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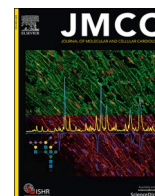
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New calcification model for intact murine aortic valves

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ABSTRACT

Calcific aortic valve disease (CAVD) is a common progressive disease of the aortic valves, for which no medical treatment exists and surgery represents currently the only therapeutic solution. The development of novel pharmacological treatments for CAVD has been hampered by the lack of suitable test-systems, which require the preservation of the complex valve structure in a mechanically and biochemical controllable system. Therefore, we aimed at establishing a model which allows the study of calcification in intact mouse aortic valves by using the Miniature Tissue Culture System (MTCS), an *ex vivo* flow model for whole mouse hearts. Aortic valves of wild-type mice were cultured in the MTCS and exposed to osteogenic medium (OSM, containing ascorbic acid, β -glycerophosphate and dexamethasone) or inorganic phosphates (PI). Osteogenic calcification occurred in the aortic valve leaflets that were cultured *ex vivo* in the presence of PI, but not of OSM. *In vitro* cultured mouse and human valvular interstitial cells calcified in both OSM and PI conditions, revealing *in vitro-ex vivo* differences. Furthermore, endochondral differentiation occurred in the aortic root of *ex vivo* cultured mouse hearts near the hinge of the aortic valve in both PI and OSM conditions. Dexamethasone was found to induce endochondral differentiation in the aortic root, but to inhibit calcification and the expression of osteogenic markers in the aortic leaflet, partly explaining the absence of calcification in the aortic valve cultured with OSM. The osteogenic calcifications in the aortic leaflet and the endochondral differentiation in the aortic root resemble calcifications found in human CAVD. In conclusion, we have established an *ex vivo* calcification model for intact wild-type murine aortic valves in which the initiation and progression of aortic valve calcification can be studied. The *in vitro-ex vivo* differences found in our studies underline the importance of *ex vivo* models to facilitate pre-clinical translational studies.

1. Introduction

Calcific aortic valve disease (CAVD) is a common progressive disease, whose prevalence, and therefore health and financial burden, is expected to significantly increase with the ageing population [1]. Currently no pharmacological therapy has shown to be effective in CAVD and when severe aortic stenosis occurs surgical or percutaneous valve replacement is the only treatment option [2]. Full understanding of the pathogenesis of CAVD is therefore imperative to find new medical treatments and research is focused on finding accurate models to elucidate the pathological process behind CAVD [3–5].

Aortic valves have a highly organized structure consisting of 3 layers of extracellular matrix (ECM) rich in collagen (fibrosa), proteoglycans (spongiosa) and elastin (ventricularis). The leaflets are covered by valvular endothelial cells (VECs) and interspersed with valvular interstitial cells (VICs). VICs are responsible for the maintenance of the valvular structure by producing, degrading and organizing the ECM [6]. In CAVD, presence of calcification is the prominent feature together with inflammation, fibrosis and lipid deposition. Calcific mineral deposition can occur through dystrophic, osteogenic and endochondral calcification and involves the activation of VICs. During dystrophic calcification, VICs adopt a myofibroblast phenotype expressing alpha-smooth muscle

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actin (α SMA) and undergo apoptosis creating a substrate on which calcium deposits form [5,7–12]. During osteogenic calcification VICs adopt an osteogenic phenotype, demonstrated by the expression of proteins such as runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP) and cyclo-oxygenase 2 (COX2), and produce calcium deposits [13–18]. During endochondral calcification, osteogenic calcification is preceded by cartilage formation [19]. These 3 types of calcification can occur simultaneously or independently in human aortic valves [9,18,20,21], where the calcifications have been described as “nodular” or “intrinsic”. Nodular calcifications have been mostly observed in the mid-tip of valve leaflets and are characterized by elastin fragmentation. “Intrinsic” calcifications have recently been described within the ECM in the hinge point of the aortic valves and are characterized by increased proteoglycan deposit as in endochondral calcification [9]. Both calcifications are mostly located at regions with high mechanical stress and disturbed flow indicating that mechanical stress has an important role in activating VICs and propagating calcification [22,23].

To study aortic valve calcification, both *in vitro* and *in vivo* models have been explored. *In vitro*, the most commonly used protocol is culturing VICs in osteogenic media (OSM) which consists of ascorbic acid, β -glycerophosphate and dexamethasone [24]. Dexamethasone has been shown to induce expression of and activate RUNX2; ascorbic acid facilitates differentiation by altering the ECM and β -glycerolphosphate provides phosphates used to create mineral depositions [25–28]. A different *in vitro* protocol induces calcification by addition of 3 mM inorganic phosphates to the medium (referred to as PI) mimicking mild hyperphosphatemia (healthy is up to 1.45 mM) [29,30]. Aside from the phosphates required to generate mineral depositions, inorganic phosphates have been shown to regulate expression of proteins such as BMP2 and osteopontin [31,32]. Studies using *in vitro* models have demonstrated the important role of mechanical stress, the influence of the matrix and the interaction between VICs, VECs and macrophages in the process of calcification [5,7,33–37]. These studies thereby demonstrated that the absence of the complete valvular structure might prevent drawing accurate conclusions for *in vivo* calcification. *In vivo* mouse models have in turn the advantage of studying the whole aortic valve under native hemodynamic conditions [38]. The induction of calcification, however, is limited in wildtype animals, and requires dietary and/or genetic modification [38–41]. In addition, in contrast to *in vitro* models, single experimental parameters cannot accurately be controlled and altered.

Because of the *in vitro* and *in vivo* limitations, it is imperative to find more suitable models to study CAVD combining both the preservation of the complex valve structure that characterizes *in vivo* modelling and a mechanically and biochemically controllable system that is facilitated by *in vitro* modelling [42]. We have previously developed the miniature tissue culture system (MTCS), which allows the controllable culture of mouse valves in their natural position in the heart and under specific hemodynamic conditions [43–45]. In this study, we have used the MTCS to develop a calcification model for the intact murine aortic valve. We

show that calcification can be induced in the intact aortic valve of wild type mice by exposing the leaflets to increased levels of PI, but not when exposed to OSM. Dexamethasone appeared to be at least partly responsible for the absence of calcification in the OSM condition. Culturing murine valvular cells *in vitro* showed calcification in both OSM and PI conditions, demonstrating clear *in vitro-ex vivo* differences. Furthermore, calcification observed in the aortic leaflets was associated with markers of osteogenic differentiation and not of dystrophic calcification. In turn, at the level of the aortic root endochondral differentiation was observed when cultured in both PI and OSM conditions. In conclusion, we developed an *ex vivo* calcification model for murine aortic valves which can facilitate pre-clinical translational studies to further advance our understanding of CAVD.

2. Materials and methods

2.1. VIC isolation and culture

Human VICs (hVICs; kindly provided by dr Hjortnaes) were cultured on 0,1% gelatin-coated wells in DMEM supplemented with 10% FBS and PenStrep (100 U/ml, Gibco).

Mouse VICs (mVICs) were isolated by pooling the aortic, pulmonary, mitral and tricuspid valves per mouse into a 48-wells coated with 0,1% gelatin in 100 μ l dissociation medium (0.125% Trypsin (Serva), 0,125% EDTA (Serva), 1,25 mg/ml Collagenase A (Roche), 46% DMEM high glucose (ThermoFisher), 1% fetal bovine serum (FBS), Insulin-Transferrin-Selenium (ITS; 10 μ g/ml insulin, 5,5 μ g/ml Transferrin, 6,7 ng/ml sodium selenite; Gibco) and incubated over night at 4 °C. Subsequently, the wells were incubated for 35 min at 37 °C and the mixture was gently dissociated using a syringe. Afterwards 300 μ l culture medium (DMEM, 10% FBS, ITS, antibiotics/antimycotics (Sigma)) was added and the cells were grown at 37 °C until confluent. Media was refreshed twice a week. DMEM contains 0,9 mM of phosphate, which is in the range of adult blood phosphate concentrations (0,87–1,45 mM).

2.2. Calcification *in vitro*

Cells were seeded for either visualization of the calcification (48-well size) or RNA isolation (12-well size). Upon confluency, the VICs were treated with calcifying medium OSM (culture medium supplemented with 10 mM β -glycerophosphate (Merck), 100 nM dexamethasone (Merck) and 50 μ g/ml ascorbic acid (Merck)) or PI (culture medium supplemented with 3 mM sodium phosphate (Sigma)) which was replaced twice a week. To quantify the calcification, the cells were fixed after 7 or 21 days using 4% paraformaldehyde solution in phosphate buffered saline (PFA/PBS, pH 7.2) for 10 min, washed with milli-Q water (MQ) and incubated with 2% Alizarin Red (Sigma, pH 4.2) in MQ for 3 min to visualize calcification. The plates were washed twice with MQ and imaged. Finally the alizarin red was dissolved by replacing the MQ with 150 μ l cetylperidiumchloride for 3 h at 37 °C and the

Table 1
Primer sequences for qPCR. * 5'end to 3'end.

Gene	Forward*	Reverse*
GAPDH	AACITTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
28S	GGCCACTTTTGGTAAGCAGA	GCGGATTCCGACTTCCAT
PECAM1	CAAAGTGAATCAAACCGTATCT	CTACAGGTGTGCCCGAG
ALP	GAACAGACCCCTCCACGAG	GTCCTCTCTTTCTCTGGCACA
COX2	AGAAGGAAATGGCTGCAGAA	GCTCGGCTTCCAGTATTGAG
PIT1	TGTGGCAAATGGGCGAGAAG	AGAAAGCAGCGGAGAGACGA
PIT2	CCATCGGCTTCTCACTCGT	AAACCAGAGGCGACAATCT
RUNX1	TCACCTCTCTCTGTCCAC	CACCATGGAGAAGTGGTAGG
RUNX2	CCACAAGGACAGATCAGATTACA	TGGCTCAGATAGGAGGGGTA
RUNX3	CCGGCAATGATGAGAACTAC	GGAGAAGGGGTTTCAGGTTTA
Aggrecan	TCTACCCCAACCAACCGG	AGGCATGGTGCTTTGACAGTG
Collagen2	TTCCACTTCAGCTATGGCGA	GACGTTAGCGGTGTTGGGAG
α SMA	ACTGGGACGACATGGAAAAG	CATCTCCAGAGTCCAGCACA

absorbance was measured in duplo at 595 nm. To study transcriptional activity, RNA was isolated using the ReliaPrep RNA cell miniprep kit (Promega) according to the manufacturer's protocol. RevertAid First Strand cDNA Synthesis (ThermoFisher Scientific) was used to generate cDNA according to the manufacturer's protocol, after which qRT-PCR was performed using GoTaq qPCR Master Mix (Promega, A6001). GAPDH and 28S were used as housekeeping genes. Primer sequences used are detailed in Table 1.

2.3. Ex vivo culture of mouse aortic valves

All animal experiments were performed in 2–6 months old mice with a mixed genetic background (B6;129) according to protocols approved by the animal welfare committee of the Leiden University Medical Center and conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Mouse hearts were cultured in the MTCS as previously described [44]. In summary and with the following modifications: mice were anesthetized with 4% isoflurane and the hearts were *in situ* perfused with salt solutions, removed and transferred to the perfusion chambers. For each heart, the inflow needle of the perfusion chamber was inserted into the aorta and ligated with a suture. Flow (1000 μ l/min) was introduced using a pump and medium (control medium, control medium with 100 nM dexamethasone, PI, PI with 100 nM dexamethasone, OSM, OSM without dexamethasone) was directed from the reservoir through the bubble trap and into the perfusion chamber where it flowed through the aorta towards the closed aortic valve into the coronary circulation (Fig. 1). The medium exited the heart via the right atrium and recirculated to the reservoir. The medium was replaced one time. After culture for 1 week the hearts were isolated and fixed overnight with 4% PFA/PBS.

Fixed mouse hearts were dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin, and sectioned at 6 μ m. The sections were deparaffinized and hydrated before subsequent staining. For visualization of the calcification, alizarin red (Sigma) and von kossa (Sigma) stainings were performed. Sections were stained with Weigert's Resorcin Fuchsin (EMS) to identify elastic fibres, alcian blue (Klinipath) to visualize glycoaminoglycans (GAGs) and counterstained with nuclear-fast red (Sigma). For immunofluorescent staining the slices were boiled for 8 or 35 min in Antigen Retrieval Buffer (10 mM Tris (pH 9)/1 mM EDTA/0.05% Tween-20) using a pressure cooker. Sections for aggrecan staining were in addition incubated for 1 h at 37 °C with chondroitinase ABC (Sigma; 200 mU/ml) diluted in 50 mM Tris (pH 8.0; Sigma) with 60 mM sodium acetate (Sigma) and 0.02% bovine serum albumin (BSA; Sigma). After blocking with 1%BSA in 0,1%Tween-PBS, sections were incubated overnight with the primary antibodies directed

against RUNX1 + RUNX3 + RUNX2 (RUNX1/2/3; Abcam; [EPR3099] ab92336; 1:100), ALP (R&D; AF2910; 1:250), COX2 (D5H5; Cell Signaling; 12282; 1:200), Platelet Endothelial Cell Adhesion Molecule (PECAM-1; R&D; 1:1000), α SMA (Sigma; A2547; 1:20.000), cleaved caspase-3 (cCasp3; Cell Signaling; #9664; 1:100), aggrecan (Millipore; AB1031; 1:200), collagen II (SouthernBiotech; 1310-01; 1:50) followed by incubation with alexa-conjugated secondary antibodies (Molecular Probes). Slides are mounted using DAPI containing ProlongGold Anti-fade reagent (Thermofisher). All slides were scanned with the Panoramic 250 slide scanner (version1.23, 3DHISTECH Ltd.) and analyzed using Caseviewer (version2.3, 3DHISTECH Ltd.).

2.4. Quantifications

To quantify the calcification in the aortic valve, the percentage of the alizarin red-positive area of the aortic valve was determined. At least 6 sections with 96 μ m interval were used per heart and the measurements were averaged. To determine the positive or elastin-positive area of the aortic root, the surface of the positive or elastin-positive area of the aortic root was determined of at least 4 sections with 96 μ m interval per heart and the measurements were averaged. To determine the percentage of RUNX1/2/3-positive cells of the aortic valve, the total number of RUNX1/2/3-positive cells were divided by the total number of nuclei as determined by DAPI-staining. Quantifications were performed using Caseviewer (version2.3, 3DHISTECH Ltd.) and ImageJ.

2.5. Statistics

Statistical analysis was performed using Graphpad Prism (version 9). Data was tested for significance as indicated in each legend using analysis of variance (ANOVA) with Tukey correction or Kruskal-Wallis with Dunn's correction for multiple groups, and Students *t*-test or Mann-Whitney test for comparison of 2 groups. Data are reported as means $\pm$ SEM. A *P*-value below 0.05 was considered significant.

3. Results

3.1. PI induces calcification in ex vivo cultured aortic valves

In order to induce calcification in intact murine aortic valve leaflets, mouse hearts were cultured in the MTCS in a continuous closed position with medium flowing from the aorta towards the closed aortic valve into the coronary circulation (Fig. 1). In this condition, the aortic valve is exposed to continuous hemodynamic stresses at its aortic side and continuous mechanical stresses throughout the valve, which are suggested to be important drivers of aortic valve calcification [22]. Hearts

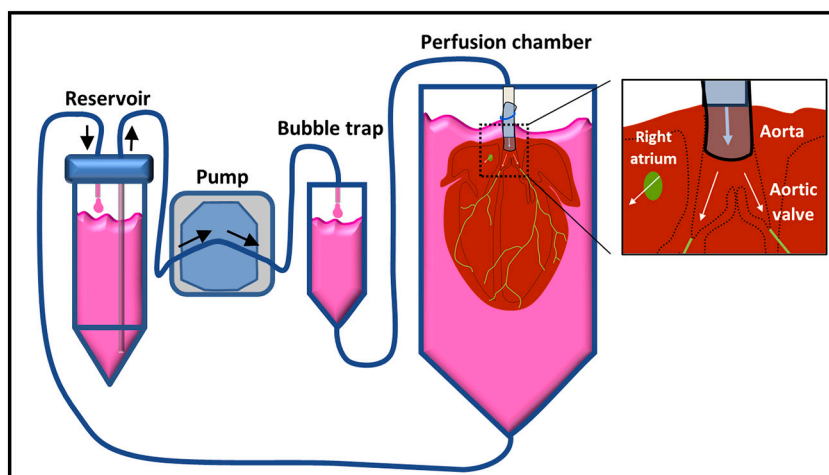


Fig. 1. The ex vivo flow system for mouse aortic valves. The MTCS is a closed flow circuit consisting of a reservoir with medium, a pump, a bubble trap and a perfusion chamber which are connected by tubings. In the perfusion chamber, the mouse heart is ligated to the inflow needle that is inserted into the aorta. The pump directs medium from the reservoir through the bubble trap into the perfusion chamber where it flows from the aorta into the coronary circulation (green lines) thereby closing the aortic valve. The medium exits the heart via the right atrium and flows back to the reservoir (modified from Kruithof et al. 2015 [44]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

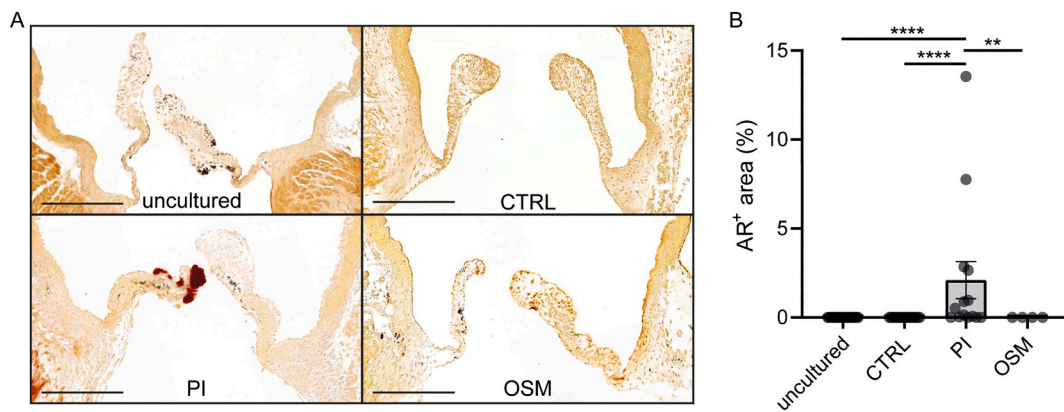


Fig. 2. Calcification of *ex vivo* cultured murine aortic valves. A) Representative pictures of alizarin red-stained aortic valves cultured for 0 days (uncultured), cultured for 7 days in control medium (CTRL), PI, or OSM. B) Quantification of the percentage of the alizarin red-positive valve area (uncultured: $n = 27$, CTRL: $n = 19$, PI: $n = 14$, OSM: $n = 4$). Data are presented as means $\pm$ SEM. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed to evaluate significant differences. **: $p < 0.01$, ****: $p < 0.0001$. Scalebar is 250 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were cultured in the presence of OSM or PI and compared to uncultured mouse hearts and control cultured mouse hearts without OSM or PI (CTRL). Calcification was identified by alizarin red staining (Fig. 2A) and confirmed with von kossa staining (not shown). The uncultured and control cultured hearts did not show any calcification (Fig. 2A,B). In the presence of PI, however, calcification was observed in the majority of the hearts (10 out of 14) after 7 days of culture with varying densities and located at the tip or body of the leaflet (Fig. 2B). In the presence of OSM, on the other hand, no calcification was observed after 7 days (Fig. 2A,B). Also after 3 weeks of culture in the presence of OSM no calcification was observed in the aortic valve ($n = 5$; not shown), indicating that the

absence of calcification in OSM cultures was not due to a delay in calcification. Therefore, calcification in intact wild type murine aortic valves could efficiently be induced *ex vivo* by PI, but not by OSM.

3.2. Both OSM and PI induce calcification *in vitro*

The inability of OSM to induce calcification in the *ex vivo* cultured mouse aortic valve is in contrast with several studies studying the *in vitro* calcification of VICs from different species [7,46–49]. To determine whether this was caused by a species or *in vitro-ex vivo* difference, mVICs of cardiac valves were isolated and cultured in PI or OSM and stained

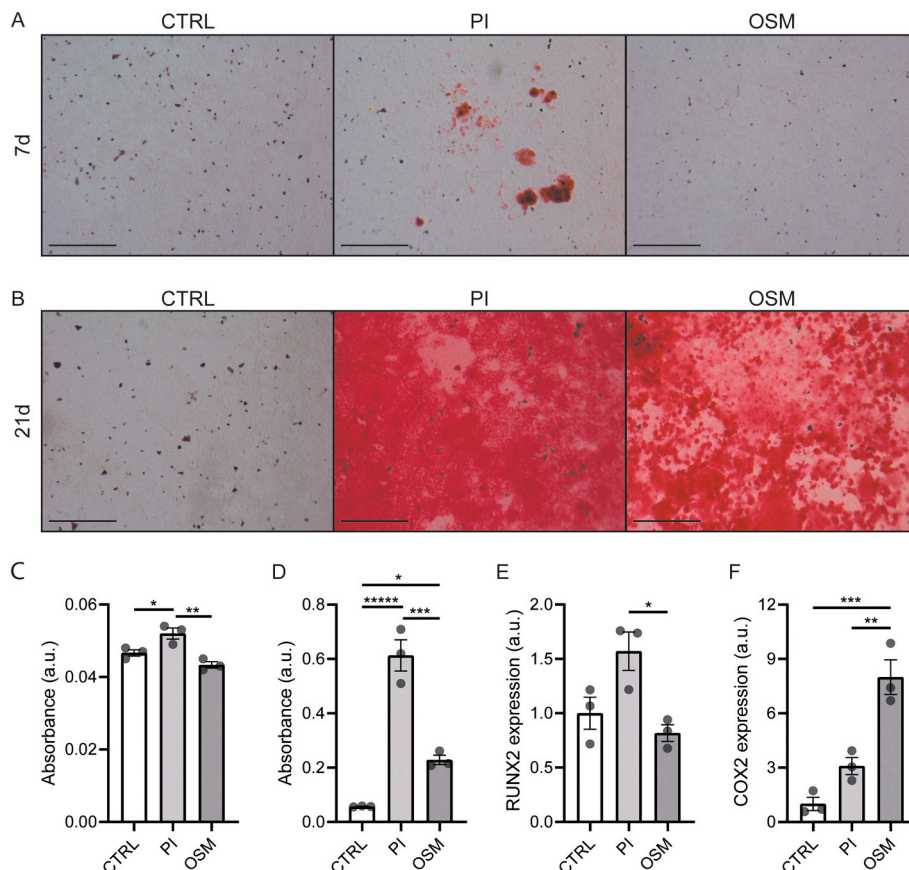


Fig. 3. *In vitro* calcification of mVICs in PI and OSM. A, B) Representative pictures of alizarin red-stained mVICs after 7 days (passage 9, A) or 21 days (passage 13, B) of culture in control (CTRL), PI or OSM medium. C, D) Quantification of the alizarin red staining in mVICs cultured for 7 (passage 9, C) or 21 days (passage 13, D) by absorbance measurements of dissolved AR staining. Note the different scales for C and D. E, F) Graphs indicating RNA expression of RUNX2 (E) and COX2 (F) in mVICs cultured for 7 days. Data are presented as means $\pm$ SEM. One-way ANOVA followed by Tukey's multiple comparisons test was performed to evaluate significant differences. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. Scale bar is 250 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using alizarin red. In the first set of experiments (passage 0 and 9), in which mVICs were cultured for 7 days, only PI was able to induce calcification (Fig. 3A,C and data not shown). In the second set of experiments, in which mVICs were cultured for 21 days (passage 0 and 13), both PI and OSM were able to induce calcification (Fig. 3B,D and data not shown). Similar results were obtained using hVICs (Fig. S1). qPCR analysis of the mVIC cultures of 7 days showed that RUNX2 expression was significantly higher after culture in PI compared to OSM, whereas

COX2 was significantly higher after culture in OSM (Fig. 3E,F). qPCR analysis of ALP1, α SMA, aggrecan, collagen II, PiT1 and PiT2 expression did not show significant differences (data not shown). Taken together, PI and OSM could both induce calcification of mVICs and hVICs *in vitro*, although by using different signaling pathways.

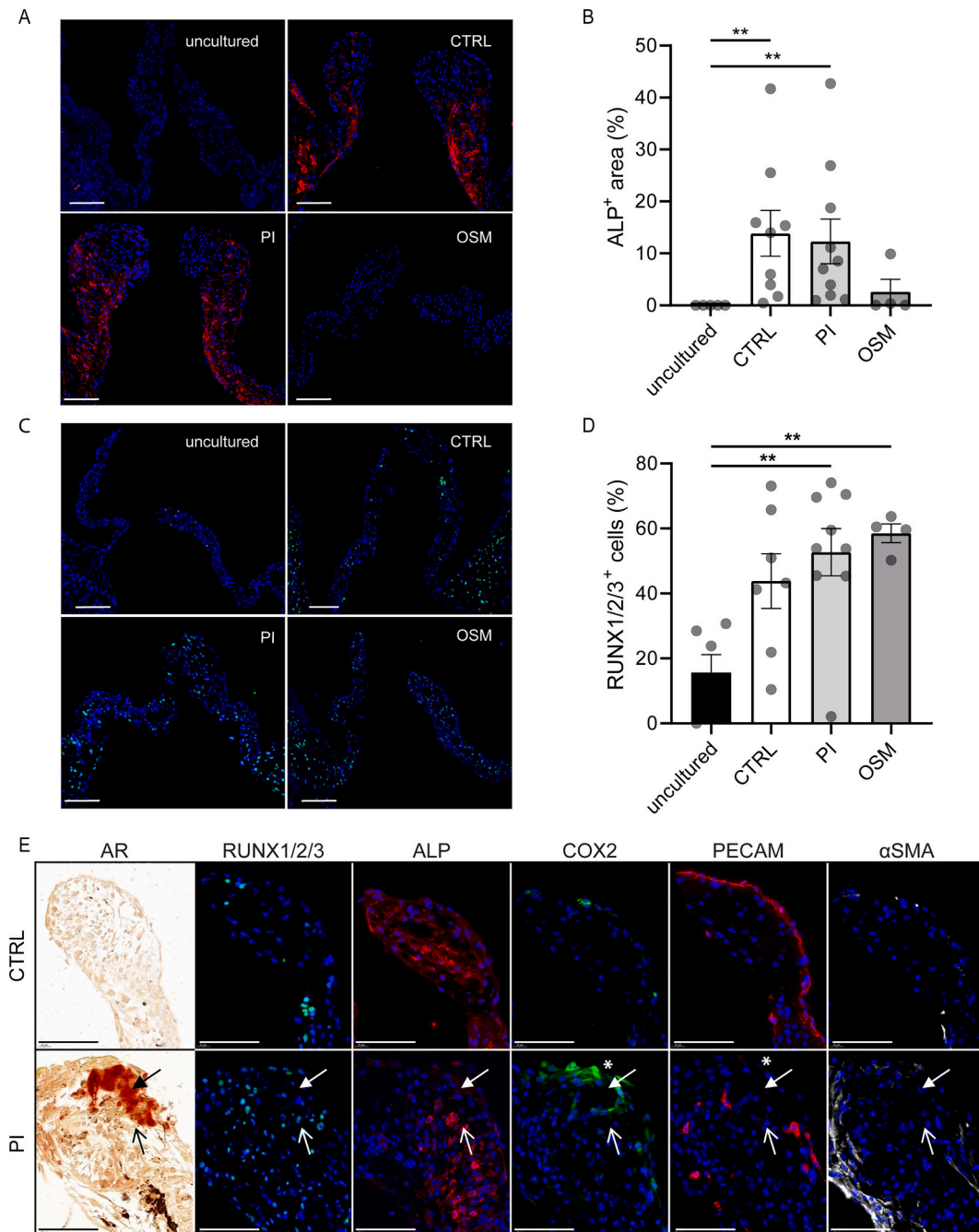


Fig. 4. Osteogenic marker expression in *ex vivo* cultured aortic valves. A,B) Representative pictures of uncultured and *ex vivo* cultured aortic valves in control medium (CTRL), PI, or OSM stained for ALP (A) and RUNX1/2/3 (B). C,D) Quantification of ALP-positive valve area (B) and percentage of RUNX1/2/3 positive cells in the aortic valve (D). E) Representative pictures of the calcified area in aortic valves cultured in PI and non-calcified aortic valves cultured in control medium (CTRL) showing stainings for alizarin red (AR), RUNX1/2/3, ALP, COX2, PECAM and α SMA. Closed arrows indicate location of calcification. Open arrows indicate area surrounding calcification. Asterisks indicate COX2 expression in PECAM-negative cells. Data are presented as means $\pm$ SEM. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed for the graph in B and One-way ANOVA followed by Tukey's multiple comparisons test was performed for the graph in D to evaluate significant differences. **: $p < 0.01$, ***: $p < 0.001$. Scalebar is 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Osteogenic differentiation in cultured aortic valves

To further characterize the calcification processes in the *ex vivo* cultured aortic valves, stainings were performed for different osteogenic markers. ALP was not expressed in the uncultured valves but was significantly upregulated in the hearts cultured in control medium or in the presence of PI, but not in the presence of OSM (Fig. 4A,B). The number of RUNX1/2/3-positive cells was low in uncultured aortic valves and showed a trend towards increase in the hearts cultured with control medium (Fig. 4C,D). The number of RUNX1/2/3-positive cells was significantly increased upon culture in PI and OSM compared to uncultured hearts (Fig. 4C,D). In PI cultured valves, at the location of the

calcification itself, the expression of RUNX1/2/3 and ALP was relatively low (closed arrow in Fig. 4E), whereas the expression was higher surrounding these calcifications (open arrow in Fig. 4E). COX2 was observed in a subset of VECs in uncultured aortic valves and aortic valves cultured in control medium and OSM. In the calcified region of the valves cultured with PI, COX2 expression was observed in addition to VECs also in non-VECs (see asterisks in Fig. 4E). A striking lack of α SMA expression was observed at the location of calcification, whereas a low signal of α SMA was present in other parts of the valve (Fig. 4E). Further, very few cleaved caspase-positive cells were observed in all groups, with no positive cells in the calcification area (data not shown). Altogether, these observations indicate that the calcifications in the

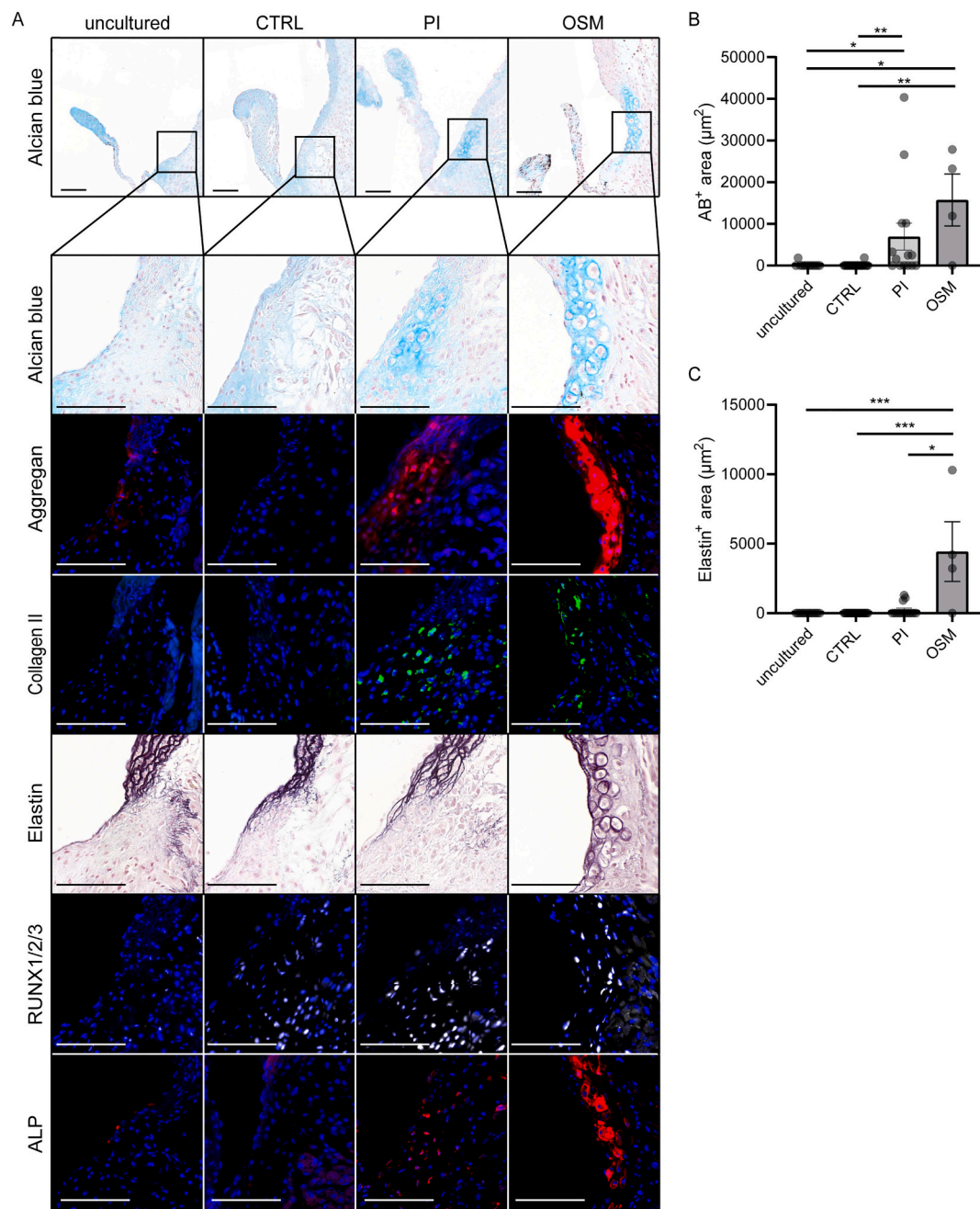


Fig. 5. Endochondral differentiation in the aortic root of *ex vivo* cultured mouse hearts. A) Representative pictures of uncultured and *ex vivo*-cultured aortic valves in control medium (CTRL), PI, or OSM stained for Alcian blue, aggrecan, collagen II, elastin, RUNX1/2/3 and ALP. The images shown for each condition are from the same heart. B,C) Quantification of the Alcian blue (B)- and elastin (C)-positive area in the aortic root. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed to evaluate significant differences. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Scalebar is 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aortic leaflets of *ex vivo* cultured mouse hearts are due to osteogenic calcification and not dystrophic calcification. In addition, differential expression of osteogenic markers is observed in PI and OSM cultures.

3.4. OSM and PI induce endochondral differentiation in the aortic root of *ex vivo* cultured mouse hearts

To assess if chondrogenic differentiation occurs during the *ex vivo* culture of the aortic valves, the expression of glycosaminoglycans (GAGs), elastin, collagen II, aggrecan and RUNX1/2/3, was determined. Alcian blue staining to visualize GAGs demonstrated large round cells surrounded by large amounts of GAGs in the aortic root, near the hinge of valves cultured for 7 days in PI and OSM (Fig. 5A,B). These areas were also positive for aggrecan and collagen II (Fig. 5A), indicating the presence of cartilage. This was not observed in the leaflets of the aortic

valve (Fig. 5A and data not shown). Furthermore, culture with OSM induced elastin expression in the cartilage region (Fig. 5A,C), indicating the formation of elastic cartilage. RUNX1/2/3 expression was observed mostly surrounding the cartilage, possibly indicating a continuing process of cartilage formation (Fig. 5A). To determine whether cartilage formation is followed by ossification as seen in bone formation (endochondral ossification), ALP expression was determined. ALP was found in both PI and OSM cultures although much higher in OSM cultures (Fig. 5A). Actual calcification in the aortic root as visualized by alizarin red was not observed after 7 days of culture (not shown and Fig. 2a). Together, these observations indicate that endochondral differentiation can be induced in the aortic root of *ex vivo* cultured mouse hearts in the presence of PI and OSM.

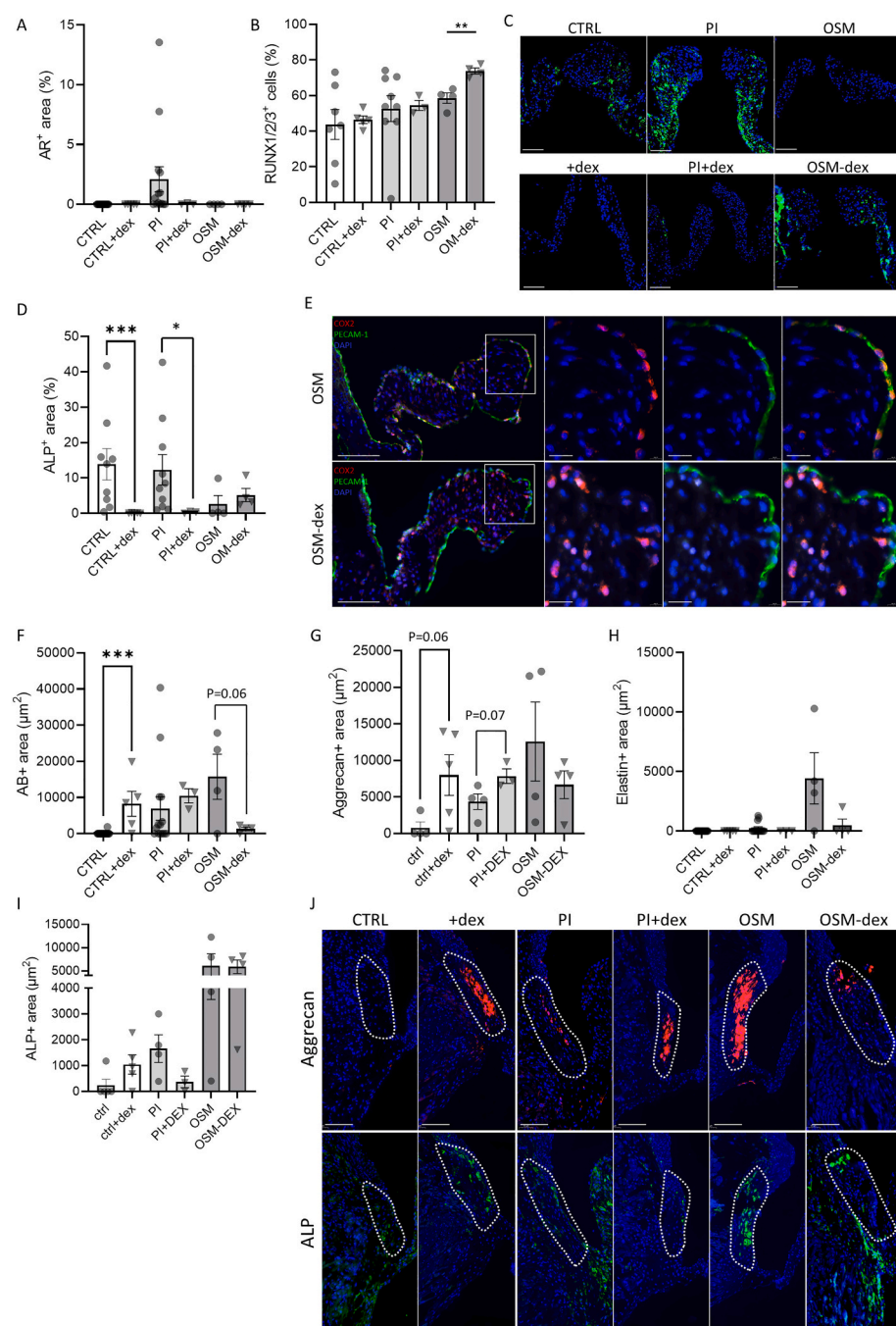


Fig. 6. The role of dexamethasone in osteogenic calcification of the aortic valve and endochondral differentiation in the aortic root of *ex vivo* cultured mouse hearts. A,B,D,F-I) Quantifications of the alizarin red-positive area (A), the percentage of RUNX1/2/3 positive cells (B) and ALP-positive valve area (D) in the aortic valve and Alcian blue (F-I), aggrecan (G), elastin- (H) and ALP- (I) positive area in the aortic root of *ex vivo* cultured mouse hearts valves in control medium (CTRL), control medium + dexamethasone (CTRL+dex), PI, PI + dexamethasone (PI+dex), OSM or OSM - dexamethasone (OSM - dex). C,E,J) Representative pictures of *ex vivo* cultured mouse hearts stained for ALP (green; C), COX2 (red) and PECAM-1 (green; E) and ALP (green) and aggrecan (red; J). Mann-Whitney test was performed for the graphs in A,D,F, G, H and I, and an unpaired *t*-test was performed for the graph in B to evaluate significant differences. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Scalebar is 100 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Dexamethasone stimulates endochondral differentiation and inhibits calcification

The OSM-component dexamethasone is an important inducer of osteogenesis in mesenchymal stem cells [25,26,28], vascular smooth muscle cells [50] and VICs [5] *in vitro*. The effect of dexamethasone on other cell types is less clear and can be contradictory [51,52]. In the *ex vivo* model, all valvular cell types are present in their native environment and it is their interaction that will determine the effect of exposure to dexamethasone. To obtain a better understanding of the effect of dexamethasone on calcification in the aortic valve and aortic root, dexamethasone was added to the control and PI cultures, and omitted from the OSM cultures (Fig. 6). In the control cultures, dexamethasone did not change the extent of RUNX1/2/3 expression, whereas ALP expression was abolished in the aortic valve (Fig. 6B–D). In the aortic root, cartilage formation was induced as assessed by alcian blue (Fig. 6F) and aggrecan expression (Fig. 6G,J) and ALP appeared to be slightly induced by dexamethasone addition (Fig. 6I,J). In the PI cultures, dexamethasone prevented calcification (Fig. 6A) and ALP expression (Fig. 6C,D), whereas RUNX1/2/3 expression was not affected (Fig. 6B). In the aortic root, dexamethasone slightly increased aggrecan expression (Fig. 6G), but inhibited ALP expression (Fig. 6I,J). In the OSM cultures, omitting dexamethasone did not result in calcification (Fig. 6A) but increased RUNX1/2/3 expression (Fig. 6B) and ALP expression (4 out of 4; Fig. 6C,D) in the aortic valve. COX2 expression, which was present mostly in the endothelial cells in the control, PI and OSM cultures and in the non-endothelial cells at places of calcification in the PI cultures (Fig. 4E, 6E), was present mostly in non-endothelial cells in the OSM cultures where dexamethasone was omitted (Fig. 6E). In the aortic root, omission of dexamethasone from the OSM cultures resulted in a decrease of cartilage formation as assessed by alcian blue (Fig. 6F), aggrecan (Fig. 6G) and elastin expression (Fig. 6H), whereas ALP expression did not change (Fig. 6I). Together, these observations indicate that in the *ex vivo* cultures of mouse hearts, dexamethasone inhibits calcification in the aortic valve and stimulates endochondral differentiation in the aortic root.

3.6. Discussion

In this study, we have established a novel *ex vivo* calcification model for intact murine aortic valves. In this model, calcification can be induced in aortic valves of wild type mice that are cultured under flow conditions in their natural position within the heart by the exposure to elevated levels of PI. The commonly used OSM was unable to induce calcification within the aortic valve leaflets in this *ex vivo*-model. In contrast, OSM did induce calcification in *in vitro* cultures of mouse and human valvular cells, indicating clear *in vitro-ex vivo* differences. Furthermore, endochondral differentiation was observed in the root of the aorta in both PI and OSM *ex vivo* culture conditions, indicating the presence of different forms and mechanisms of calcification in *ex vivo* cultured aortic valves. Finally, the OSM-component dexamethasone was able to inhibit calcification in the aortic valve and stimulate endochondral differentiation in the aortic root.

A clear understanding of the pathology of valvular calcification has been hampered by insufficient *in vitro* and *in vivo* test-models. *In vitro* models lack the complex valvular structure and the simulation of the hemodynamic condition whereas *in vivo* systems require genetic modification, long incubation times and have limited mechanical and biochemical controllability [5,7,33–35,38]. By culturing the mouse hearts in the MTCS [43] under calcifying conditions, we have created a highly controllable culture model in which calcification can be induced within 1 week in the intact aortic valve. We observed 2 types of aortic valve calcification present at distinct locations, which coincide with the 2 types of calcification found in human aortic leaflets, *i.e.* the nodular form found on the more distal part of the aortic leaflets and the intrinsic form found at the leaflet hinge [9].

Nodular calcification was only observed in the PI cultures and was mostly present in the body and near the coaptation part of the aortic leaflet characterized by relative low levels of the early osteogenic markers RUNX1/2/3 and ALP and by specific expression of COX2 in the calcified area. The region surrounding the calcification displayed higher levels of RUNX1/2/3 and ALP, indicating ongoing osteogenic differentiation. The absence of α SMA within and directly surrounding the calcification area suggests that a transient myofibroblast stage before calcification did not take place, which has been shown for VICs *in vitro* [8] and *in vivo* [16]. The lack of apoptosis in these regions after 7 days of culture further suggested the absence of dystrophic calcification. Calcification in the aortic leaflet was not observed in the OSM cultures. A possible explanation might be the virtual absence of ALP in the aortic valves cultured in OSM as ALP activity is required for OSM induced calcification [48,53]. ALP is present in the cultures without OSM (control cultures), indicating that a component of OSM inhibits ALP expression. The OSM-component dexamethasone is a synthetic glucocorticoid steroid that has been shown to increase the expression of ALP and other osteogenic marker in cell cultures [7,48,50,54]. Dexamethasone, however, has also been shown to inhibit COX2 expression [55,56], which is required for calcification in the aortic valves of the *klf1*-deficient mice harboring elevated levels of serum phosphate secondary to kidney failure [16,57]. Whereas we observed non-endothelial COX2 expression in the calcified areas of the aortic valve in the PI cultures, COX2 was mostly absent from the non-endothelial cells, and present in the endothelial cells. To understand the role of dexamethasone in the *ex vivo* cultures of the aortic valve and to find a potential reason for the difference between the effects of PI and OSM, we added dexamethasone to the control and PI cultures and omitted dexamethasone from the OSM cultures. Interestingly, dexamethasone inhibited ALP expression in the control and PI cultures and prevented calcification in the PI cultures, illustrating clear differences between published *in vitro* studies indicating the stimulation of calcification by dexamethasone and our *ex vivo* study showing the inhibition of calcification and calcification pathways by dexamethasone. Omission of dexamethasone did not result in calcification indicating that the presence of dexamethasone in the OSM is not solely responsible for the absence of calcification in the OSM cultures. ALP expression, however, was induced and COX2 expression was present in the non-endothelial cells indicating that dexamethasone does inhibit calcification pathways in the OSM cultures. Although calcification was not observed in the control *ex vivo* cultures, RUNX1/2/3 and ALP were found to be upregulated suggesting osteoblastic activity [58]. Since mechanical stresses may play a role in the activation of VICs and propagation of calcification [22], the altered mechanical stress and flow conditions in the *ex vivo* model might have resulted in induction of early osteogenic markers. Subsequent moderately increased levels of phosphates in the PI cultures allowed calcification to take place.

The second type of calcification observed in the *ex vivo* cultured aortic valves is the endochondral differentiation that was observed in the root of the aorta near the hinge of the aortic valve. Cartilage, as evidenced by its morphological features and high alcian blue, collagen II and aggrecan staining, was bordered by the osteogenic markers ALP and RUNX1/2/3. Previously, osteochondrogenic gene expression has been shown to precede calcification in the same region in the *klf1*-deficient mice [19]. Moreover, cartilage formation was observed in the aortic sinus of an inbred family of Syrian hamster and was suggested to be the result of locally intense mechanical stimulation [59]. These observations suggest that the “intrinsic” calcification found in the human aortic sinus is formed by the process of endochondral calcification and can be recapitulated in wild type mouse hearts using our *ex vivo* flow model. Interestingly, in contrast to the osteogenic calcification in the aortic leaflets, endochondral differentiation was found both in PI and OSM conditions, although the type of cartilage formed differed with elastic cartilage being present in the OSM cultures. Addition of dexamethasone to the control cultures induced cartilage formation and increased the

cartilage formation in the PI cultures. Omission of dexamethasone from the OSM cultures decreased the cartilage formation. Together, these observations show that dexamethasone can induce cartilage formation in the aortic root. PI cultures and OSM without dexamethasone, however, do show cartilage formation indicating that dexamethasone is not solely responsible for the endochondral differentiation process.

Differences between the effects of PI and OSM on VICs are also found in the *in vitro* cultures. After 7 days of *in vitro* culture in OSM, the expression of COX2 was increased, which is in contrast with the PI *in vitro* cultures and the OSM *ex vivo* cultures. Furthermore, RUNX2 expression was increased in PI, but not in OSM *in vitro* cultures. These differences in gene expression between the PI and OSM *in vitro* cultures might explain why PI treatment is more potent in inducing *in vitro* calcification than OSM, showing calcification already after 7 days of culture. Interestingly, in our *in vitro* cultures the valvular cells of aortic, pulmonary, mitral and tricuspid valves were combined and showed after 3 weeks of culture, virtually complete calcification indicating that VICs of all cardiac valves are able to calcify.

The inconsistencies of the observations between the *in vitro* and *ex vivo* experiments are likely caused by the many differences between the culture conditions. First, the hemodynamic condition in the *ex vivo* cultures resembles the diastolic phase of the cardiac cycle and therefore creates similar types of mechanical stresses on the valve as in the *in vivo* situation. Second, the complex valve structure is preserved, including the heterogeneous composition of the matrix and valvular cells whose interactions create multiple levels of regulation, which are lost in cell culture studies [7,33–35,46,49,60]. It is therefore of utmost importance to approach the native environment of the valvular cells as much as possible to obtain the response most representative of the *in vivo* situation. *Ex vivo* systems can fulfill most of these requirements and should be considered an important tool for translational studies.

Some limitations should be mentioned about the culture conditions used in this study. The aortic valves were subjected to flow stress in a continuous closed position, mainly simulating the stresses present during diastole. However, the continuous exposure to retrograde flow from the aorta into the coronary arteries created a mechanical environment favoring calcification to take place already after 7 days of culture in the presence of PI. Culturing the valve in the open position or in presence of pulsatile flow might give further insight in the mechanical regulation of calcification. Furthermore, the valves are cultured in the absence of blood, which contains multiple cell types and factors potentially crucial in the regulation of calcification. The *ex vivo* flow model, however, provides the possibility to add cells and factors back to the culture medium, allowing the controlled study of the involvement of specific cell types and factors in the regulation of valvular calcification.

In conclusion, with the MTCS we can now study the initiation and progression of osteogenic and endochondral calcification in the intact murine aortic valves and root. This provides the possibility to elucidate the mechanical, cellular and molecular mechanisms underlying CAVD, and therefore may set the stage to identify new targets for effective pharmacological therapies in this common and progressive disease.

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Disclosures

None.

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