Exploiting novel valve interstitial cell lines to study calcific aortic valve disease

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Abstract. Calcific aortic valve disease (CAVD) involves progressive valve leaflet thickening and severe calcification, impairing leaflet motion. The *in vitro* calcification of primary rat, human, porcine and bovine aortic valve interstitial cells (VICs) is commonly employed to investigate CAVD mechanisms. However, to date, no published studies have utilised cell lines to investigate this process. The present study has therefore generated and evaluated the calcification potential of immortalized cell lines derived from sheep and rat VICs. Immortalised sheep (SAVIC) and rat (RAVIC) cell lines were produced by transduction with a recombinant lentivirus encoding the Simian virus (SV40) large and small T antigens (sheep), or large T antigen only (rat), which expressed markers of VICs (vimentin and α-smooth muscle actin). Calcification was induced in the presence of calcium (Ca; 2.7 mM) in SAVICs (1.9 fold; $P<0.001$) and RAVICs (4.6 fold; $P<0.01$). Furthermore, a synergistic effect of calcium and phosphate was observed (2.7 mM Ca/2.0 mM Pi) on VIC calcification in the two cell lines ($P<0.001$). Analysis of SAVICs revealed significant increases in the mRNA expression of two key genes associated with vascular calcification in cells cultured under calcifying conditions, runt related transcription factor-2 (RUNX2;1.3 fold; $P<0.05$ in 4.5 mM Ca) and sodium-dependent phosphate transporter-1 (PiT1; 1.2 fold; $P<0.05$ in 5.4 mM Ca). A concomitant decrease in the expression of the calcification inhibitor matrix Gla protein (MGP) was noted at 3.6 mM Ca (1.3 fold; $P<0.01$). Assessment of RAVICs revealed alterations in Runx2, Pit1 and Mgp mRNA expression levels ($P<0.01$). Furthermore, a significant reduction in calcification was observed in SAVICs following treatment with established calcification inhibitors, pyrophosphate (1.8 fold; $P<0.01$) and etidronate (3.2 fold; $P<0.01$). Overall, the present study demonstrated that the use of immortalised sheep and rat VIC cell lines is a convenient and cost effective system to investigate CAVD *in vitro*, and will make a useful contribution to increasing current understanding of the pathophysiological process.

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Abbreviations: CAVD, calcific aortic valve disease; VIC, valve interstitial cell; SAVIC, sheep aortic valve interstitial cell line; RAVIC, rat aortic valve interstitial cell line; RUNX2/Runx2, Runt-related transcription factor 2; PiT1/Pit1, sodium-dependent phosphate transporter 1; MGP/Mgp, matrix Gla protein; PPi, pyrophosphate; α-SMA, alpha-smooth muscle actin; CD31, cluster of differentiation 31; VEC, valve endothelial cell; DMEM, Dulbecco's modified eagles media; FBS, foetal bovine serum; PFA, paraformaldehyde; Ca, calcium; Pi, phosphate

Key words: calcification, valve interstitial cell, calcific aortic valve disease, *in vitro* model, large animal model

Introduction

Calcific aortic valve disease (CAVD) involves gradual thickening of the aortic valve leaflet (aortic sclerosis) and severe calcification, impairing leaflet motion (aortic stenosis) (1). CAVD is a prevalent heart valve disease, present in almost 30% of adults over 65 years, increasing to around 40-50% in those over 75 years (2-4). Dysfunctional heart valves frequently require surgical replacement using mechanical or bioprosthetic valves, however these are prone to failure over time due to structural or thrombosis-related problems (5).

Presently, CAVD is considered an actively regulated and progressive disease (6). The development of this disease is thought to be initiated by injury, inflammation and lipid deposition in the valve, followed by a propagation phase in which factors promoting calcification and osteogenesis drive disease progression (7,8). The increased mechanical stress and injury caused by this early calcification event may then elicit further calcification, leading to a continuous cycle of valve calcification (9).

Valve interstitial cells (VICs) are the predominant cell type in the aortic valves, and play a major role in CAVD progression (7,10). The underlying mechanisms of CAVD share many similarities with that of physiological bone formation (11). VICs are thought to acquire osteoblastic characteristics during the propagation phase of aortic stenosis, following inflammation (7,9). A number of studies have established the ability of VICs to undergo osteogenic trans-differentiation and calcification (12-14). Despite this knowledge, the pathways underlying the initiation and progression of CAVD remain unclear, and studies are needed to elucidate the mechanisms underpinning early disease pathogenesis.

The *in vitro* calcification of primary porcine (15-17), human (14,18,19), rat (20-22) and bovine (23,24) VICs is commonly used as models of aortic valve calcification. However, to date, the application of a cell line to interrogate VIC function has not been reported. Cell lines offer a valuable alternative to primary cells isolated directly from animals, reducing experimental variation and animal use. To our knowledge this is the first study reporting the generation and evaluation of the calcification potential of immortalised VIC lines derived from sheep (SAVIC) and rat (RAVIC).

Materials and methods

Ethics statement. All animal work was approved by The Roslin Institute's and the University of Edinburgh's Protocols and Ethics Committees. The animals were maintained in accordance with UK Home Office guidelines under the regulations of the Animal (Scientific Procedures) Act 1986.

Establishment of sheep and rat valve interstitial cell lines. Sheep primary aortic VICs were harvested from a 4-year-old Scottish mule sheep (generated from a Bluefaced Leicester sire and Scottish Blackface dam cross; Dryden Farm, Midlothian, UK). Rat aortic VICs were isolated from aortic valve leaflets dissected from the hearts of eight 5-week-old male Sprague Dawley rats as previously described (22). Sheep and rat valve leaflets were digested in 0.6 mg/ml collagenase Type II (Worthington, New Jersey USA) for 30 min and washed in Hanks' Balanced Salt Solution (HBSS; Life Technologies, Paisley, UK) to remove valve endothelial cells. The leaflets were subsequently digested with 0.6 mg/ml collagenase Type II for a further 1 h to release the VICs. Cells were pelleted at 300 x g for 5 min, before resuspension in growth medium consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Life Technologies) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Life Technologies) and 1% gentamicin (Life Technologies), and cultured at 37˚C in a humidified atmosphere of 95% air/5% CO_2 and grown for four passages.

Immortalised cell lines were established by Capital Biosciences (Gaithersburg, Maryland, USA) from the primary sheep and rat VICs through transduction with recombinant lentivirus encoding Simian virus (SV40) large and small T antigens (sheep), or large T antigen only (rat). The non-clonal cell lines were derived from multiple founder cells. Following continuous culture to 10 passages, transgene expression was confirmed by real-time quantitative PCR (RT-qPCR) for the expression of SV40 large T antigen (Capital Biosciences). The resulting cell lines were designated SAVIC (sheep; SVIC-SVTta) and RAVIC (rat; RVIC-SV40T).

SAVIC and RAVIC cell culture. SAVIC and RAVIC cells were seeded in growth media in multi-well plates at a density of 1.11×10^4 cells/cm^2. Calcification was induced as described previously (22,25). Cells were grown to 80% confluence (Day 0), before treating with control (1.05 mM Ca/0.95 mM Pi) or test media: 1.5 to 3.6 mM calcium (Ca) and/or 1.5 to 2.5 mM phosphate (Pi). $CaCl_2$ and Na_2HPO_4/NaH_2PO_4 (Sigma-Aldrich, Dorset, UK) were used to supplement ionic calcium and phosphate in the media. To study the effect of calcification inhibitors and bisphosphonates on VIC calcification, SAVICs were exposed to inorganic pyrophosphate (PPi) and etidronate (both 0.1 mM; Sigma-Aldrich). Cells were incubated for up to 7 days in a humidified atmosphere of 95% air/5% CO_2, and the medium was changed every second/third day.

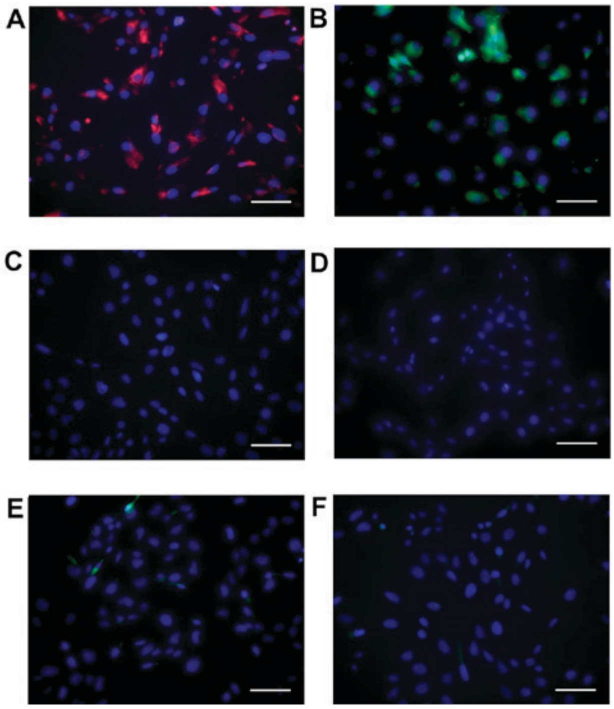

Figure 1. Expression of valve interstitial cell markers in SAVICs. Positive fluorescent staining for (A) vimentin and (B) alpha-smooth muscle actin (α-SMA) in the sheep aortic valve interstitial cell line (SAVIC). (C) No expression of valve endothelial cell (VEC) marker, CD31, was observed. Representative images of (D) mouse IgG and (E and F) rabbit IgG negative controls. DAPI was used as a counterstain. Scale bar=100 μm.

Detection of calcification. Calcium deposition was quantified based on a previously described method (26,27). Cells were washed twice with phosphate buffered saline (PBS) and decalcified with 0.6 M HCl at room temperature for 2 h. Free calcium was determined colorimetrically by a stable interaction with phenolsulphonethalein, using a commercially available kit (Randox Laboratories Ltd., County Antrim, UK), and corrected for total protein concentration (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) following solubilisation with 0.1 M NaOH/0.1% SDS. Absorbances were measured using a Synergy HT microplate reader (BioTek, Swindon UK) at 570 nm (calcium) and at 690 nm (protein).

Fluorescent immunocytochemical staining. To confirm retention of mesenchymal phenotype, cell monolayers cultured on glass coverslips were fixed with 4% paraformaldehyde (PFA) and washed with phosphate-buffered saline (PBS). Fixed cells were permeabilised with 0.3% Triton X-100 (Sigma-Aldrich) and incubated with rabbit polyclonal anti-α-smooth muscle actin (α-SMA; catalogue #ab5694; 1:100; Abcam, Cambridge, UK), mouse monoclonal anti-vimentin (catalogue #V6384;

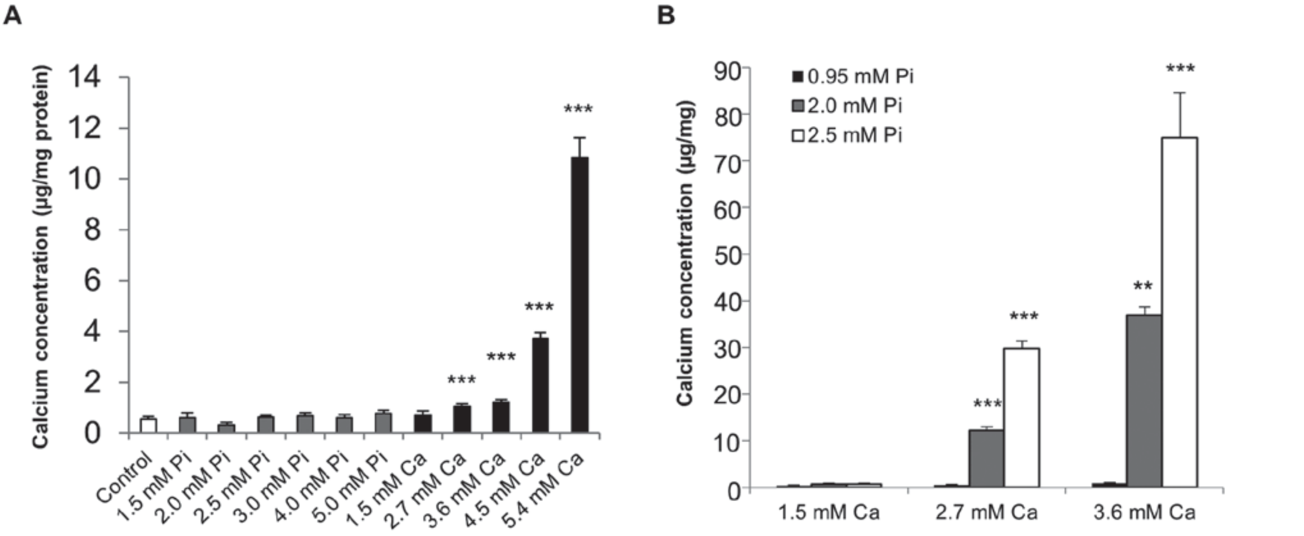

Figure 2. SAVICs undergo calcification *in vitro*. Calcium deposition determined by HCl leaching (µg/mg protein) in VICs treated with (A) calcium (Ca) alone (1.5-5.4 mM) and phosphate (Pi) alone (1.5-5.0 mM), and (B) calcium and phosphate in combination (1.5 to 3.6 mM Ca/0.95 to 2.5 mM Pi). Results are presented as mean ± SEM. **P<0.01; ***P<0.001 compared with control; n=6.

1:900; Sigma-Aldrich) or rabbit polyclonal anti-cluster of differentiation 31 (CD31; 1:900; Abcam, Cambridge, UK) overnight at 4˚C. After washing, cells were incubated with Alexa Fluor® 488 donkey-anti-rabbit antibody (catalogue #A-21,206; 1:250; ThermoFisher Scientific) or Alexa Fluor® 594 goat-anti-mouse antibody (catalogue #A-110,055; 1:250; ThermoFisher Scientific) for 1 h in the dark. Glass coverslips were mounted onto slides with Prolong Gold Anti-Fade Reagent containing DAPI (Life Technologies). Slides were examined using a Leica DMLB fluorescence microscope (Leica Geosystems, Milton Keynes, UK). In place of the primary antibody, control cells were incubated with non-immune mouse or rabbit IgG (2 µg IgG/ml, Sigma-Aldrich).

Real-time quantitative PCR. RNA extraction was performed using the RNeasy Mini kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions. RNA abundance was quantified, RNA was reverse transcribed, and the expression of selected genes were quantified via RT-qPCR employing the SYBR green detection method (PrecisionPLUS mastermix, Primerdesign Ltd, Southampton, UK), measured on a Stratagene Mx3000P (Agilent Technologies, Stockport, UK), as previously reported (28,29). Sheep primers for Runt-related transcription factor 2 (*RUNX2*; Forward 5'-CTCCTCCATCCA TCCACTCC-3'; Reverse 5'-CAGAGGCAGAAGTCAGAG GT-3') and Matrix Gla protein (*MGP*; Forward 5'-ACAACA GAGATGGAGAGCGA-3'; Reverse 5'-CGGAAATAACGG TCGTAGGC-3') were designed via Primer3 (http://primer3.ut.ee/) to span exon-exon junctions, and obtained from Invitrogen (Paisley, UK). Primers for sheep glyceraldehyde 3-phosphate dehydrogenase (*GAPDH;* sequences not disclosed), tyrosine 3-monooxygenase (*YWHAZ;* sequences not disclosed), and sodium-dependent phosphate transporter 1 (*PiT1*; also known as *SLC20A1*; Forward 5'-ACATCTTGA ACGCCGCTA-3'; Reverse 5'-AGTAGCAGCAATAGCAGT GGTA-3') were obtained from Primerdesign Ltd. Sheep expression data were normalised against the geometric mean of *GAPDH* and *YWHAZ*. Rat primers for *Gapdh*, *Mgp*, and *Runx2* were obtained from Qiagen (sequences not disclosed; QuantiTect primers, Qiagen). Rat *Pit1* primers were acquired from Primerdesign Ltd. (sequences not disclosed). Rat expression data were normalised against *Gapdh*. The ΔΔCq method was used to analyse relative gene expression (30).

Statistical analysis. All statistical analyses were performed using Minitab 17 (Minitab Inc., Coventry, UK). General Linear Model (GLM) analysis incorporating pairwise comparisons and the Student's t-test were used to assess the data. Data are presented as mean ± standard error of the mean (SEM). P<0.05 was considered to be statistically significant, and P-values are represented as: *P<0.05; **P<0.01; ***P<0.001.

Results

SAVICs express VIC markers. Cells showed positive immunohistochemical staining for vimentin (Fig. 1A) and α-SMA (Fig. 1B), in agreement with previous reports of primary VIC cultures (31,32). Cells were negative for the endothelial marker CD31 (Fig. 1C).

Calcification of SAVICs. Initial studies were undertaken to determine whether the calcification of SAVICs could be induced when cultured in the presence of calcifying medium containing Ca and/or Pi (Fig. 2A). Ca potently induced the calcification of SAVICs from 2.7 mM (1.9 fold; P<0.001; n=6; Fig. 2A) whereas Pi treatment alone had no effect (Fig. 2A). The treatment of VICs with Ca and Pi together had a synergistic effect on VIC calcification from 2.7 mM Ca/2.0 mM Pi (22.2 fold; P<0.001; n=6; Fig. 2B).

Gene expression in early calcification in vitro. Next, gene expression studies were undertaken in calcifying SAVICs to investigate the expression profile of key genes associated with vascular calcification. Expression of the gene encoding the

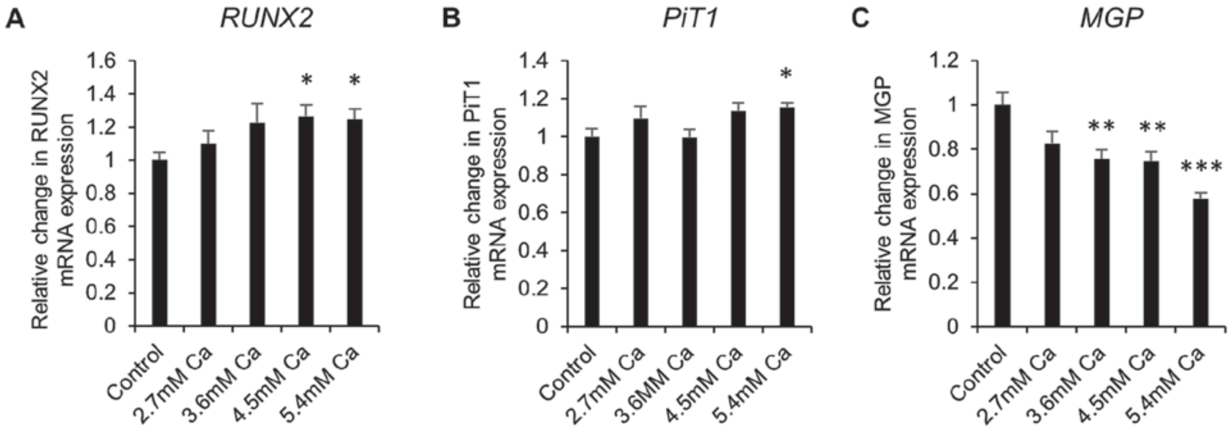

Figure 3. Upregulation of osteogenic gene marker expression in calcifying SAVICs. Fold change in the mRNA expression of (A) *RUNX2*, (B) *PIT1*, and (C) *MGP* in sheep aortic valve interstitial cell line (SAVIC) treated with Ca (2.7-5.4 mM) for 48 h. Results are presented as mean ± SEM. *P<0.05, **P<0.01; ***P<0.001 compared to control; n=6.

master osteoblastic transcription factor, *RUNX2*, was significantly increased from 4.5 mM Ca (1.3 fold; P<0.05; n=6; Fig. 3A) compared to control culture conditions. Expression of sodium-dependent phosphate transporter 1 gene (*PiT1*) was significantly increased at 5.4 mM Ca (1.2 fold; P<0.05; n=6; Fig. 3B). In contrast, a decrease in the expression of the calcification inhibitor Matrix Gla Protein gene (*MGP*) was noted from 3.6 mM Ca (1.3 fold; P<0.01; n=6; Fig. 3C).

Inhibition of SAVICs calcification by pyrophosphate and etidronate. Further experiments investigated whether the calcification of SAVICs could be reduced using recognised inhibitors of calcification.

As CAVD is a consequence of hydroxyapatite formation and deposition in the aortic valve, we examined the effects of PPi (0.01-1 mM; Fig. 4A) and the bisphosphonate etidronate (0.01-1 mM; Fig. 4B), both established inhibitors of hydroxyapatite formation (33-35) on SAVIC calcification. A significant decrease in calcium deposition was observed at 1 mM PPi (1.8 fold; P<0.01; n=6; Fig. 4A). Additionally, following exposure to 1 mM etidronate, a significant reduction in calcium deposition (3.2 fold; n=6; P<0.01) was observed, confirming the inhibitory effect of this bisphosphonate on valve calcification *in vitro* (Fig. 4B).

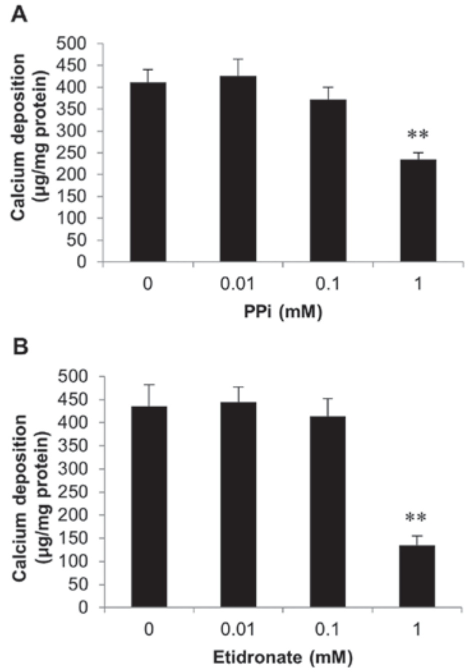

Figure 4. Modulation of SAVIC calcification with established calcification inhibitors. Effect of (A) pyrophosphate (PPi) and (B) etidronate exposure on SAVIC calcification *in vitro*, following culture for 7 days under calcifying conditions (2.7 mM Ca/2.0 mM Pi). Results are presented as mean ± SEM; **P<0.01; n=6.

Additional RAVIC studies. We further corroborated the application of immortalised VICs as an *in vitro* model of aortic valve calcification through the generation of a rat immortalised VIC cell line, RAVIC. A presented in Fig. 5, RAVICs also showed positive immunohistochemical staining for vimentin (Fig. 5A) and α-SMA (Fig. 5B), and were negative for the endothelial marker CD31 (data not shown).

Analogous to our findings with the SAVICs, Ca markedly induced the calcification of RAVICs from 2.7 mM (4.6 fold; P<0.01; n=6; Fig. 5E) and Pi treatment alone had no effect (Fig. 5E). Furthermore, the treatment of RAVICs with Ca and Pi together had a synergistic effect on cell calcification (2.7 mM Ca/2.0 mM Pi; 82.2 fold; P<0.001; n=6; Fig. 5F). 5.4 mM Ca treatment induced a significant increase in the mRNA expression of *Runx2* and *Pit1* (1.5 fold; P<0.01, and 4.7 fold; P<0.001, respectively; Fig. 5G and H), with a concomitant reduction in *Mgp* expression (3.2 fold; P<0.001; Fig. 5I).

Discussion

The calcification of primary porcine (15-17), human (14,18,19), rat (20-22) and bovine (23,24) VICs in 10% serum, inducing an activated phenotype, is frequently employed to produce *in vitro* models of aortic valve calcification. Compared to cell lines, primary cultures exhibit slow growth and cannot be used beyond a limited number of passages due to senescence and phenotypic changes that occur during culture. Furthermore, the use of primary cells requires animal sacrifice

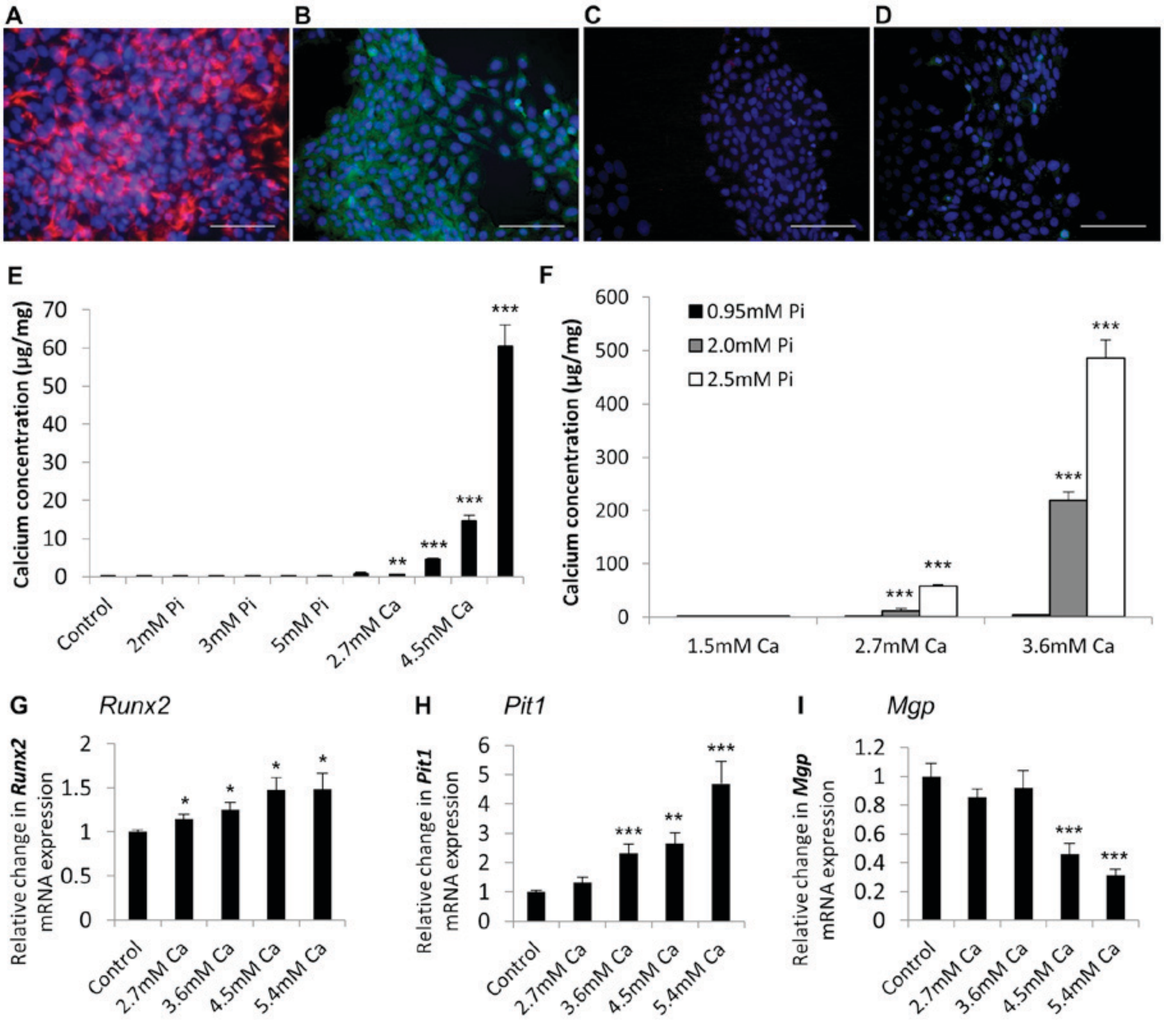

Figure 5. Characterisation of RAVICs confirms the use of immortalised VICs as an *in vitro* CAVD model. Positive staining for (A) vimentin and (B) α-SMA in the rat aortic valve interstitial cell line (RAVIC). Representative images of (C) mouse and (D) rabbit IgG negative control. DAPI was used as a counterstain. Scale bar=100 μm. Calcium deposition determined by HCl leaching (μg/mg protein) in VICs treated with (E) calcium (Ca) alone (1.5-5.4 mM) and phosphate (Pi) alone (1.5-5.0 mM) and (F) calcium and phosphate in combination (1.5 to 3.6 mM Ca/0.95 to 2.5 mM Pi). Fold change in the mRNA expression of (G) *Runx2* (H) *PiT1* and (I) *Mgp* in VICs treated with Ca (2.7-5.4 mM) for 48 h. Results are presented as mean ± SEM. *P<0.05, **P<0.01; ***P<0.001 compared to control; n=6.

and is labour intensive, costly, and time consuming (36). A number of studies have employed the mouse vascular smooth muscle cell line, MOVAS-1, to investigate arterial calcification *in vitro* (28,37,38). As yet, however, no published studies have employed immortalized VIC cell lines to investigate aortic valve calcification. In the present study, we have characterized the *in vitro* calcification potential of immortalised sheep (SAVIC) and rat (RAVIC) VIC cell lines.

Our data have established that calcification of SAVICs and RAVICs can be induced in the presence of calcifying medium containing high concentrations of calcium and phosphate. This was demonstrated through standard assays of *in vitro* vascular calcification (28,39,40), verifying these cell lines as a feasible and relevant *in vitro* model of aortic valve calcification.

Calcified SAVICs and RAVICs showed increased gene expression of *RUNX2* and *PiT1*, recognized markers of vascular calcification, with a concomitant reduction in the expression of *MGP*, an established calcification inhibitor. RUNX2 is an early marker of vascular calcification, initiating osteoblastic differentiation via the upregulation of mineralization proteins, including osteopontin and osteocalcin (10,41). Increased *PiT1* expression leads to elevated intracellular phosphate and induces the osteogenic conversion of vascular smooth muscle cells (VSMCs) (42). Conversely, down-regulation of *PiT1* gene expression by siRNA silencing has been shown to reduce phosphate uptake and inhibit phosphate-induced VSMC phenotypic transition and calcification (42). MGP is a γ-carboxyglutamic acid-rich and vitamin K-dependent protein, and is proposed to block calcification by antagonising bone morphogenetic protein signaling (10,43). Mouse models that lack MGP develop arterial calcification that results in blood vessel rupture, as well as ectopic cartilage calcification (44). Additionally, circulating MGP levels have been shown to be reduced in aortic valve disease patients (45). Taken together, our data suggests that the culture of SAVICs and RAVICs in calcifying medium is an appropriate *in vitro* model with which to study the processes leading to aortic valve calcification.

Using known molecular inhibitors, we have also shown that functional studies can be performed in SAVICs. Pyrophosphate is a potent inhibitor of the calcification of primary VSMCs (46) and VICs (23). The bisphosphonate etidronate, an inhibitor of hydroxyapatite formation and a non-hydrolysable analogue of PPi, has also been reported to inhibit the calcification of MOVAS-1 cells and NIH3T3 cells (28,47). The reduction in calcification observed in SAVICs following treatment with PPi and etidronate therefore establishes this cell line as highly appropriate for modeling aortic valve calcification.

The severe clinical implications of CAVD are widely recognized. Nonetheless, the underlying mechanisms have not been fully determined and effective therapeutic strategies that may prevent or possibly reverse aortic valve calcification have yet to be developed. The SAVIC and RAVIC lines are convenient and inexpensive models in which to investigate aortic valve calcification *in vitro*, and will make a valuable contribution to expanding our knowledge of this pathological process.

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References

1. Freeman RV and Otto CM: Spectrum of calcific aortic valve disease: Pathogenesis, disease progression and treatment strategies. Circulation 111: 3316-3326, 2005.
2. Stewart BF, Siscovick D, Lind BK, Gardin JM, Gottdiener JS, Smith VE, Kitzman DW and Otto CM: Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. J Am Coll Cardiol 29: 630-634, 1997.
3. Coffey S, Cox B and Williams MJ: The prevalence, incidence, progression and risks of aortic valve sclerosis: A systematic review and meta-analysis. J Am Coll Cardiol 63: 2852-2861, 2014.
4. Lindman BR, Clavel MA, Mathieu P, Gardin JM, Gottdiener JS, Smith VE, Kitzman DW and Otto CM: Calcific aortic stenosis. Nat Rev Dis Primers 2: 16006, 2016.
5. Balaoing LR, Post AD, Liu H, Minn KT and Grande-Allen KJ: Age-related changes in aortic valve hemostatic protein regulation. Arterioscler Thromb Vasc Biol 34: 72-80, 2014.
6. Yutzey KE, Demer LL, Body SC, Huggins GS, Towler DA, Giachelli CM, Hofmann-Bowman MA, Mortlock DP, Rogers MB, Sadeghi MM and Aikawa E: Calcific aortic valve disease: A consensus summary from the alliance of investigators on calcific aortic valve disease. Arterioscler Thromb Vasc Biol 34: 2387-2393, 2014.
7. Pawade TA, Newby DE and Dweck MR: Calcification in aortic stenosis: The skeleton key. J Am Coll Cardiol 66: 561-577, 2015.
8. New SE and Aikawa E: Molecular imaging insights into early inflammatory stages of arterial and aortic valve calcification. Circ Res 108: 1381-1391, 2011.
9. Dweck MR, Pawade TA and Newby DE: Aortic stenosis begets aortic stenosis: Between a rock and a hard place? Heart 101: 919-920, 2015.
10. Leopold JA: Cellular mechanisms of aortic valve calcification. Circ Cardiovasc Interv 5: 605-614, 2012.
11. Mohler ER III, Gannon F, Reynolds C, Zimmerman R, Keane MG and Kaplan FS: Bone formation and inflammation in cardiac valves. Circulation 103: 1522-1528, 2001.
12. Poggio P, Sainger R, Branchetti E, Grau JB, Lai EK, Gorman RC, Sacks MS, Parolari A, Bavaria JE and Ferrari G: Noggin attenuates the osteogenic activation of human valve interstitial cells in aortic valve sclerosis. Cardiovasc Res 98: 402-410, 2013.
13. Monzack EL and Masters KS: Can valvular interstitial cells become true osteoblasts? A side-by-side comparison. J Heart Valve Dis 20: 449-463, 2011.
14. Osman L, Yacoub MH, Latif N, Amrani M and Chester AH: Role of human valve interstitial cells in valve calcification and their response to atorvastatin. Circulation 114: I547-I552, 2006.
15. Yip CY, Chen JH, Zhao R and Simmons CA: Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix. Arterioscler Thromb Vasc Biol 29: 936-942, 2009.
16. Cloyd KL, El-Hamamsy I, Boonrungsiman S, Grau JB, Lai EK, Gorman RC, Sacks MS, Parolari A, Bavaria JE and Ferrari G: Characterization of porcine aortic valvular interstitial cell 'calcified' nodules. PLoS One 7: e48154, 2012.
17. Gomez-Stallons MV, Wirrig-Schwendeman EE, Hassel KR, Conway SJ and Yutzey KE: Bone morphogenetic protein signaling is required for aortic valve calcification. Arterioscler Thromb Vasc Biol 36: 1398-1405, 2016.
18. Côté N, El Husseini D, Pépin A, Guauque-Olarte S, Ducharme V, Bouchard-Cannon P, Audet A, Fournier D, Gaudreault N, Derbali H, *et al*: ATP acts as a survival signal and prevents the mineralization of aortic valve. J Mol Cell Cardiol 52: 1191-1202, 2012.
19. El Husseini D, Boulanger MC, Fournier D, Mahmut A, Bossé Y, Pibarot P and Mathieu P: High expression of the Pi-transporter SLC20A1/Pit1 in calcific aortic valve disease promotes mineralization through regulation of Akt-1. PLoS One 8: e53393, 2013.
20. Seya K, Yu Z, Kanemaru K, Daitoku K, Akemoto Y, Shibuya H, Fukuda I, Okumura K, Motomura S and Furukawa K: Contribution of bone morphogenetic protein-2 to aortic valve calcification in aged rat. J Pharmacol Sci 115: 8-14, 2011.
21. Acharya A, Hans CP, Koenig SN, Nichols HA, Galindo CL, Garner HR, Merrill WH, Hinton RB and Garg V: Inhibitory role of Notch1 in calcific aortic valve disease. PLoS One 6: e27743, 2011.
22. Cui L, Rashdan NA, Zhu D, Milne EM, Ajuh P, Milne G, Helfrich MH, Lim K, Prasad S, Lerman DA, *et al*: End stage renal disease-induced hypercalcemia may promotes aortic valve calcification via Annexin VI enrichment of valve interstitial cell-derived matrix vesicles. J Cell Physiol 232: 2985-2995, 2017.
23. Rattazzi M, Bertacco E, Iop L, D'Andrea S, Puato M, Buso G, Causin V, Gerosa G, Faggin E and Pauletto P: Extracellular pyrophosphate is reduced in aortic interstitial valve cells acquiring a calcifying profile: Implications for aortic valve calcification. Atherosclerosis 237: 568-576, 2014.
24. Rattazzi M, Iop L, Faggin E, Bertacco E, Zoppellaro G, Baesso I, Puato M, Torregrossa G, Fadini GP, Agostini C, *et al*: Clones of interstitial cells from bovine aortic valve exhibit different calcifying potential when exposed to endotoxin and phosphate. Arterioscler Thromb Vasc Biol 28: 2165-2172, 2008.
25. Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, Jahnen-Dechent W, Weissberg PL and Shanahan CM: Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: A potential mechanism for accelerated vascular calcification in ESRD. J Am Soc Nephrol 15: 2857-2867, 2004.
26. Zhu D, Mackenzie NC, Millan JL, Farquharson C and Macrae VE: Upregulation of IGF2 expression during vascular calcification. J Mol Endocrinol 52: 77-85, 2014.
27. Zhu D, Mackenzie NC, Shanahan CM, Shroff RC, Farquharson C and MacRae VE: BMP-9 regulates the osteoblastic differentiation and calcification of vascular smooth muscle cells through an ALK1 mediated pathway. J Cell Mol Med 19: 165-174, 2015.
28. Mackenzie NC, Zhu D, Longley L, Patterson CS, Kommareddy S and MacRae VE: MOVAS-1 cell line: A new in vitro model of vascular calcification. Int J Mol Med 27: 663-668, 2011.
29. Staines KA, Zhu D, Farquharson C and MacRae VE: Identification of novel regulators of osteoblast matrix mineralization by time series transcriptional profiling. J Bone Miner Metab 32: 240-251, 2014.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
31. Liu AC, Joag VR and Gotlieb AI: The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology. Am J Pathol 171: 1407-1418, 2007.
32. Latif N, Quillon A, Sarathchandra P, McCormack A, Lozanoski A, Yacoub MH and Chester AH: Modulation of human valve interstitial cell phenotype and function using a fibroblast growth factor 2 formulation. PLoS One 10: e0127844, 2015.
33. Register TC and Wuthier RE: Effect of pyrophosphate and two diphosphonates on 45Ca and 32Pi uptake and mineralization by matrix vesicle-enriched fractions and by hydroxyapatite. Bone 6: 307-312, 1985.

34. Lomashvili KA, Garg P, Narisawa S, Millan JL and O'Neill WC: Upregulation of alkaline phosphatase and pyrophosphate hydrolysis: Potential mechanism for uremic vascular calcification. Kidney Int 73: 1024-1030, 2008.
35. Lomashvili KA, Monier-Faugere MC, Wang X, Malluche HH and O'Neill WC: Effect of bisphosphonates on vascular calcification and bone metabolism in experimental renal failure. Kidney Int 75: 617-625, 2009.
36. Afroze T, Yang LL, Wang C, Gros R, Kalair W, Hoque AN, Mungrue IN, Zhu Z and Husain M: Calcineurin-independent regulation of plasma membrane Ca^{2+} ATPase-4 in the vascular smooth muscle cell cycle. Am J Physiol Cell Physiol 285: C88-C95, 2003.
37. Idelevich A, Rais Y and Monsonego-Ornan E: Bone Gla protein increases HIF-1alpha-dependent glucose metabolism and induces cartilage and vascular calcification. Arterioscler Thromb Vasc Biol 31: e55-e71, 2011.
38. Kelynack KJ and Holt SG: An in vitro murine model of vascular smooth muscle cell mineralization. Methods Mol Biol 1397: 209-220, 2016.
39. Zhu D, Rashdan NA, Chapman KE, Hadoke PW and MacRae VE: A novel role for the mineralocorticoid receptor in glucocorticoid driven vascular calcification. Vascul Pharmacol 86: 87-93, 2016.
40. Zhu D, Hadoke PW, Wu J, Vesey AT, Lerman DA, Dweck MR, Newby DE, Smith LB and MacRae VE: Ablation of the androgen receptor from vascular smooth muscle cells demonstrates a role for testosterone in vascular calcification. Sci Rep 6: 24807, 2016.
41. Johnson RC, Leopold JA and Loscalzo J: Vascular calcification: Pathobiological mechanisms and clinical implications. Circ Res 99: 1044-1059, 2006.
42. Li X, Yang HY and Giachelli CM: Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. Circ Res 98: 905-912, 2006.
43. Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ and Boström KI: Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. Circ Res 107: 485-494, 2010.
44. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR and Karsenty G: Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature 386: 78-81, 1997.
45. Koos R, Krueger T, Westenfeld R, Kühl HP, Brandenburg V, Mahnken AH, Stanzel S, Vermeer C, Cranenburg EC, Floege J, *et al*: Relation of circulating Matrix Gla-Protein and anticoagulation status in patients with aortic valve calcification. Thromb Haemost 101: 706-713, 2009.
46. Villa-Bellosta R, Wang X, Millan JL, Dubyak GR and O'Neill WC: Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle. Am J Physiol Heart Circ Physiol 301: H61-H68, 2011.
47. Li Q, Kingman J, Sundberg JP, Levine MA and Uitto J: Dual effects of bisphosphonates on ectopic skin and vascular soft tissue mineralization versus bone microarchitecture in a mouse model of generalized arterial calcification of infancy. J Invest Dermatol 136: 275-283, 2016.