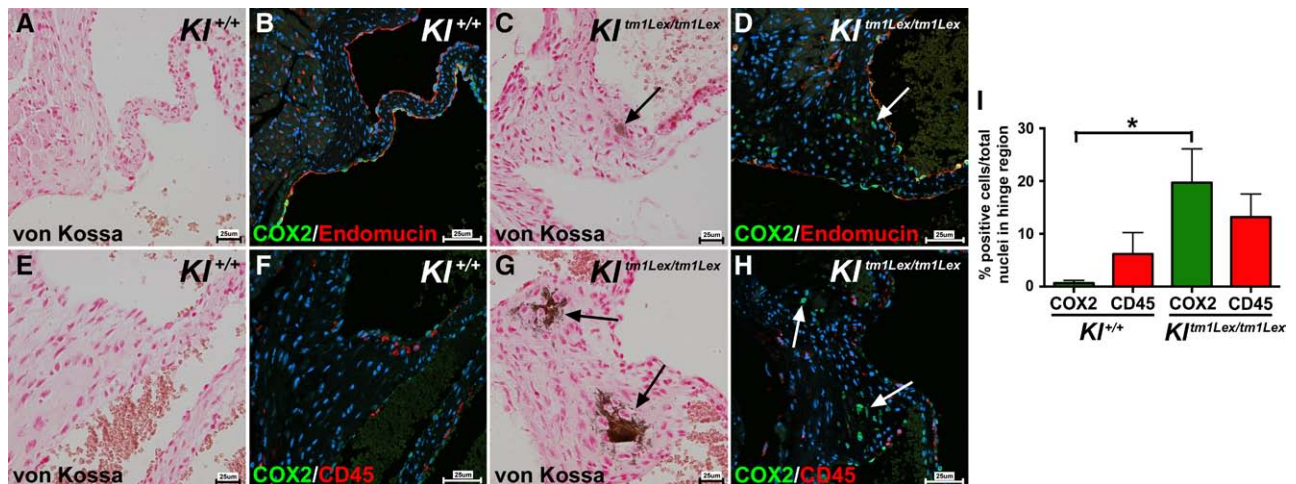
diseased and control AoVs demonstrated that the majority of COX2-expressing cells are not in close proximity to or colocalized with  $\alpha$ SMA-expressing cells although limited regions of coexpression were observed (Figure I in the online-only Data Supplement). Thus, COX2 expression is increased in human CAVD and, in some cases, is localized in proximity to calcified nodules in the absence of inflammation as occurs in the *klotho*-deficient mice.

### Aortic VICs Induce COX2 Expression Before the Initiation of Valve Calcification

To determine the timing of COX2 induction in aortic VICs relative to the progression of CAVD, COX2 protein expression was examined in *klotho*-deficient mice ( $Kl^{tm1Lex/tm1Lex}$ ) at 2, 3, 4, and 5 weeks. Although the timing is variable, *klotho*-deficient mice typically develop AoV calcification between 4 and 5 weeks, as indicated by von Kossa staining (Figure IIA–IID in the online-only Data Supplement). As expected, COX2 immunofluorescence is detected in the endothelial cells lining both *klotho*-deficient and wild-type valves at all time points investigated (Figure 1B; Figure IIE–IIH in the online-only Data Supplement and data not shown). In addition, COX2 expression can be detected as early as 3 weeks in aortic VICs of *klotho*-deficient animals (Figure IIF in the online-only Data Supplement). Before calcification, the COX2-expressing VICs are in the AoV hinge region where calcific lesions ultimately form, and COX2 expression colocalizes with the region of calcification by 5 weeks (Figure IIE–IIH in the online-only Data Supplement). Thus, COX2 is expressed in the VICs of the AoV hinge region in *klotho*-deficient mice before the initiation of valve calcification.

### Calcified VICs Express COX2

In wild-type mice ( $Kl^{+/+}$ ) at 6 to 6.5 weeks, COX2 expression in the heart is limited to endothelial cells (Figure 1B and 1B'). To confirm that increased COX2 expression in the AoV hinge region of *klotho*-deficient mice ( $Kl^{tm1Lex/tm1Lex}$ ) is separate from COX2 expression in endothelial cells, coexpression immunofluorescence of COX2 and endomucin was performed. Endomucin marks the endothelial cells lining the AoVs in both wild-type and *klotho*-deficient mice, and a subset of endomucin-positive cells also expresses COX2 (Figure 3B and 3D). However, VICs that express COX2 in the calcified valve regions of *klotho*-deficient mice are separate from the valve endothelial cells, as they do not express endomucin (Figure 3C and 3D). COX2 expression is often associated with immune cell infiltration and an inflammatory response.<sup>24,25</sup> Few infiltrating immune cells or apoptotic cells, indicative of dystrophic calcification, are observed in the calcific lesions of *klotho*-deficient mice (Figure III in the online-only Data Supplement).<sup>14</sup> To demonstrate that COX2-expressing cells in the valve interstitium are not leukocytes or other hematopoietic-derived cells, COX2 expression was compared with CD45 expression (Figure 3F and 3H).<sup>28</sup> No overlap between CD45 and COX2 expression was observed in the region of AoV calcification in *klotho*-deficient mice (Figure 3G and 3H). Similarly, there was no overlap between CD45 and COX2 expression in  $Kl^{+/+}$  mice (Figure 3E and 3F). Quantification of COX2-positive cells and CD45-positive cells revealed that COX2 expression is significantly increased in the VICs of the AoV hinge region in *klotho*-deficient mice, whereas the percentage of CD45-positive cells was similar to that of wild-type controls (Figure 3I). Together, the data



**Figure 3.** Valvular interstitial cells (VICs) expressing cyclooxygenase 2 (COX2) do not coexpress endothelial or leukocyte/hematopoietic cell markers in *klotho*-deficient mice. COX2 (green staining) is coexpressed with the endothelial marker endomucin (red staining) in endothelial cells in wild-type animals ( $Kl^{+/+}$ ) (B). In *klotho*-deficient mice ( $Kl^{tm1Lex/tm1Lex}$ ), in addition to endothelial cell expression, COX2 is evident in VICs in regions of calcification (COX2 expression indicated by white arrow in D), which are not positive for endomucin.  $Kl^{+/+}$  mice exhibit dispersed expression of the leukocyte/hematopoietic cell marker CD45 (red staining in F). In  $Kl^{tm1Lex/tm1Lex}$  mice, coimmunofluorescent staining of CD45 (red staining) and COX2 (green staining) shows that COX2-expressing cells do not express CD45 (COX2 staining is indicated by white arrows in H). Quantification of COX2-positive (green bars) and CD45-positive (red bars) VICs in the aortic valve (AoV) hinge region demonstrates a significant increase in COX2 expression in  $Kl^{tm1Lex/tm1Lex}$  compared with wild-type animals, whereas CD45 expression was unchanged (I). AoV calcification is detected by von Kossa staining in adjacent sections of the same specimens (brown staining indicated by black arrows in A, C, E, and G; sections are counterstained with nuclear fast red). Nuclei are counterstained with ToPro3 (blue staining in B, D, F, and H). A 1-way ANOVA with a post hoc multiple comparisons test was used to compare expression of COX2 and CD45 in wild-type and *klotho*-deficient mice, a significant difference is indicated by an asterisk ( $P < 0.05$ ), and error bars represent the SD (I).



suggest that COX2-positive cells are not immune infiltrate nor cells of hematopoietic origin, but rather are resident VICs. To further support the idea that COX2-expressing cells are resident fibroblast-like VICs, expression of the fibroblast markers, type 1 collagen, and vimentin was analyzed in *klotho*-deficient and wild-type mice. Calcified VICs in the AoVs of *klotho*-deficient mice express both type 1 collagen and vimentin, similar to surrounding noncalcified VICs and normal VICs in wild-type mice (Figure IV in the online-only Data Supplement). Myofibroblast-like VICs, characterized by expression of  $\alpha$ SMA, have been implicated in CAVD.<sup>3</sup> To determine whether there is a myofibroblast intermediate preceding VIC calcification, *klotho*-deficient mice were crossed with a tamoxifen-inducible  $\alpha$ SMAcreER transgenic line with a cre-dependent ROSA membrane Tomato/membrane enhanced green fluorescent protein (EGFP) reporter. Mice were administered tamoxifen to induce SMAcreER activity before observable calcification. Analysis of recombined EGFP-positive cells revealed that calcified VICs did not arise from a myofibroblast intermediate or do they have smooth muscle cell characteristics (Figure V in the online-only Data Supplement).<sup>29</sup> Thus, in *klotho*-deficient mice, COX2-expressing VICs are resident interstitial fibroblast-like cells localized to the calcified area.

### COX2 Inhibition Reduces Osteogenic Gene Induction and Calcification in Cultured Porcine Aortic VICs

To examine whether COX2 activity is necessary for osteogenic gene induction and calcification in aortic VICs, a porcine VIC culture system was used. On treatment of VICs with osteogenic media, expression of the osteogenic genes *OCN* and *BSP* is increased, as detected by quantitative polymerase chain reaction (Figure 4B and 4C). In addition, *COX2* gene expression is also increased upon osteogenic media treatment, showing that *COX2* mRNA expression is induced concomitant with osteogenic gene expression (Figure 4A). VICs were treated with the specific COX2 inhibitor celecoxib, which inhibits COX2 enzyme activity by binding to the active site of the protein, but does not affect *COX2* gene expression (Figure 4A).<sup>30</sup> The requirement for COX2 activity in osteogenic gene induction was examined in VICs treated with osteogenic media in the presence of 15  $\mu$ mol/L celecoxib. In these experiments, expression levels of *OCN* and *BSP* were significantly reduced when compared with VICs treated with osteogenic media alone (Figure 4B and 4C). Therefore, COX2 inhibition reduces osteogenic gene induction in cultured VICs. In addition to gene expression, calcific nodule formation was assessed. VICs were treated with control or osteogenic media in low serum (2%), in combination with celecoxib treatment, and stained with alizarin red (Figure 4D–4F) and von Kossa (Figure 4G–4I) to detect calcification. The cellular response to osteogenic media and COX2 inhibition by celecoxib was measured by quantifying the number of precalcified (as detected by orange alizarin red staining) and calcified (as detected by red alizarin red staining and positive von Kossa staining) nodules (Figure 4D–4K). In response to osteogenic media, VIC calcific nodule formation is increased (Figure 4E, 4H, 4J, and 4K) when compared with cells treated with control media (Figure 4D, 4G, 4I, and 4K).

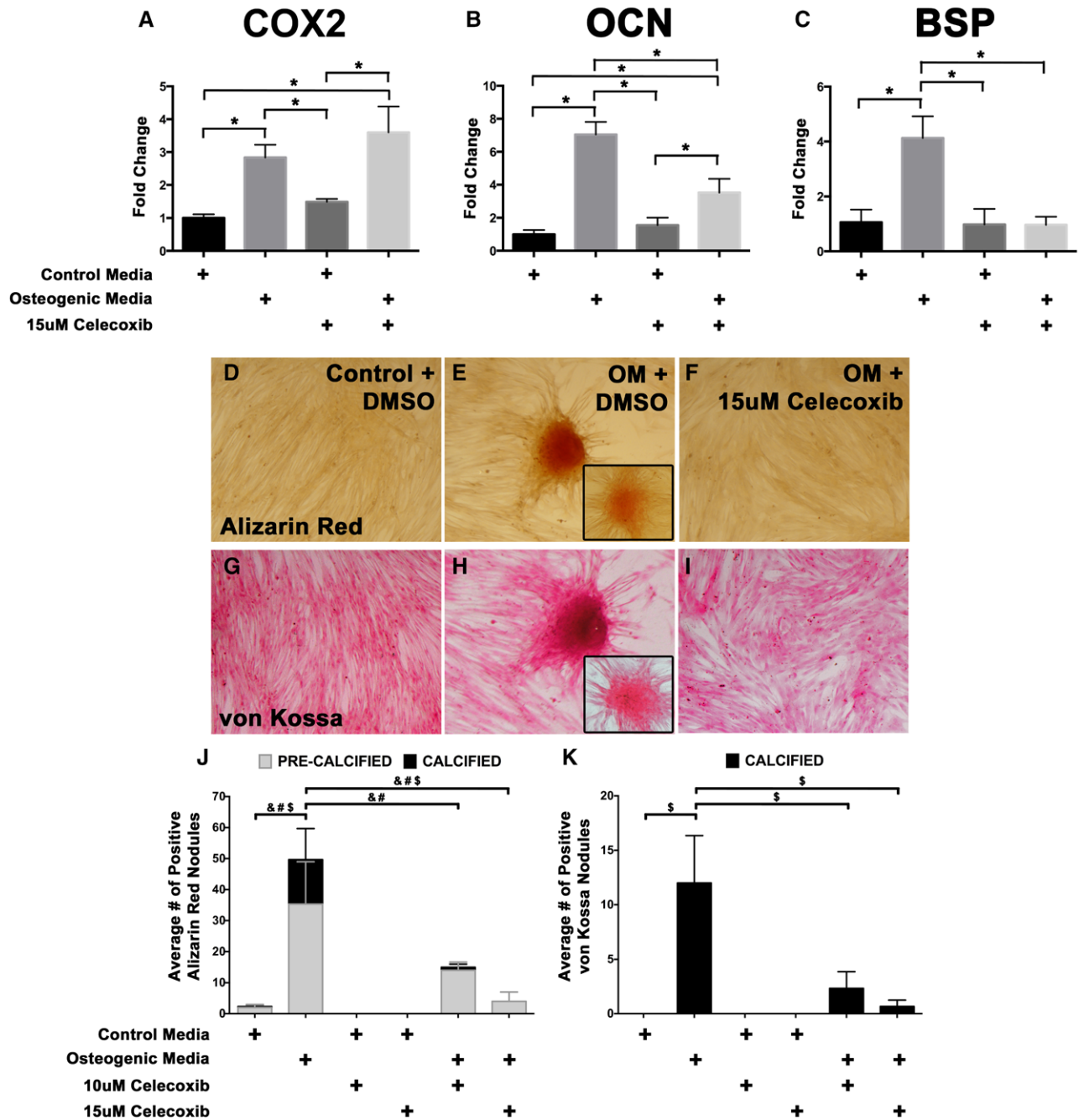
VICs treated with 2 different concentrations of celecoxib (10 or 15  $\mu$ mol/L) to inhibit COX2 activity had significantly fewer nodules (Figure 4F, 4I, 4J, and 4K). Together, the data show that osteogenic media treatment stimulates *COX2* mRNA induction, osteogenic gene expression, and calcific nodule formation. Thus, COX2 inhibition is sufficient to reduce osteogenic gene expression and cell calcification in isolated porcine VICs.

### Genetic COX2-Deficiency Reduces AoV Calcification in *klotho*-Deficient Mice

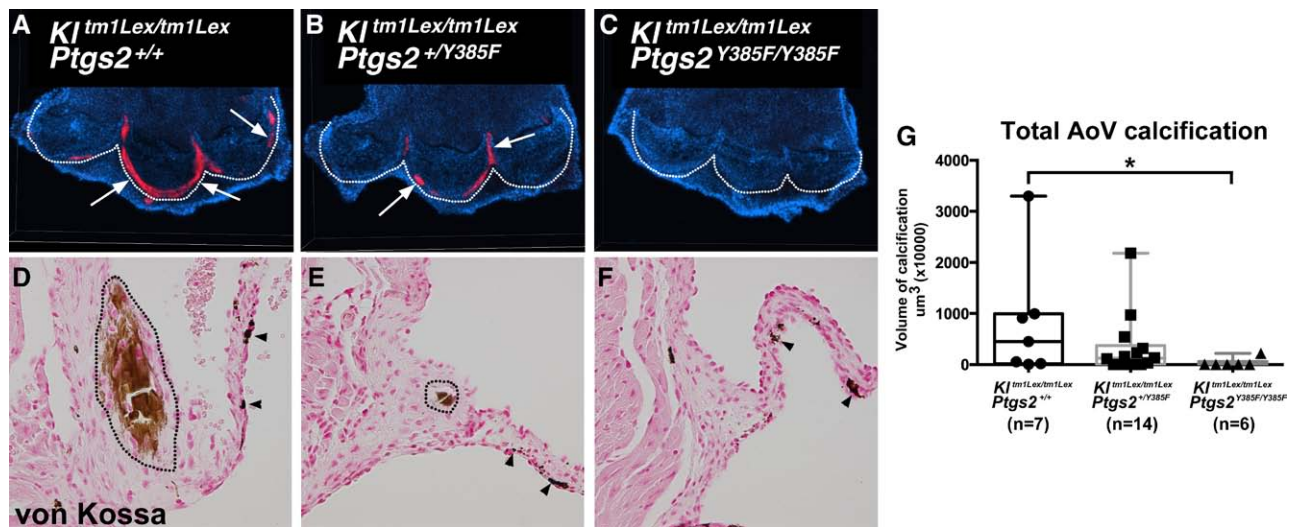
To determine whether the loss of COX2 function can inhibit AoV calcification in vivo, *COX2* mutant mice (B6.129S6(FVB)-*Ptgs2*<sup>tm1.1Fun/J</sup>) were bred with *klotho*-deficient mice.<sup>31</sup> *COX2* mutant mice possess a point mutation in exon 8 of the *Ptgs2* gene, which disrupts the cyclooxygenase activity and mimics the effect of COX2 inhibition by selective COX2 inhibitor drugs.<sup>31</sup> *Klotho*-deficient littermates with *Ptgs2* wild-type (*Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+/+</sup>), heterozygous (*Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+/-Y385F</sup>), or homozygous (*Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>Y385F/Y385F</sup>) alleles were harvested at 6 weeks-of-age. AoV calcification was assessed by whole mount alizarin red staining, and positive staining was quantified for each genotype to obtain AoV calcification volumes (Figure 5A–5C, and 5G). Aortic wall calcification was excluded from this analysis. Although the extent of calcification is variable, *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+/+</sup> mice with intact *Ptgs2* alleles demonstrate the highest level of calcification (Figure 5A and 5G; n=7). In comparison, mice with 2 mutated copies of the *Ptgs2* gene (*Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>Y385F/Y385F</sup>) have significantly lower amounts of AoV calcification, and 67% of animals had no detectable AoV calcification (Figure 5C and 5G; n=6; *P*=0.039). There was no statistically significant difference in the amount of AoV calcification observed in *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+/-Y385F</sup> mice (Figure 5B and 5G; n=14). Histological assessment of the AoV calcification by von Kossa staining confirms the reduced calcification in *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>Y385F/Y385F</sup> mice (Figure 5D–5F). Together, these findings show that genetic loss of COX2 cyclooxygenase activity in vivo prevents AoV calcification in *klotho*-deficient mice.

### Inhibition of COX2 Activity by Celecoxib Treatment Reduces AoV Calcification and Osteogenic Gene Expression in *klotho*-Deficient Mice

To determine whether pharmacological inhibition of COX2 activity is sufficient to reduce AoV calcification in vivo, *klotho*-deficient mice were treated with the selective COX2 inhibitor celecoxib. Increased COX2 expression is present in aortic VICs as early as 3 weeks in *klotho*-deficient mice (Figure IIF in the online-only Data Supplement). Therefore, animals were started on celecoxib diet at this stage. *Klotho*-deficient mice were split into 2 groups: the control group was fed a normal chow diet, whereas the treatment group was fed chow containing 1000 ppm celecoxib ad libitum from 3 to 6 weeks. Animals were harvested at 6 weeks-of-age, and whole mount alizarin red staining was performed to obtain AoV calcification volumes, excluding aortic wall calcification (Figure 6A–6C). Although variable, *klotho*-deficient mice fed normal chow diet have obvious AoV calcification (Figure 6A and 6C; n=12). In comparison, *klotho*-deficient mice that were



**Figure 4.** Cyclooxygenase 2 (COX2) inhibition in porcine aortic valvular interstitial cells (VICs) reduces osteogenic gene expression and cell calcification. For gene expression studies, isolated VICs were cultured for 6 days in control or osteogenic media (OM) with vehicle (DMSO) or the COX2 inhibitor celecoxib (15  $\mu$ mol/L; **A–C**). Quantitative polymerase chain reaction analysis demonstrates that *COX2* mRNA expression (**A**) and the osteogenic gene markers *osteocalcin* (*OCN*; **B**) and *bone sialoprotein* (*BSP*; **C**) are induced with OM treatment. COX2 inhibition by celecoxib reduces the expression of both *OCN* and *BSP* in cells induced with OM (**B** and **C**), whereas *COX2* expression is unchanged (**A**). Statistical analysis was performed using ANOVA with a multiple comparison post hoc test, and significance was determined when  $P < 0.05$  (indicated by \* in **A–C**). For calcification studies, VICs were cultured for 9 days in control or OM media with DMSO or 2 different concentrations of celecoxib (10 or 15  $\mu$ mol/L). Precalcified nodules (orange staining, inset in **E**) and calcified nodules (red staining, **E**) were identified by alizarin red staining in OM-treated cells (**E**), when compared with control cells (**D**). COX2 inhibition by celecoxib treatment reduces the number of alizarin red positive nodules upon OM treatment (**F**). A similar effect was observed in nodules stained by von Kossa to detect calcification (**G–I**; **H**, a positive von Kossa nodule [brown staining]; the inset in **H** indicates a nodule that is not calcified and was excluded from the analysis). The average number of alizarin red precalcified (gray bars) and calcified (black bars) nodules (**J**), as well as average number of positive von Kossa nodules (**K**, black bars) in cultured VICs was quantified. Statistical significance as determined by ANOVA with post hoc multiple comparisons tests ( $P < 0.05$ ) is indicated by & for the average number of precalcified nodules, # average number of alizarin red positive calcified nodules; \$ total nodules positive for alizarin red (**J**) and von Kossa staining (**K**).



**Figure 5.** Genetic loss of cyclooxygenase 2 (COX2) activity in *klotho*-deficient mice reduces aortic valve (AoV) calcification. Whole mount alizarin red staining reveals AoV calcification in *klotho*-deficient mice (*Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+/+</sup>; n=7; **A**), *klotho*-deficient mice with 1 mutated allele of the COX2 gene (*Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+Y385F</sup>; n=14; **B**), and *klotho*-deficient mice with 2 mutated alleles of the COX2 gene (*Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>Y385F/Y385F</sup>; n=6; **C**). Alizarin red staining is indicated by white arrows, nuclei are counterstained with ToPro3 (blue), and the base of the AoV cusps is outlined with dotted lines. Von Kossa staining confirms valve calcification in *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+/+</sup> (**D**), and *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+Y385F</sup> (**E**) but not *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>Y385F/Y385F</sup> (**F**) mice (calcific regions outlined by black dotted lines in **D** and **E**, dark melanocytes are indicated by black arrowheads). AoV calcification, exclusive of aortic wall calcification, was quantified from alizarin red whole mount stained specimens and volumes are represented in the graph where the horizontal bar represents the median values and the error bars represent the minimum and maximum values obtained (**G**). The Kruskal–Wallis statistical test reveals significantly lower volumes of AoV calcification in *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>Y385F/Y385F</sup> mice compared with *Kl*<sup>tm1Lex/tm1Lex</sup>; *Ptgs2*<sup>+/+</sup> mice (**G**, *P*=0.039).

fed celecoxib-containing diet have significantly reduced levels of AoV calcification (Figure 6B and 6C; n=12; *P*=0.038). Furthermore, celecoxib diet treatment significantly reduced mRNA expression of the osteogenic gene markers *OPN* and *Runx2* in the AoVs of *klotho*-deficient mice (Figure 6E and 6F). Thus, pharmacological inhibition of COX2 activity in vivo is sufficient to reduce osteogenic gene expression, in addition to valve calcification. As expected, *COX2* mRNA expression is increased in *klotho*-deficient mice, but it is not affected by celecoxib treatment (Figure 6D). Vascular calcification in the valve sinus and ascending aorta was unchanged between animals treated with normal diet versus celecoxib-containing diet, suggesting that vascular calcification is relatively COX2-independent (Figure VI in the online-only Data Supplement). Serum phosphate levels were comparable in untreated and celecoxib-treated animals (Figure VII in the online-only Data Supplement), demonstrating that reduced AoV calcification by COX2 inhibition is not the result of normalized serum phosphate levels in *klotho*-deficient mice. Thus, COX2 inhibition by administration of celecoxib in vivo specifically reduces AoV calcification and osteogenic gene induction in *klotho*-deficient mice.

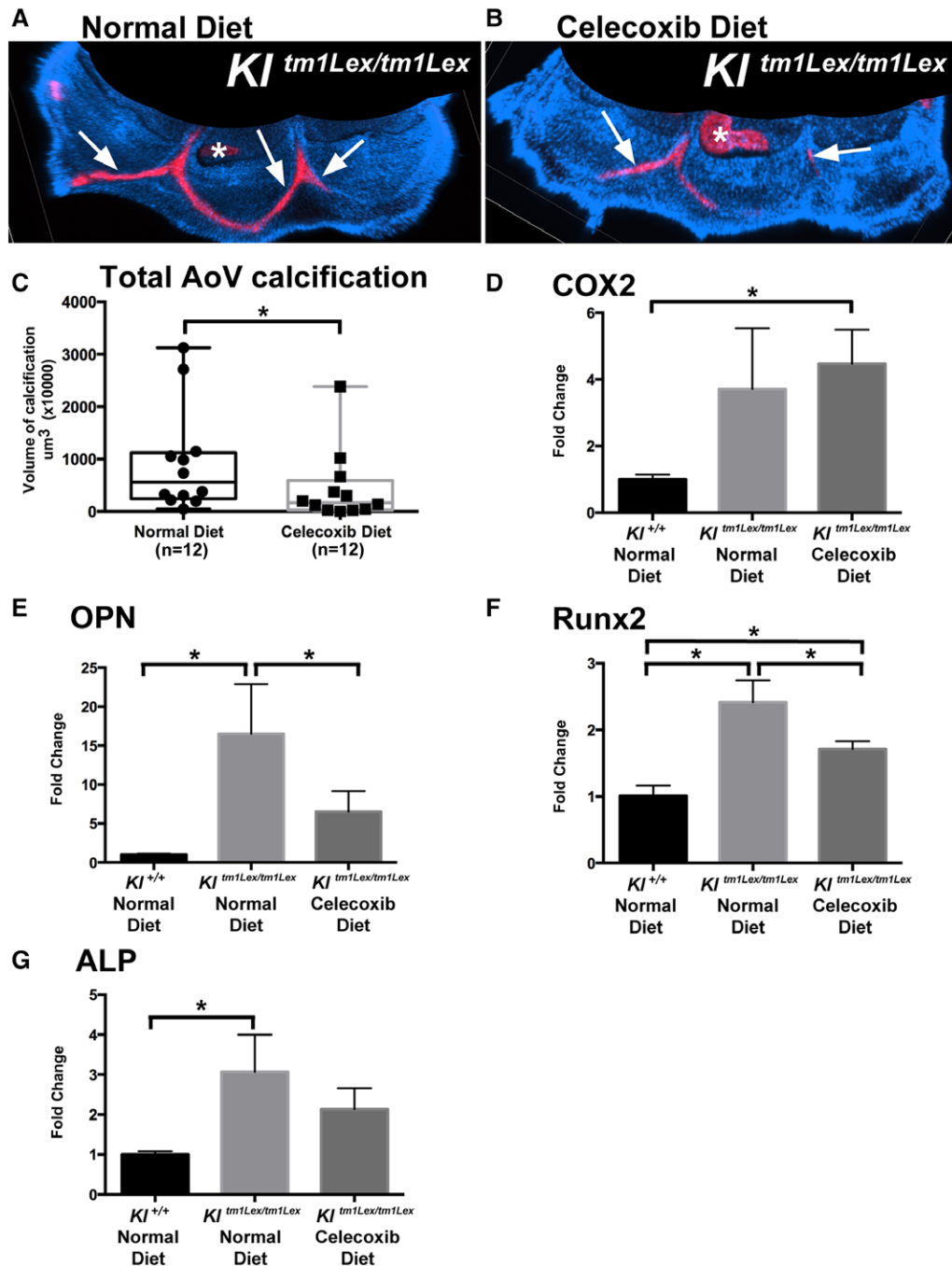
## Discussion

Here, we demonstrate that COX2 inhibition reduces osteogenic gene induction and AoV calcification in a mouse model of CAVD. COX2 expression is increased in calcified AoVs of *klotho*-deficient mice and is localized to the VICs before the formation of AoV calcification, which occurs in the context of minimal inflammation. In human CAVD, COX2 expression is increased and is present in regions where the valve leaflet is calcified. In porcine VICs, *COX2* mRNA expression is

induced upon treatment with osteogenic media, and COX2 inhibition reduces osteogenic gene induction and calcification, supporting a direct role for COX2 in VIC mineralization. Furthermore, COX2 inhibition through genetic or pharmacological manipulation is sufficient to reduce AoV calcification and osteogenic gene expression in *klotho*-deficient mice. Thus, inhibition of COX2 activity is sufficient to reduce AoV calcification in vivo in mice.

COX2 has not previously been associated with the process of calcification in CAVD, but it is necessary for bone homeostasis and fracture healing. *COX2* knockout mice display abnormal bone density and reduced ability to heal after fracture, and nonselective COX inhibitors are associated with delayed or inhibited bone fracture healing in humans.<sup>20,22,23,32</sup> Conversely, lentivirus-mediated COX2 overexpression has been used to promote bone fracture healing in mice.<sup>33</sup> COX2 activity and its prostaglandin metabolites play a key role in osteogenic gene induction during bone formation and maintenance. In osteoblast cultures, prostaglandin treatment induces the expression of the osteogenic genes *BMP2*, *OCN*, and *Runx2*, while promoting matrix mineralization, and injection of prostaglandins in mice and rats induces bone formation in vivo.<sup>23,34–38</sup> Osteogenic gene induction also occurs during valve calcification in human diseased valves and in *klotho*-deficient mice.<sup>8,9,14</sup> In the current study, we show that COX2 inhibition reduces the expression of *OCN* and *BSP* in cultured porcine VICs stimulated with osteogenic media and also reduces the expression of *Runx2* and *OPN* in *klotho*-deficient mice treated with celecoxib diet. Furthermore, we show that COX2 expression is induced in the interstitial cells of *klotho*-deficient mice before the initiation of valve calcification. Thus, COX2 is activated before valve calcification and is required for osteogenic gene expression in the





**Figure 6.** Cyclooxygenase 2 (COX2) inhibition with celecoxib treatment reduces aortic valve (AoV) calcification and osteogenic gene expression in *klotho*-deficient mice. *Klotho*-deficient mice (*KI* *tm1Lex/tm1Lex*) were fed either normal chow diet (**A**; n=12) or diet containing 1000 ppm celecoxib (**B**; n=12). AoV calcification was detected by whole mount alizarin red staining (**A** and **B**, valve annulus calcification is indicated by white arrows; nuclei are counterstained with ToPro3 in blue). The white \* indicates aortic wall calcification in the valve sinus that was excluded from analysis (**A** and **B**). AoV calcification was quantified and plotted with the horizontal bar representing the median values, and the error bars representing the minimum and maximum values (**C**). A Mann-Whitney test determined that treatment with celecoxib-containing diet significantly reduces the amount of AoV hinge region calcification in *klotho*-deficient mice (**C**;  $P=0.038$ , indicated by \*). COX2 mRNA expression is increased in *klotho*-deficient mice, and its expression is not changed with celecoxib treatment (**D**). mRNA expression of the osteogenic genes *osteopontin* (OPN; **E**) and *Runx2* (**F**) is significantly reduced, and *alkaline phosphatase* (ALP; **G**) is trending downward by treatment with celecoxib diet in *klotho*-deficient mice (**E** and **F**). Statistical analysis was performed using ANOVA with post hoc multiple comparisons test, and  $P<0.05$  was considered significant (designated by \*).

valves because COX2 inhibition is able to block the expression of these factors and reduce valve calcification.

CAVD can result from abnormal valve development, inflammation due to increased lipid deposition, or hyperphosphatemia

secondary to chronic kidney disease, and the cellular processes that lead to valve calcification may differ depending on the underlying factors of the disease.<sup>16,19,39,40</sup> *Klotho*-deficient mice develop hyperphosphatemia secondary to kidney failure, and



valve calcification in these animals is likely related to chronic high serum phosphate.<sup>17,18</sup> Similarly, humans with chronic kidney disease or elevated serum phosphate levels have a higher risk of developing AoV calcification.<sup>16,19</sup> Valve endothelial injury, leading to the recruitment of infiltrating immune cells and subsequent VIC myofibroblast activation, has been implicated in the initial stages of CAVD.<sup>3</sup> In the current study, we show that there is little evidence of macrophage infiltration in the AoVs of *klotho*-deficient mice, and COX2 expression in VICs does not overlap with CD45 expression, suggesting that COX2-expressing cells are not leukocytes (Figure 3; Figure III in the online-only Data Supplement). In addition, calcified VICs do not transition through a myofibroblast intermediate before calcifying in *klotho*-deficient mice, as indicated by lack of SMACreER lineage-positive calcified cells (Figures V and VI in the online-only Data Supplement). Likewise,  $\alpha$ SMA (ACTA2) gene expression was not increased in cultured porcine VICs treated with osteogenic media, consistent with published studies (data not shown).<sup>41</sup> Human diseased AoVs also exhibit calcification and COX2 expression in regions with little immune infiltration or myofibroblast activation, suggesting that valve calcification can occur by multiple cellular mechanisms (Figure 2; Figure I in the online-only Data Supplement). Thus, there may be distinctive differences in the process of AoV calcification depending on the underlying factors contributing to the disease.

Nonselective COX1/COX2 inhibitors are nonsteroidal anti-inflammatory drugs commonly used to treat inflammation associated with joint or muscle pain.<sup>24</sup> The only currently Food and Drug Administration–approved selective COX2 inhibitor, celecoxib (Celebrex), is used to treat pain associated with osteoarthritis, but it is contraindicated for patients with heart disease risk factors.<sup>26,27,42</sup> The current study demonstrates that COX2 inhibition via celecoxib treatment reduces valve calcification in *klotho*-deficient mice and also reduces osteogenic gene induction and mineralization in cultured porcine VICs. This approach may not be feasible in human patients because of the increased cardiovascular risk associated with the use of COX2 inhibitors.<sup>26,27</sup> A recent observational study in the Multi-Ethnic Study of Atherosclerosis (MESA) participants showed that use of specific or nonspecific COX inhibitors was not associated with lower AoV calcification scores.<sup>43</sup> However, this was not a placebo-controlled trial, and additional research is necessary to determine the effectiveness of COX inhibitors in human CAVD.

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### Disclosures

None.

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## Significance

Calcific aortic valve disease is a significant cause of morbidity and mortality. Understanding the molecular mechanisms that contribute to the formation and progression of valve calcification may lead to new pharmacotherapies as alternatives to surgery. In the *klotho*-deficient mouse model of calcific aortic valve disease, we show that COX2 expression is increased in valvular interstitial cells at the hinge region of the aortic valves and localizes to the region of calcification once the calcific lesions have formed. We also show that human explanted calcific aortic valve disease tissues have increased COX2 expression when compared with healthy controls. Blockade of COX2 activity in vitro blocks osteogenic gene induction and mineralization in valvular interstitial cells. Furthermore, genetic mutation or pharmacological inhibition of COX2 activity in vivo is sufficient to reduce AoV calcification in the *klotho*-mouse model of calcific aortic valve disease. This study shows that COX2 inhibition reduces osteogenic gene induction and AoV calcification in vivo.