Investigating novel regulators and inhibitors of aortic valve calcification

Lerman D 1,2, Mackenzie NCW1, Zhu D1,2, Prasad S3, Walker W3, Dweck M2, Newby D2, MacRae VE1
1 The Roslin Institute and Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Easter Bush, Roslin, Midlothian, EH25 9RG, Scotland, UK
2 Centre for Cardiovascular Science, University of Edinburgh, 47 Little France Crescent, Edinburgh, Midlothian, EH16 4TJ, Scotland, UK
3 Royal infirmary of Edinburgh (NHS Lothian), 51 Little France Crescent, Edinburgh, Midlothian EH16 4SA, Scotland, UK

Introduction
Calcific aortic stenosis (CAS) is the third greatest risk factor for cardiovascular disease (CVD) and the most common cause of valve replacement surgery in Europe and North America (Akat et al., 2009) affecting 25% of individuals over age 65 years and > 50% of those over age 85 years (Stewart et al., 1997). Recent evidence suggests that CAS is due to an active inflammatory process affecting the valve and leading to osteoblastic transformation in valve interstitial cells (VICs) by activation of the receptor activator of nuclear factor kappa-B (RANK). Denosumab is a human monoclonal antibody with high affinity to RANKL (Receptor activator of NF-kB Ligand), which blocks its interaction with RANK and inhibits differentiation and activation of osteoclast cells. As these processes are poorly understood, the characterisation of gene expression in pig VICs during in vitro calcification and the effect of Denosumab as a potential inhibitor of VICs activation were analysed.

Aims
• Characterisation of vascular and bone markers in porcine VICs during spontaneous calcification.
• To determine the effects of Denosumab in porcine VICs during the calcification process.

Methodology
Molecular studies in porcine valve interstitial cells (VICs)
mRNA from porcine aortic valves was extracted, and reverse transcribed to cDNA. qPCR analysis assessed baseline expression levels of a number of markers of vascular calcification. Initial studies involved the establishment and validation of cell isolation, culture and calcification procedures. In vitro calcification was determined using standard staining and enzyme activity assay. Induced calcification in pig VICs was carried out with sodium phosphate and the effect of Denosumab was assessed in vitro. Six well plates were seeded at a density of 1.5 x 10^5 cells per well and incubated 3-4 days until confluent. Cells were then treated with 3mM NaPi +/- Denosumab (0.5ug/ml, 5ug/ml and 50ug/ml) and samples taken at day 0, 7 and 14 for molecular studies. All linear variables were compared using the two group t-test. A p value of <0.05 was considered statistically significant.

Results
Porcine VICs calcify spontaneously. Calcium and Collagen levels detected by Alizarin red and Sirius red staining respectively continued to rise up to day 14th (P <0.001) Fig.2). ALP activity showed a significant growth between day 0 and 14th (P <0.05). No alterations in cell viability were observed (Fig 1).

mRNA levels were analysed by qPCR during spontaneous calcification (Fig. 2). This showed significant changes in markers of VIC cell trans-differentiation through myofibroblasts to osteoblasts. α-Actin, a marker of myofibroblast phenotype, showed a 1.7 fold increase by day 14 (P <0.05). RhoA, a regulator of node formation in myofibroblasts, was increased 4.6 fold by day 14 (P <0.001). Similarly, there was a 1.3 fold increase in Runx2, a major regulator of osteoblast differentiation, by day 14 (P <0.05) and TGFβ 1 a promoter of osteogenesis, showed a 1.5 fold increase by day 7 (P <0.01) and a 3.2 fold increase by day 14 (P <0.001). There were no significant changes in levels of RANKL, a key regulator of bone and the target molecule for Denosumab. Calponin an inhibitor of osteoblast decreased 0.7 fold by day 14P <0.05).

Fig.2. qPCR analysis of porcine VICs during spontaneous calcification. Showing relative changes in the expression of the following genes: α-Actin (A), Calponin (B), Runx2 (C), TGFβ (E) and RhoA (F), RANKL(E) remained stable.

Induced calcification with Sodium Phosphate in porcine VICs showed a significant increase in calcification against the control after 2 weeks time (P <0.05). Experiments on pig (VICs) indicated that denosumab inhibits calcification of cells induced with NaPi at a concentration of 50 microgram/ml (P <0.05) (Fig 3).

Fig 3. The effects of Denosumab on calcification of VICs. Showing levels of calcium build up determined by (A) Alizarin red staining of VICs treated with alpha MEM based calcifying medium with 5ug/ml or 50ug/ml of Denosumab and (B) quantification of Alizarin binding from treated cells.

Conclusions
1) Porcine VICs will spontaneously calcify in vitro, providing a useful model for studying the calcification of valve cells. During this process a change in gene expression profile indicates a change in cellular phenotype.
2) Denosumab inhibits calcification of VICs.
3) A fuller understanding of the actions of Denosumab may identify a novel therapeutic strategy for clinical intervention against calcific aortic valve stenosis.

Future work
1) To determine changes in gene expression in VICs treated with NaPi.
2) Deeper analysis of the effect of Denosumab on the calcification of VICs.
3) Derive a cell culture model from human aortic valves leaflets.

References

Acknowledgements