

1 **Gene expression in the cardiovascular system of the domestic sheep (*Ovis aries*); a new tool**  
2 **to advance our understanding of cardiovascular disease.**

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20 seq, network analysis, vascular calcification

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23

24 **Abstract**

25

26 Large animal models are of increasing importance in cardiovascular disease research as they  
27 demonstrate more similar cardiovascular features (in terms of anatomy, physiology and size) to  
28 humans than do rodent species. The maintenance of a healthy cardiovascular system requires  
29 expression of genes that contribute to essential biological activities and repression of those that are  
30 associated with functions likely to be detrimental to cardiovascular homeostasis. In this study we have  
31 used the transcriptome of the sheep, which has been utilised extensively to model human physiology  
32 and disease, to explore genes implicated in the process of vascular calcification. Vascular calcification  
33 is a major disruption to cardiovascular homeostasis where tissues of the cardiovascular system  
34 undergo ectopic calcification and consequent dysfunction. We investigate the gene expression  
35 profiles of genes involved in vascular calcification in a wide array of cardiovascular tissues and across  
36 multiple developmental stages, using RT-qPCR. The majority of transcriptomic studies on the  
37 mammalian cardiovascular system to date have focused on regional expression of specific genes.  
38 Here we also use RNA sequencing results from the sheep heart and cardiac valves to further explore  
39 the transcriptome of the cardiovascular system in this large animal. Our results demonstrate that there  
40 is a balance between genes that promote and those that suppress mineralisation during development  
41 and across cardiovascular tissues. We show extensive expression of genes encoding proteins  
42 involved in formation and maintenance of the extracellular matrix in cardiovascular tissues, and high  
43 expression of haematopoietic genes in the cardiac valves. Our analysis will support future research  
44 into the functions of implicated genes in the development of vascular calcification, and increase the  
45 utility of the sheep as a large animal model for understanding cardiovascular disease. This study  
46 provides a foundation to explore the transcriptome of the developing cardiovascular system and is a  
47 valuable resource for the fields of mammalian genomics and cardiovascular research.

48

49

## 50 Introduction

51

52 The cardiovascular system plays a crucial role not only in the distribution of nutrients to the various  
53 cells, tissues and organs within the mammalian body, but also in the removal of waste products.  
54 Extensive regulatory mechanisms are required to support this functional system, with perturbations  
55 likely to lead to abnormalities, and thus give rise to cardiovascular-related diseases. Cardiovascular  
56 disease (CVD) is a major cause of morbidity and mortality worldwide, with an estimated 17.3 to 17.5  
57 million deaths per year (Townsend et al. 2016; WHO, 2017). The major cardiovascular-related causes  
58 of premature death include coronary heart disease (CHD) and stroke (Townsend et al. 2016; WHO,  
59 2017). In addition, cardiac valvulopathies are becoming increasingly prevalent in the ageing  
60 population (Nkomo et al. 2006). A recent United Kingdom study of nearly 80,000 adult patients  
61 referred for echocardiography found that 50% had some degree of cardiac valve dysfunction  
62 (Marciniak et al. 2017). In contrast, only 12% of the same patient group had left ventricular systolic  
63 dysfunction. Aortic aneurysm is also an increasing health care burden, particularly in elderly males  
64 (reviewed by Hohneck et al, 2019) who found an incidence of 7% in elderly male patients hospitalised  
65 for cardiopulmonary symptoms). Thus cardiovascular disease is a major socioeconomic burden and  
66 understanding of the cardiovascular system is important in developing treatments for the range of  
67 conditions.

68

69 In recent decades, both non-invasive and invasive CVD therapies have advanced considerably. This  
70 advancement has been underpinned by basic research, with animal models of CVD being of key  
71 importance. Of growing value is the use of large animal models of CVD research (reviewed in Tsang  
72 et al. 2016). Sheep and pigs, for example, are more similar in terms of their cardiovascular features  
73 (in terms of anatomy, genetics, physiology and size) to humans than are rodent species. Evidence  
74 suggests there are significant phenotypic differences between mouse and human stem cells (Ginis et  
75 al. 2004; Gabdoulline et al. 2015) and large animals might therefore provide greater similarity at the  
76 cellular and molecular level. Early developmental stages can be studied in detail in large animal  
77 models, which is limited in scope in both human and mouse (Emmert et al. 2013). Finer resolution of  
78 regions of the cardiovascular system is also possible with the increased size of the heart and vessels  
79 of the large animal models. Sufficient RNA for transcriptomic studies can be obtained from a single  
80 animal, so that inter-animal variability can be assessed. The major benefit of large animals in CVD  
81 clinical research remains however their application in the development of interventional technologies  
82 and implantable devices (reviewed in Tsang et al. 2016). Characterising the normal transcriptome of  
83 the healthy mammalian cardiovascular system will allow better understanding of the cellular changes  
84 induced by these treatments.

85

86 The maintenance of a healthy cardiovascular system requires expression of genes that contribute to  
87 essential biological activities and repression of those that are associated with functions likely to be  
88 detrimental to cardiovascular homeostasis. A major pathological process that disrupts cardiovascular  
89 homeostasis is vascular calcification (VC), which is associated with aging, hypertension and

90 atherosclerosis (Abedin, Tintut, & Demer, 2004; Towler, 2008; Zhu, Mackenzie, Farquharson, &  
91 Macrae, 2012, Tsang et al. 2016). VC is a disease of abnormal mineral metabolism, involving the  
92 deposition of calcium phosphate, in the form of hydroxyapatite (HA), in cardiovascular tissues, most  
93 critically in the arteries and cardiac valves, and is a significant, independent risk factor of  
94 cardiovascular mortality (Giachelli, 2004; Li, Yang, & Giachelli, 2006; Zhu et al. 2012). Most  
95 individuals above 60 years of age have gradually enlarging calcium deposits in their major arteries  
96 (Allison, Criqui, & Wright, 2004; Demer & Tintut, 2008). VC is a highly regulated, active process  
97 involving a variety of signalling pathways, with evidence suggesting the involvement of mechanisms  
98 similarly observed in bone formation (Boström et al. 1993; Lanzer et al. 2014). However, the exact  
99 molecular basis underpinning the complex process of VC, particularly the dysregulated expression of  
100 genes involved in cardiovascular function, has yet to be fully defined.

101

102 A number of factors have been implicated in the phenotypic transition of vascular smooth muscle cells  
103 (VSMCs) into osteocytic-, osteoblastic- and chondrocytic-like cells (Yang et al. 2004; Li et al. 2006;  
104 Giachelli, 2009; Zhu et al. 2011; Zhu et al. 2012). These include osteochondrogenic markers,  
105 including *TNAP* (*ALPL* gene), osteopontin (also known as secreted phosphoprotein 1; *SPP1* gene),  
106 and the transcription factor *RUNX2* (Steitz et al. 2001; Rajamannan et al. 2003; Lomashvili et al.  
107 2004; Speer et al. 2009; Yang et al. 2009; Zhu et al. 2012), as well as mineralisation inhibitors,  
108 including *ENPP1*, *MGP*, ecto-5'-nucleotidase *NT5E* (also known as cluster of differentiation 73,  
109 *CD73*), and *FBN1* (Luo et al. 1997; Schurgers et al. 2008; Rutsch et al. 2011; St Hilaire et al. 2011;  
110 Albright et al. 2015). During the calcification process VSMCs enter a synthetic state with abundant  
111 production of extracellular matrix (ECM) proteins (Hruska et al, 2005) followed by matrix vesicle-  
112 mediated calcification (Giachelli, 2009; Leopold, 2015). Indeed recent comparative transcription  
113 profiling has identified over 50 ECM genes identically regulated by calcifying VSMCs and bone-  
114 forming osteoblasts (Alves et al., 2014), with ECM proteins likely acting in concert with each other to  
115 determine the extent of calcification. Nevertheless, although the sequence of events conducting  
116 normal bone mineralisation is better understood, the specific mechanisms by which VC occurs  
117 remains ambiguous, as vascular cells may still retain their overall identity, despite acquiring  
118 osteoblastic properties (Frink, 2002; Zhu et al. 2012; Alves et al. 2014).

119

120 In recent years, the use of high-throughput technologies such as RNA sequencing (RNA-seq), has  
121 been continually expanding the number of gene expression datasets available for specific tissues and  
122 cells. In order to investigate the transcriptional landscape of the mammalian genome, various high-  
123 throughput mammalian gene expression profiling studies have been performed at different levels  
124 including varying cellular, tissue and whole organism levels. Atlases of gene expression have been  
125 generated for the majority of tissues, and a small number of cell types, in ovine (Jiang et al. 2014;  
126 Clark et al. 2017), caprine (Muriuki et al. 2019), bovine (Harhay et al. 2010), porcine (Freeman et al.  
127 2012; Summers et al. 2020), equine (Mansour et al. 2017), murine, and human (Lein et al. 2007;  
128 Siddiqui et al. 2005; Su et al. 2002). Large-scale and collaborative projects have also been formed to  
129 generate big datasets on the mammalian transcriptome, across multiple tissues and cell types and

130 including many individuals. These include the Functional Annotation of Animal Genomes (FAANG)  
131 Consortium (Andersson et al. 2015), Encyclopedia of DNA Elements (ENCODE) Consortium (Encode  
132 Project Consortium et al. 2007), the Functional ANnoTation Of the Mammalian genome (FANTOM5)  
133 consortium (Andersson et al. 2014; Fantom Consortium et al. 2014; Lizio et al. 2015), and the  
134 Genotype-Tissue Expression (GTEx) Consortium (Mele et al. 2015). The majority of transcriptomic  
135 studies on the mammalian cardiovascular system have to date only looked in detail at tissue-specific  
136 expression of single genes, or a subset of genes of interest (Gaborit et al. 2007; Potter, Abbey-Hosch,  
137 & Dickey, 2006), rather than providing wider resolution of the cardiovascular transcriptome across  
138 multiple tissues. Although there are various public resources, the transcriptomic data available for the  
139 mammalian cardiovascular system are generally limited to the “heart” or ventricular tissue, such as in  
140 BioGPS (<http://biogps.org/>) and the Expression Atlas online database (EMBL-EBI;  
141 <https://www.ebi.ac.uk/gxa>). In the human GTEx Project (Mele et al. 2015), for example, two  
142 cardiovascular tissues are included (Heart – Atrial Appendage and Heart – Left Ventricle) from a large  
143 number of individuals (n=372 and n=386 respectively). RNA-seq has been used to greatly enhance  
144 resolution of cardiovascular disease in humans (reviewed in Wirka et al. 2018) and generate baseline  
145 estimates of gene expression in developing cardiovascular tissues (Pervolaraki et al. 2018). However,  
146 comparable resources were not available for large animal models, which could be used to develop  
147 interventions and other treatments for cardiovascular disease.

148

149 This study describes gene expression in the mammalian cardiovascular system, supporting and  
150 extending the high resolution gene expression atlas for sheep (Clark et al. 2017). We use reverse  
151 transcriptase quantitative PCR (RT-qPCR) to measure myocardial and arterial tissue gene expression  
152 during development in the sheep and investigate expression of vascular calcification (VC) inhibitors in  
153 the healthy cardiovascular system. We also present tissue-specific gene expression profiles in the  
154 heart muscle and cardiac valves using RNA-Seq. The study provides a foundation to explore the  
155 transcriptome of the developing cardiovascular system and provides a valuable resource for the fields  
156 of mammalian genomics and cardiovascular research.

157

## 158 **Materials and methods**

159

### 160 **Reverse transcriptase quantitative PCR (RT-qPCR)**

161 RT-qPCR was performed to measure the expression of twenty-five genes involved in cardiovascular  
162 and skeletal muscle function and VC across five developmental stages from Texel x Scottish  
163 Blackface sheep: 100-day gestation (foetal), newborn, 1 week, 8 weeks and 2 years (n=3-5 per  
164 group). Details of the samples included in each of the three sets of analyses (RNA-seq of eight  
165 tissues, developmental stage expression profiles and VC gene expression profiles) are included in  
166 Table 1. Samples were collected within an hour and thirty minutes post euthanasia. 17 different  
167 tissues were collected from adults; the equivalent tissues were collected from fetuses at day 100 of  
168 gestation and young lambs where possible. Detailed dissection of tissues was performed by the same  
169 two researchers, for all sheep, in order to standardise tissue sampling. After dissection, tissues of

170 interest were placed into RNeasy (Thermo Fisher Scientific) and stored according to the  
171 manufacturer's instructions. RNA was extracted from tissues using TRIzol (Thermo Fisher Scientific)  
172 as described in Clark et al. 2017. RNA integrity (RIN<sup>e</sup>) was estimated on an Agilent 2200 TapeStation  
173 System (Agilent Genomics) and only samples with RIN<sup>e</sup> > 7 were included in the analysis. RT-qPCR  
174 reactions were performed using PrecisionPLUS-MX-SY Mastermix (containing SYBR Green;  
175 Primerdesign Ltd) following the manufacturer's protocol. Details of the twenty-five sheep-specific  
176 primers used are listed in Supplementary Table 1. Primers were designed using the current version of  
177 the sheep genome Oar v3.1 ([https://www.ensembl.org/Ovis\\_aries/Info/Annotation](https://www.ensembl.org/Ovis_aries/Info/Annotation)) with Primer3  
178 software (<http://primer3.ut.ee/>) to span exon-exon junctions, and obtained from Invitrogen (Paisley,  
179 UK) and Primerdesign Ltd (Eastleigh, UK). Because of the limitations of the sheep genome sequence  
180 it was not possible to design primers for all genes of interest.

181

## 182 **RNA-seq**

183 The RNA-seq analysis we present in this manuscript is based on a subset of data, from seven  
184 cardiovascular and one skeletal muscle tissue (Table 1), from our high resolution atlas of gene  
185 expression for domestic sheep (Clark et al. 2017). These tissues were collected from three male and  
186 three female adult Texel x Scottish Blackface sheep at 2 years of age (n=6 in total). RNA extraction,  
187 RNA-seq library preparation, sequencing and bioinformatic analysis of the RNA-seq data were  
188 described in Clark et al 2017. After quality control a small number of samples, despite multiple  
189 extraction attempts, were of insufficient quality for RNA-Seq, and as such a small proportion of the  
190 tissues have less than six biological replicates (Table 1).

191

192 RNA-seq libraries were generated by Edinburgh Genomics (Edinburgh, UK). All libraries were 125bp  
193 paired end stranded, sequenced at a depth of >25 million reads per sample, and prepared using the  
194 Illumina TruSeq mRNA library preparation protocol (Illumina; Part: 15031047 Revision E). The only  
195 exceptions were the left ventricle samples that were prepared using the Illumina TruSeq total RNA  
196 library preparation protocol (Illumina; Part: 15031048, Revision E) and sequenced at a depth of >100  
197 million reads per sample. Supplementary Table 2 includes the details of the cardiovascular tissues  
198 included in the sheep atlas dataset and analysed further here.

199

200 The raw RNA-seq data are deposited in the European Nucleotide Archive (ENA) under study  
201 accession number PRJEB19199 (<http://www.ebi.ac.uk/ena/data/view/PRJEB19199>). For each tissue  
202 a set of expression estimates (averaged across biological replicates from n=6 adult sheep), as  
203 transcripts per million (TPM), were obtained using the transcript quantification tool Kallisto v0.43.0  
204 (Bray et al. 2016), as described in Clark et al. 2017. It was necessary to normalise these estimates  
205 according to the methods described in (Bush et al. 2017) to account for the two different library types  
206 (ribo-depleted total RNA (left ventricle) and poly-A selected mRNA (all other tissues)). The gene  
207 expression estimates for the sheep gene expression atlas dataset are publicly available on BioGPS  
208 ([http://biogps.org/dataset/BDS\\_00015/sheep-atlas/](http://biogps.org/dataset/BDS_00015/sheep-atlas/)), and we have included the gene expression  
209 estimates for the subset of tissues re-analysed here as Supplemental Dataset 1.

210

## 211 **Visualisation and analysis of the gene expression estimates**

212 Expression estimates from Kallisto for each gene were analysed using the network visualisation tool,  
213 Graphia Professional (Kajeka Ltd, Edinburgh, UK; <https://kajeka.com/graphia/>) (Livigni et al. 2018).  
214 Briefly, similarities between individual gene expression profiles, averaged across N=6 biological  
215 replicates (3 male and 3 female adult sheep) where possible for each tissue, were determined by the  
216 calculation of a Pearson correlation matrix for both sample-to-sample and gene-to-gene comparisons.  
217 During this process the dataset was filtered to remove relationships where the Pearson correlation  
218 coefficient (which is the statistical measure of the strength of a linear relationship between paired  
219 data) was below a threshold of  $r \geq 0.91$  and  $r \geq 0.99$ , respectively. The Markov clustering algorithm  
220 (MCL) was applied at the default inflation value (to determine cluster granularity) of 2.2 (van Dongen  
221 & Abreu-Goodger, 2012) to identify groups of transcripts with closely related expression patterns.  
222 Clusters were numbered in order of decreasing cluster size. The online Database for Annotation,  
223 Visualization and Integrated Discovery (DAVID) Functional Annotation tool (<https://david.ncifcrf.gov/>)  
224 was used for Gene Ontology (GO) analysis.

225

## 226 **Statistical analyses**

227 Statistical analyses were performed using Minitab 17 (Coventry, UK). The Kolmogorov-Smirnov  
228 normality test was performed to check whether experimental data were normally distributed. In this  
229 study, one-way analysis of variance (ANOVA) using a general linear model incorporating Fisher's  
230 least significant difference (LSD) method was used for pairwise comparisons. Gene expression data  
231 in this study are expressed as mean  $\pm$  standard deviation (SD), and p-value  $<0.05$  was considered  
232 significant. Dotplots were made in R v3.2.2 (<https://www.r-project.org/>), using the R package 'ggplot2'  
233 with error bars showing mean  $\pm$  SD. Individual data points are also included in the dotplots.

234

## 235 **Results**

236

237 To explore the gene expression differences in the whole cardiovascular system, we used RT-qPCR to  
238 analyse selected genes involved in extracellular matrix (ECM) composition and in maintenance of  
239 calcium homeostasis, in a range of samples from pre- and post-natal developmental stages of the  
240 sheep. Genes which are the focus of studies in our group because they are associated with  
241 cardiovascular pathology and/or ectopic calcification were examined. The genes and the diseases  
242 associated with them are listed in Supplementary Table 3. A summary of the results for the  
243 developmental stages is presented in Table 2 and the full results with significance levels can be seen  
244 in Supplementary Figures 1-6. Results for different tissues in the adult sheep are summarised in  
245 Figure 1 and full results with significance levels can be found in Supplementary Figures 7-9.

246

## 247 **Myocardial tissue gene expression during development**

248 In the left ventricle free wall, the ECM protein-encoding genes showed significant decreases in  
249 relative expression during development from 100 days gestation to 2 years of age, although the timing

250 varied, with *BGN* and *TIMP1* declining before birth while *COL1A1*, *MMP2* and *FBN2* decreased after  
251 birth. *FBN1* dropped rapidly before birth but then increased at 8 weeks (Table 2; Supplementary  
252 Figure 1). The expression of *SPP1* (also known as *OPN*), which promotes calcification, expression  
253 decreased overall with age while *RUNX2*, which is also involved in mineralisation increased during  
254 development. The mineralisation inhibitor *ANKH* showed increases in relative mRNA expression,  
255 while *TNFRSF11B* (also known as *OPG* and thought to restrict calcification) showed a significant  
256 decrease in its expression levels after birth. Overall, the expression levels of *RUNX2* and  
257 *TNFRSF11B* were low compared to the other tested genes and *MMP2*, *BGN* and *MGP* were the  
258 highest compared to the other genes in the left ventricle (Table 2; Supplementary Figure 1).

259

260 In contrast, in the interventricular septum, the ECM genes *COL1A1*, *BGN* and *MMP2* were largely  
261 unchanged during development while *FBN1* and *FBN2* exhibited decreases in mRNA expression as  
262 development progressed (Table 2; Supplementary Figure 2). In general, *SPP1* expression decreased  
263 with age in the interventricular septum although higher expression was observed in the 1 week old  
264 lambs compared to newborn lambs ( $p < 0.05$ ). As in the left ventricle, the expression of *ANKH* showed  
265 an increasing trend with age, but with a significant increase between the foetal and newborn lamb  
266 samples ( $p < 0.05$ ). Overall, the expression of *MGP* (mineralisation inhibitor) did not change, although  
267 the 1 week old lambs showed statistically significant higher expression compared to the foetal lambs  
268 ( $p < 0.05$ ). Genes that were most highly expressed in the interventricular septum were *BGN*, *MGP* and  
269 *FBN1*, whereas *TNFRSF11B* showed the lowest levels of expression within this tissue (Table 2).

270

#### 271 **Arterial tissue expression during development**

272 It was not possible to obtain samples from the foetal animals for the arteries, but we examined gene  
273 expression changes from newborn to adult. In the pulmonary artery, *FBN2* expression decreased and  
274 *TIMP1* expression increased between birth and 2 years of age (Table 2; Supplementary Figure 3).  
275 Expression of both *RUNX2* and *ENPP1* (likely to have opposing effects on mineralisation) was  
276 significantly higher in the 2-year old sheep ( $p < 0.01$ ; Table 2; Supplementary Figure 3). *SPP1* was  
277 significantly lower in the 2-year old sheep compared to both newborn and 8-week old lambs ( $p < 0.01$ ).  
278 In the pulmonary artery, *MGP* was the most highly expressed of the genes tested, followed by *BGN*,  
279 *MMP2*, *ENPP1* and *COL1A1*, with *RUNX2* as the gene with the lowest expression levels (Table 2).

280

281 In the aortic root, all the significant changes involved a decrease from young lambs to 2-year-old  
282 adults. Both the ECM protein genes (*COL1A1*, *BGN*, *MMP2*, *FBN1* and *FBN2*) and the genes with  
283 opposing effects on mineralisation (*ENPP1* and *SPP1*) showed decreases in their expression (Table  
284 2), and were significantly lower at 2 years of age compared to (Supplementary Figure 4). The ion  
285 channel gene *KCNK3* also showed significantly lower expression in the 2-year old sheep compared to  
286 newborn and 1 week old lambs ( $p < 0.05$ ). Overall, in the aortic root, *MGP* followed by *BGN*, *MMP2*  
287 and *FBN1* showed the highest levels of expression, whereas the lowest levels of expression were  
288 observed for *RUNX2* and *SPP1* and *ANKH* in some adult samples (Table 2).

289



290 In the aortic arch many of the selected genes were not significantly changed through development  
291 (Table 2; Supplementary Figure 5) *FBN2* expression decreased in 2-year old sheep compared to  
292 newborn and 1 week old lambs ( $p<0.01$ ). *SPP1* expression was also significantly lower in adult sheep  
293 compared to newborn and 1 week old lambs ( $p<0.05$ ). In contrast the mineralisation inhibitor genes  
294 *TNFRSF11B* and *MGP* increased with age as did *RUNX2*. Within the aortic arch, the highest levels of  
295 expression were seen in *MGP*, *BGN* and *ENPP1*, and the lowest in *RUNX2* and *TNFRSF11B*  
296 (newborn lambs) and *FBN2* and *SPP1* (2-year old adults) (Table 2).

297

298 In the abdominal aorta, expression of the mRNAs of ECM protein-encoding genes *COL1A1* and *FBN1*  
299 peaked in 8-week old lambs (Supplementary Figure 6) while *FBN2* expression decreased from 8  
300 weeks of age to 2 years of age ( $p<0.01$ ). *TIMP1* expression was found to increase with age. For the  
301 key calcification genes, *SPP1* showed a reduction in its expression from 8-week old lambs to 2-year  
302 old sheep ( $p<0.05$ ), and *RUNX2* was decreased from newborn to 8-week old lambs ( $p<0.05$ ;  
303 Supplementary Figure 6). *ANKH* and *TNFRSF11B* expression was significantly increased in the 8-  
304 week old and 2-year old sheep compared to newborn lambs ( $p<0.05$ ). In the abdominal aorta, the  
305 highest levels of expression were observed for *MMP2*, *MGP*, *BGN* and *FBN1*, and the lowest overall  
306 for *RUNX2* (Table 2).

307

### 308 **Vascular calcification (VC) inhibitors expressed in the healthy adult cardiovascular system**

309 Using RT-qPCR the gene profiles of various key calcification inhibitors were investigated in different  
310 cardiovascular regions, in the six adult sheep. Figure 1 summarises where these genes were highly  
311 expressed in the cardiovascular tissues. Overall, the key VC genes tended to be more highly  
312 expressed in the cardiac valves than the other tissues; expression in the myocardium was lowest  
313 (Figure 1).

314

315 Of the genes examined, *FBN1* and *TNFRSF11B* showed the greatest variation across the  
316 cardiovascular system. *FBN1* was most highly expressed in the valves, which had significantly higher  
317 expression than the myocardium and vena cava tissues ( $p<0.01$ ; Figure 3). Overall, *FBN1* expression  
318 was approximately 4-fold lower in the aortic samples than the cardiac valves ( $p<0.05$ ). There was no  
319 significant difference between the aortic arch compared to the left AV valve, the abdominal aorta  
320 compared to the left AV (mitral), right AV (tricuspid) and pulmonary valves, and the pulmonary artery  
321 compared to the left AV valve (Figure 2). Expression of *FBN1* in the arteries was in general higher (4  
322 fold) than in the myocardium and cranial vena cava ( $p<0.05$ ). *TNFRSF11B* expression was highest in  
323 the arteries and cardiac valves compared to the myocardium and cranial vena cava (Figure 2). The  
324 levels of *TNFRSF11B* expression in the aortic samples, pulmonary artery, the aortic valve and left AV  
325 valve were significantly higher compared to the myocardium and cranial vena cava ( $p<0.05$ ; Figure 2).  
326 The expression of *TNFRSF11B* was generally very low in the myocardium (approximately 1000-fold  
327 lower than in the arteries) (Figure 2).

328

329 Of the other VC genes investigated, *MGP* showed the highest levels of expression compared to the  
330 other tested genes with expression being similar in all tissues, although the aortic valve showed  
331 higher expression than the aortic arch, the cranial vena cava and the myocardial tissues ( $p < 0.05$ ;  
332 Supplementary Figure 7). The expression of *ANKH* was similar in all tested tissues, but the pulmonary  
333 artery showed significantly higher expression levels than the right atrium and left auricle ( $p < 0.05$ ;  
334 Supplementary Figure 7). *NT5E* expression was found to be higher in the cardiac valves and the  
335 pulmonary artery compared to the other tissues included in this study ( $p < 0.05$ ; Supplementary Figure  
336 8). Some arterial tissues exhibited higher expression than the myocardial samples, including the  
337 aortic root, aortic arch and abdominal aorta compared to the right atrium, left ventricle and  
338 interventricular septum ( $p < 0.05$ ; Supplementary Figure 8). The expression of *ENPP1* was significantly  
339 higher in the cardiac valves and aortic arch compared to the myocardium and vena cava ( $p < 0.05$ ;  
340 Supplementary Figure 9). The remaining aortic samples showed intermediate expression between the  
341 valves and myocardium (Supplementary Figure 9). Similarly the expression of *SPP1* was found to be  
342 higher in the cardiac valves compared to the myocardium and the aortic root ( $p < 0.05$ ; Supplementary  
343 Figure 9). Other than in the cardiac valves, *SPP1* expression was generally very low in the tested  
344 cardiovascular tissues, reaching levels similar to that of the bone marker *RUNX2* (Supplementary  
345 Figure 9). The expression of *RUNX2* was low in all tested samples. However, the aortic valve showed  
346 higher *RUNX2* expression compared to the myocardium, the cranial vena cava and aortic root  
347 ( $p < 0.05$ ; Supplementary Figure 8).

348

#### 349 **Gene expression profiles reflect anatomical structure**

350 In addition to analysis of selected ECM and VC genes across a wide range of tissues and  
351 developmental stages, we took advantage of the RNA-seq data from the sheep atlas project (Clark et  
352 al. 2017) to explore more broadly the transcriptome of the heart and cardiac valves. The 3D network  
353 visualisation for sample-to-sample analysis is similar to a principal components analysis and grouped  
354 the tissue samples (averaged across biological replicates from up to 6 adult sheep; Supplemental  
355 Dataset 1) together based on highly similar expression profiles. The resultant graph contained all 8  
356 nodes (tissue samples) that were connected by 10 edges (connections between nodes at a  
357 correlation coefficient of  $\geq 0.91$ ; Figure 3A). Two distinct elements were identified: a group containing  
358 the five myocardium/skeletal muscle samples and a cardiac valve group (Figure 3A). The network  
359 indicated that there were close similarities in the overall expression profiles of genes in skeletal  
360 muscle and heart muscle, which were distinct from the cardiac valve tissues. Similar grouping of  
361 skeletal muscle and heart muscle tissues was previously observed by (Lukk et al. 2010).

362

#### 363 **Tissue-specific expression clusters in the cardiovascular system**

364 Network-based gene-to-gene analysis of Supplemental Dataset 1 grouped genes according to their  
365 expression pattern across the muscle and valve samples, producing a gene co-expression network  
366 (GCN) (Gaiteri et al. 2014). A high correlation coefficient of  $r \geq 0.99$  was necessary to discriminate  
367 expression patterns due to the similarity of expression in the relatively small set of samples being  
368 analysed. The resultant graph (Figure 3B) included 11,341 nodes (genes) with 938,652 edges

369 (correlations at  $r \geq 0.99$  between them). MCL (inflation = 2.2) clustering of the graph resulted in 555  
370 clusters containing >3 genes. Details of the genes included in the 40 largest clusters are included in  
371 Supplemental Dataset 2. For most of the clusters, expression was highest in the three valve samples.  
372 Some of these (where the difference in average expression was less than 4-fold) contained  
373 housekeeping genes and were considered ubiquitous, while others (where the difference was 10-fold  
374 or more) were considered to be valve specific. Several clusters contained genes that were high in  
375 skeletal muscle only and some were high in the myocardium samples only. There was no cluster of  
376 genes that were high in the ventricles alone, or in a single myocardium sample, but a small number of  
377 clusters contained genes that were high in the auricles. Interestingly there were some clusters of  
378 genes with higher expression in the skeletal muscle and heart valves than the myocardium or with  
379 higher expression in the skeletal muscle and left ventricle. There were also some differences between  
380 the different valves. Expression profiles of a subset of ECM, VC and heart function genes are shown  
381 in Supplementary Figure 10..

382  
383 Cluster 1 contained 3543 genes, with 529 of those unannotated (Figure 4A). Genes in this cluster  
384 showed approximately a 3-fold greater expression in the cardiac valves than in the other tissues. A  
385 wide variety of genes was included in this cluster. The cluster contained genes enriched for GO terms  
386 associated with cell structure, such as *COL1A1* (encoding collagen type I alpha 1) and *COL3A1*  
387 (collagen type III alpha 1), *MMP2*, -9, -19, -20 and -28 (matrix metalloproteinases), *FBN2* (fibrillin-2)  
388 and *TIMP1* (tissue inhibitor of matrix metalloproteinases 1) (Table 3). There were also multiple genes  
389 expressed specifically by macrophages in sheep (Clark et al. 2017) and other species (Fantom  
390 Consortium et al. 2014; Summers et al. 2020), including *CSF1R*, *AIF1* and *SPI1*, *CSF2RA/B* and a  
391 number of interleukin and interferon responsive genes. In addition, members of the smoothed  
392 signalling pathway (*SMO*, *GLI1-3*) involved in cilium formation and function, genes associated with  
393 RNA transcription and processing (for example POLR genes) and some genes associated with cell  
394 proliferation (for example centromere protein genes) were in this cluster. Cluster 1 also contained  
395 genes associated with TGF beta signalling, including *TGFB3*, *TGFB1*, *TGFBR2* and *TGFBR3*.

396  
397 Cluster 3 (Figure 4B) contained 192 genes with highest expression in the cardiac valves, particularly  
398 the aortic valve, at approximately 4.5 to 18-fold higher than the myocardial and skeletal muscle  
399 tissues. This cluster was enriched for GO terms associated with extracellular matrix organisation,  
400 skeletal system development, osteoblast differentiation and cartilage morphogenesis. Genes in this  
401 cluster included those encoding bone morphogenetic protein 4 (*BMP4*), collagen type I alpha 2  
402 (*COL1A2*) and bone gamma-carboxyglutamate protein (*BGLAP*, also known as osteocalcin) (Table  
403 3). Other valve specific genes in this cluster included *MYH10* (myosin heavy chain 10), Potassium  
404 channel gene *KCNU1* and *BGN* (ECM protein biglycan) which was 15- to 20-fold higher in the valves.  
405 Genes encoding various transcription factors were also contained within this cluster, including FOS  
406 like 2, AP-1 transcription factor subunit (*FOSL2*), Twist basic helix-loop-helix transcription factor 2  
407 (*TWIST2*), Snail family transcriptional repressor 1 and 2 (*SNAI1 and -2*) and Sry homeobox 8 (*SOX8*).  
408 In cluster 3, 42/192 genes were unannotated and not included in the input into DAVID.

409

410 Like cluster 1, the 27 genes in cluster 22 (Figure 4C) also showed relatively ubiquitous expression,  
411 with smaller differential between the cardiac valves and the myocardium and skeletal muscle than  
412 those in cluster 1 (approximately 2 to 3-fold difference) (Figure 4C). Genes in cluster 22 included  
413 *ENPP1* (ectonucleotide pyrophosphate/phosphodiesterase 1), *ADAMTS6* (ADAM metalloproteinase  
414 with thrombospondin type 1 motif 6) and *SMAD2* (SMAD family member 2). Some of the genes in this  
415 cluster are annotated as immune-related, including C-C motif chemokine receptor 6 (*CCR6*),  
416 interleukin6 signal transducer (*IL6ST*) and nuclear factor, interleukin 3 (*NFIL3*) (Table 3) but these  
417 genes are less specific to hematopoietic cells than those in Cluster 1  
418 (<https://www.bioGPS.org/sheepatlas>; Clark et al. 2017).

419

420 The 25 genes in cluster 24 (Figure 4D) were also more highly expressed in the cardiac valves, at up  
421 to 16-fold higher than the myocardium, and approximately 4 to 5-fold higher than the bicep  
422 (representing skeletal muscle). Genes encoding proteins involved in transcriptional regulation were  
423 included in this cluster, including mesenchyme homeobox 1 (*MEOX1*), the GATA-regulated gene  
424 repressor transcriptional repressor GATA Binding 1 (*TRPS1*) and recombination signal binding protein  
425 for immunoglobulin kappa J region (*RBPJ*) (Table 3). ECM protein encoding genes were also  
426 included, such as *FBN1* (fibrillin-1), *FMOD* (fibromodulin), and *COL6A1* (collagen type VI alpha 1) as  
427 well as the macrophage specific gene NLR family pyrin domain containing 3 (*NLRP3*) (Table 3).

428

429 The 20 genes in cluster 36 (Figure 4E) showed high expression in the auricles, at up to 2,000-fold  
430 higher than the other tissues. Genes included in this cluster were involved in muscle contraction  
431 through potassium ion transport ion transport (Table 3) e.g. potassium voltage-gated channel  
432 subfamily Q member 3 (*KCNQ3*), subfamily J member 3 (*KCNJ3*) and subfamily K member 3  
433 (*KCNK3*), as well as peptidylglycine alpha-amidating monooxygenase (*PAM*) and myosin light chains  
434 4 and 7 (*MYL4 and -7*) (Table 3). Other genes to note include natriuretic peptide A (*NPPA*) and  
435 Dickkopf WNT signalling pathway inhibitor 3 (*DKK3*) (Table 3). Examination of the wider sheep gene  
436 expression atlas (<https://www.biogps.org/sheepatlas>) showed that many of the genes in this cluster  
437 were also highly expressed in brain regions, consistent with the function of many as ion transport  
438 channels.

439

440 A number of clusters contained genes that were high in both bicep (representing skeletal muscle) and  
441 the heart regions and 2- to 3-fold lower in the valves. As might be expected, these clusters were  
442 enriched for genes involved in mitochondrial function, reflecting the energy requirements of muscle  
443 tissue. In contrast, Cluster 4 (164 nodes) expression was high only in bicep and contained genes  
444 specific to skeletal muscle such as a range of troponin and myosin genes, the ryanodine receptor 1  
445 gene (*RYR1*) and sodium, potassium and calcium ion channels genes. Other bicep-specific clusters  
446 included Clusters 23 (25 genes) and 28 (24 genes). These three clusters were comprised of genes  
447 encoding proteins for zinc-dependent proteases e.g. *ADAMTS20* (ADAM metalloproteinase with  
448 thrombospondin type 1 motif 20) and genes involved in actin binding and motor activity e.g. *MYH15*

449 (myosin heavy chain 15). A small proportion of clusters exhibited expression patterns that were  
450 specific to skeletal muscle (bicep) and left ventricle. The largest of these clusters was cluster 13 (48  
451 genes), which included genes encoding proteins involved in hydrolysis of extracellular nucleotides  
452 e.g. ectonucleotide pyrophosphatase/phosphodiesterase 3 (*ENPP3*) and transcription factors e.g.  
453 caudal type homeobox 1 (*CDX1*), solute carriers e.g. (*SLC16A4* and *SLC26A3*). Several smaller  
454 clusters, 66 (15 genes), 83 (13 genes) and 90 (13 genes), exhibited left ventricle specific expression  
455 profiles and were comprised of genes with a similar function to those within cluster 13.

456

457 In summary, the cardiac valves showed more consistent expression of ECM genes, while both valves  
458 and the muscle samples had detectable levels of many transcripts associated with resident  
459 macrophage populations. The transcriptome of cardiac muscle was similar to skeletal muscle in this  
460 analysis, although some tissue specific gene expression could be seen. For example, the potassium  
461 channel genes *KCNJ3*, *KCNK3* and *KCNQ3*, the myosin light chain genes *MYL4* and *MYL7* and a  
462 natriuretic peptide gene, *NPPA* were auricle-specific (Cluster 36).

463

#### 464 **Functional annotation of unannotated genes**

465 Each cluster included a number of genes, which had no informative gene name. While many of these  
466 genes had low expression, some were highly expressed. For example, *ENSOARG00000020353* had  
467 a maximum of nearly 6,000 TPM in aortic valve. It is described as a novel gene with a 24 amino acid  
468 match to parathymosin (*PTMS*) in the bovine. According to the 'guilt by association' principle (Oliver et  
469 al. 2000) since this gene was found within a cluster of genes with high expression in the cardiac valve  
470 samples it may well have a similar function to other annotated genes that are co-expressed in Cluster  
471 1. Other examples include *ENSOARG00000005484* in Cluster 36, which was expressed at around 18  
472 TPM in left and right auricle. This gene has some homology to *FAM155A* and *FAM155B* (*Tmem28* in  
473 mouse), probably a transmembrane calcium ion transporter (UniProtKB B1AL88 and O75949  
474 respectively). Further exploration of the 923 ENSOARG genes that were included in the cluster  
475 analysis should allow attribution of putative functions based on their presence in a cluster of genes of  
476 known function.

477

#### 478 **Discussion**

479

480 The maintenance of a healthy cardiovascular system requires expression of genes that contribute to  
481 essential biological activities and repression of those that are associated with functions likely to be  
482 detrimental to cardiovascular homeostasis. As cardiovascular disease (CVD) is of major clinical  
483 importance, understanding the roles of genes in co-expression networks and their associated  
484 molecular pathways will be useful in understanding their dysregulation in pathological events. Detailed  
485 analysis of gene expression of tissues and cell types in the cardiovascular system provides a powerful  
486 resource for investigation of healthy cardiovascular system function (reviewed in Wirke et al. 2018).  
487 However, analysis of the cardiovascular system in humans is often jeopardised due to tissues being  
488 only available post-mortem, where frequently the health status of the individual is not known and the

489 quality of the RNA may be poor (Ferreira et al. 2017). A large animal model, where tissues can be  
490 collected quickly post mortem from healthy animals, offers the opportunity to perform a detailed  
491 characterisation of the mammalian cardiovascular transcriptome. The recently published sheep gene  
492 expression atlas (<https://www.bioGPS.org/sheepatlas>; Clark et al. 2017) allowed us to examine  
493 individual components of the cardiovascular system in the sheep, which are similar to humans in their  
494 physiology and genetics (reviewed in Hamernik 2019). Additional tissues and developmental stages,  
495 from the same animals, but not initially presented as part of the sheep transcriptional atlas, were also  
496 examined in this study, using RT-qPCR. The insights from this novel analysis of the sheep  
497 cardiovascular system will be valuable in understanding the mechanisms behind many  
498 cardiovascular-related diseases and will help to facilitate the development of clinical and therapeutic  
499 approaches for the prevention and treatment of cardiovascular diseases.

500

501 Our results demonstrate that there is extensive expression of genes encoding proteins involved in  
502 formation and maintenance of the extracellular matrix (ECM) in cardiovascular tissues. The ECM is an  
503 important provider of structural and biomechanical support, and helps to regulate molecular  
504 interactions between growth factors and cell surface receptors (Davis & Summers, 2012; Kim,  
505 Turnbull, & Guimond, 2011). The cardiac valves showed higher expression of a range of ECM genes  
506 relative to cardiac muscle, both by RNA-seq and by RT-qPCR. This is consistent with continuous  
507 remodelling of the ECM in cardiac valves due to the normal functional stresses (mechanical and  
508 blood-flow induced shear) that the valve is subjected to. During development, most ECM genes  
509 decreased in expression, particularly in the left ventricle, intraventricular septum and aortic root. This  
510 probably reflects the completion of organ development, after which the requirement for expression  
511 would depend on the turnover of the proteins to maintain ECM homeostasis. However, with ageing,  
512 expression and *de novo* synthesis may not be sufficient to balance turnover of the proteins, leading to  
513 a loss of structural support in the ECM over time. Different ECM genes were activated at different  
514 times during development. For example, two members of the fibrillin family, key components of the  
515 ECM (Davis and Summers 2012; Ramirez & Pereira, 1999; Sakai, Keene, & Engvall, 1986) were  
516 expressed in the cardiovascular system. The gene encoding FBN2, traditionally regarded as a fetal  
517 protein which is involved at the beginning of elastogenesis and early morphogenesis (Zhang et al.  
518 1995), appeared to be activated earlier in cardiovascular development than the gene for FBN1, which  
519 has been attributed functions late in morphogenesis and organogenesis (Zhang et al. 1995). Both  
520 genes exhibited highest expression in the cardiac valves and decreased in expression during  
521 cardiovascular development. Expression of *FBN1* in the aorta supports the role of FBN1 in  
522 maintaining the structural integrity of this major artery. In Marfan Syndrome, the dysfunction of FBN1  
523 leads to aortic aneurysms and elastic fibre calcification (Pereira et al. 1999; Bunton et al. 2001). Other  
524 ECM genes that decreased with age included *COL1A1* and *BGN*. The ECM proteases known as  
525 matrix metalloproteinases (MMPs) and their tissue inhibitors, TIMPs, are important modulators of  
526 matrix protein turnover (Elmore, Keister, Franklin, Youkey, & Carey, 1998; Hughes & Jacobs, 2017). It  
527 is thought that alterations of the balance between MMPs and TIMPs are critical in the formation of  
528 aortic aneurysms and age-associated physiological changes in the cardiovascular system (Meschiari,

529 Ero, Pan, Finkel, & Lindsey, 2017; Rabkin, 2014). In this study, the level of *MMP2* expression was  
530 amongst the highest of the genes examined in the abdominal aorta, and *TIMP1* expression was found  
531 to increase with age in this tissue. The increase in *TIMP1* expression may help prevent the  
532 development of abdominal aortic aneurysms in a healthy animal by inhibiting degradation of ECM  
533 structural proteins. MMPs and TIMPs may also be crucial in myocardial function, where increases in  
534 their levels have been found to correlate with age in human and mouse (Meschiari et al. 2017).  
535 Furthermore, these ECM regulators have also been reported to be important in the remodelling  
536 process in the left ventricle after experimentally induced myocardial infarction in mice, where the local  
537 endogenous control of MMPs by TIMP1 was suggested to be important for the ECM structure, as well  
538 as myocardial function and myocyte growth (Creemers et al. 2003). Additional studies on the  
539 expression of other MMPs and TIMPs may be useful to determine their involvement in the  
540 development of CVD.

541

542 We detected transcripts associated with macrophages in all samples, notably enriched in the valves.  
543 The homeostatic functions of resident macrophages in arterial and cardiac tissue have been widely  
544 studied (Swirski et al. 2016; Lim et al. 2018). The presence of resident macrophages in human and  
545 mouse valve tissue has also been recognised previously (Sraeyes et al. 2018). In the mouse,  
546 heterogeneous resident valve macrophage populations are established in the postnatal period and  
547 the population is expanded by monocyte recruitment in a model of myxomatous disease (Hulin et al.  
548 2018). Damaged cardiac valves are prone to life-threatening infectious and non-infectious  
549 endocarditis (Yang and Frazee 2018), which is common in elderly humans, and ongoing surveillance  
550 and repair are necessary to prevent pathological outcomes. The sheep is an ideal animal to  
551 investigate the ageing heart further, since the sheep life span is around 10 years  
552 (<http://www.sheep101.info>) and elderly animals can be obtained from commercial sources at the end  
553 their productive life, rather than needing to be aged for the investigation.

554

555 A striking finding of this analysis was the expression of genes associated with bone formation and VC  
556 in the cardiovascular system of healthy sheep throughout development. These included both genes  
557 encoding proteins that promote bone formation and calcification (such as *SPP1*, *SPARC*, *BMP4* and  
558 *BGLAP*) and those which suppress mineralisation (such as *ENPP1*, *ANKH*, *FBN1*, *MGP*,  
559 *TNFRSF11B* and *NT5E*). VC can develop in various tissues, although many reports include the aorta  
560 and the aortic valve as sites of VC (L. L. Demer & Tintut, 2009; New & Aikawa, 2011). The expression  
561 of genes associated with suppression of bone formation would likely be advantageous in preventing  
562 VC, but the predisposing factors and pathways that infer the susceptibility of specific tissues to  
563 calcification are still unknown. Moreover, differences in the mechanisms behind intimal, median and  
564 valvular calcification may exist (Côté et al. 2012; Patel et al. 2017; Qian et al. 2017). Expression of  
565 *ENPP1* decreased throughout development. *ENPP1* has a role in regulating extracellular nucleotide  
566 levels and potentially a dual role in VC (Côté et al. 2012). *ENPP1* may contribute to normal  
567 cardiovascular function through the regulation of extracellular ATP concentrations and the generation  
568 of the calcification inhibitor PPI (Côté et al. 2012; Nam et al. 2011). Deficiency of *ENPP1* leads to

569 generalised arterial calcification (Mackenzie et al. 2012). *ANKH* mRNA was also increased. *ANKH*  
570 transports cytoplasmic PPI out of the cell (Harmey et al. 2004). *ANKH* may provide a protective effect  
571 against the development of VC, since patients with VC have been found to have decreased *ANKH*  
572 expression (Zhao et al. 2012). MGP is also a calcification inhibitor, possibly via its ability to block BMP  
573 signalling (Yao et al. 2010; Zebboudj, Imura, & Bostrom, 2002). Expression of *BMP4* in the cardiac  
574 valves in the sheep gene expression atlas dataset analysed here, supports the importance of  
575 calcification inhibitors like MGP in preventing the development of calcification, especially in tissues  
576 which express genes associated with bone development. The expression of *MGP* was consistently  
577 high in all the different ages and tissues investigated and this factor may play a cardioprotective role  
578 against the development of calcification. *SPP1* encodes secreted phosphoprotein 1, also known as  
579 osteopontin which is associated with bone formation and calcification, and is a constituent of normal  
580 elastic fibres in the aorta and skin (Rutsch et al. 2011). *SPP1* in the valves is likely to be associated  
581 with the resident macrophages, since it was the most highly-expressed transcript in isolated  
582 macrophages in the sheep atlas, at least 100-fold higher than in any tissue other than placenta  
583 (<https://www.bioGPS.org/sheepatlas>; Clark et al. 2017). *SPP1* is similarly macrophage-enriched in  
584 humans (Fantom Consortium et al. 2014) and pig (Summers et al. 2020). Increased *SPP1* mRNA  
585 expression and plasma osteopontin levels have been linked with Cardiac Allograft Vascular Disease  
586 (CAVD) (Rajamannan et al. 2003; Yu et al. 2009), whereas it has been reported to have inhibitory  
587 effects on arterial calcification (Speer et al. 2002; Wada, McKee, Steitz, & Giachelli, 1999). Examples  
588 of its reported roles include bone remodelling, anti-apoptotic signalling and inflammatory regulation  
589 (Denhardt, Noda, O'Regan, Pavlin, & Berman, 2001). *SPP1* can exist in different states  
590 (phosphorylated and glycosylated), and it is thought that these specific forms have distinct functions  
591 (Denhardt et al. 2001). There was a decrease in expression of *SPP1* mRNA with age in the sheep  
592 cardiovascular tissues. Increased expression of *SPP1* has been implicated in VC development, as  
593 well as coronary artery disease and heart failure (Dai et al. 2014; New & Aikawa, 2011; Rosenberg et  
594 al. 2008). Our results suggest that *SPP1* is important in the earlier stages of cardiovascular  
595 development, whereas higher expression in later life may lead to these adverse clinical outcomes.  
596 *ENPP1* was also strongly macrophage-enriched in the wider sheep atlas  
597 (<https://www.bioGPS.org/sheepatlas>; Clark et al. 2017). The expression profiles of *SPP1* and *ENPP1*  
598 were very similar suggesting that they contribute to a balance between promotion and suppression of  
599 calcification in cardiovascular tissues. *MGP* expression was high compared to the other genes in this  
600 study. Although it has been established that *MGP* has a role in the inhibition of VC, its particular role  
601 within the cardiovascular system is still unclear. As with *SPP1*, *MGP* can exist in different states, and  
602 the levels of these different states are thought to affect the CVD risk of an individual (Dalmeijer et al.  
603 2013). Elevated dephosphorylated *MGP* (*dpMGP*) has been found in patients with chronic kidney  
604 disease (CKD), heart failure, CAVD, aortic stenosis and other CVD events (Mayer et al. 2014;  
605 Schurgers, Cranenburg, & Vermeer, 2008; Vassalle & Iervasi, 2014). The locally produced active form  
606 of MGP (phosphorylated and gammacarboxylated) has been implicated to have cardioprotective  
607 effects (El Asmar, Naoum, & Arbid, 2014; Y. P. Liu et al. 2015; Schurgers et al. 2010) such as through  
608 its inhibition of VC, where it has been reported to inhibit BMP signalling (Yao et al. 2010; Zebboudj et



609 al. 2002). In addition, decreased active MGP was found in aortic valvular interstitial cells (VICs)  
610 derived from patients with CAVD (Venardos et al. 2015). More studies into the numerous genes that  
611 have been implicated in VC are required in order to understand their expression patterns within the  
612 cardiovascular system, and to gain additional insights into their physiological functions.

613

614 One outcome of this study is the functional annotation of previously novel genes. At present, there are  
615 many predicted mammalian protein-coding loci and non-protein-coding genes that are yet to have  
616 informative annotation (Oliver 2000; Klomp & Furge, 2012). Protein-coding genes that contribute to  
617 common generic and cell-specific cellular processes or pathways generally form co-expression  
618 clusters, allowing the inference of the function of a gene (of previously unknown function) using the  
619 'guilt-by-association' principle (Oliver 2000; Freeman et al. 2012; Klomp & Furge, 2012). Martherus et  
620 al. 2010, for example, used this method effectively to identify heart enriched mitochondrial genes. In  
621 our study a number of co-expression clusters were found that distinguished the cardiac valves from  
622 heart muscle. The novel (unannotated) genes within the tissue-specific clusters described here  
623 potentially have the same functions as other genes in the cluster, which allows for functional  
624 annotation of these genes. For example, the gene *ENSOARG0000005484* from Cluster 36 encodes  
625 a protein involved in calcium ion transport across membranes, consistent with the other ion channel  
626 genes in this cluster. The high level of expression of some of these novel genes suggests that they  
627 are an important part of the process of development and differentiation in the cardiovascular system.  
628 As such they warrant further investigation using knock out animals or functional validation in relevant  
629 cell lines using CRISPR to examine consequences of their dysfunction (as reviewed in Van Kampen  
630 and Rooij 2019).

631

632 As the RNA-seq analysis we present here was only performed for seven cardiovascular tissues (three  
633 valves and the four chambers of the heart), we were not able to define gene expression clusters  
634 associated with other cardiovascular tissues, such as the veins, arteries and other regions of the  
635 heart. We used RT-qPCR to examine a limited number of genes in the extended cardiovascular  
636 system at several developmental stages. Transcriptomic analysis using RNA-seq of a wider sub-set of  
637 samples, including more tissue types and developmental stages, would identify specific expression  
638 patterns, for example for different parts of the aorta. In addition, we did not cluster the cardiovascular  
639 samples with other tissues (other than a representative of skeletal muscle) from the wider sheep gene  
640 expression atlas dataset (<https://www.bioGPS.org/sheepatlas>; Clark et al. 2017).

641

642 In summary we have used RNA-seq results from the sheep heart and cardiac valves to further  
643 explore the transcriptome of the cardiovascular system in this large animal. These data provide initial  
644 insights into tissue-specific expression of key genes, which will be useful in understanding their  
645 physiological function in a healthy mammal. This study will support future research into the functions  
646 of implicated genes in the development of VC, and increase the utility of the sheep as a model in  
647 cardiovascular research. The analysis of further tissues and developmental stages, such as a wider  
648 range of prenatal ages and elderly animals would provide further insight into the gene expression

649 patterns of key genes implicated in the progression of important cardiovascular functions or disease  
650 with age, and is feasible using the sheep as a model. Here we have built a foundation to explore the  
651 transcriptome of the developing and ageing cardiovascular system and provided a highly useful  
652 comprehensive resource. Recent advances in single cell RNA-seq technology provide a new frontier  
653 to understand cell type specific gene expression and will allow us to further de-convolute expression  
654 patterns in cardiovascular tissues (Chaudhry et al. 2019). Further in-depth studies will be necessary to  
655 understand the gene expression networks and molecular pathways that exist in the different  
656 cardiovascular structures, and how they develop and change as the cardiovascular system matures.

657

#### 658 **Data Availability**

659 The datasets from the sheep gene expression atlas (Clark et al. 2017) supporting the conclusions of  
660 this article are available in the following locations. The raw read data is deposited in the European  
661 Nucleotide Archive (ENA) under study accession number PRJEB19199  
662 (<http://www.ebi.ac.uk/ena/data/view/PRJEB19199>). The expression estimates (averaged across  
663 biological replicates) from Supplemental Dataset 1 can also be viewed and downloaded via BioGPS  
664 ([http://biogps.org/dataset/BDS\\_00015/sheep-atlas/](http://biogps.org/dataset/BDS_00015/sheep-atlas/)). Sample metadata for all the tissue samples  
665 collected has been deposited in the EBI BioSamples database under project identifier GSB-718  
666 (<https://www.ebi.ac.uk/biosamples/groups/SAMEG317052>).

667

#### 668 **Ethics Statement**

669 All animal work was approved by The Roslin Institute's Animal Welfare and Ethical Review Body  
670 (AWERB). Animals were maintained in accordance with UK Home Office guidelines and experiments  
671 were carried out under the authority of UK Home Office Project Licenses under the regulations of the  
672 Animal (Scientific Procedures) Act 1986.

673

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695 portal.

696

697 **Authors Contributions**

698 DAH acquired the funding for the sheep gene expression atlas. ELC coordinated and designed the  
699 sheep gene expression atlas with assistance from DAH and KMS. HGT, ELC, and KMS performed  
700 sample collection from sheep. HGT and ELC performed the RNA extractions. SJB performed all  
701 bioinformatic analyses. HGT and KMS performed the network cluster analysis. HGT performed the  
702 RT-qPCR analysis. Results were interpreted by HGT with VM, BMC and KMS. HGT wrote the  
703 manuscript with GRM, ELC, KMS and VEM. All authors read and approved the final manuscript.

704

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1058 Table 1. Details of the samples, number of biological replicates and developmental stage of all  
1059 samples included in the analyses.

| Tissue                                | No. of replicates | Developmental stage                           |
|---------------------------------------|-------------------|---|
| <b>RNA-seq</b>                        |                   |   |
| Aortic Valve                          | 4                 | Adult (2 years)                               |
| Mitral Valve                          | 4                 | Adult (2 years)                               |
| Tricuspid Valve                       | 4                 | Adult (2 years)                               |
| Left Auricle                          | 4                 | Adult (2 years)                               |
| Right Auricle                         | 5                 | Adult (2 years)                               |
| Left Ventricle                        | 6                 | Adult (2 years)                               |
| Right Ventricle                       | 6                 | Adult (2 years)                               |
| Skeletal Muscle (Bicep)               | 6                 | Adult (2 years)                               |
| <b>RT-qPCR (developmental stages)</b> |                   |   |
| Left Ventricle                        | 3-5               | Fetus d100, newborn, 8 weeks, 2 years         |
| Interventricular septum               | 3-5               | Fetus d100, newborn, 1 week, 8 weeks, 2 years |
| Aortic Root                           | 3-5               | Newborn, 1 week, 8 weeks, 2 years             |
| Aortic Arch                           | 3-5               | Newborn, one week, 2 years                    |
| Abdominal Aorta                       | 3-5               | Newborn, 8 weeks, 2 years                     |
| Pulmonary Artery                      | 3-5               | Newborn, 8 weeks, 2 years                     |
| <b>RT-qPCR (VC genes)</b>             |                   |   |
| Left Auricle                          | 6                 | Adult (2 years)                               |
| Left Atrium                           | 6                 | Adult (2 years)                               |
| Right Auricle                         | 6                 | Adult (2 years)                               |
| Right Atrium                          | 6                 | Adult (2 years)                               |
| Right Ventricle                       | 6                 | Adult (2 years)                               |
| Cranial Vena Cava                     | 6                 | Adult (2 years)                               |
| Aortic Valve                          | 6                 | Adult (2 years)                               |
| Mitral Valve                          | 6                 | Adult (2 years)                               |
| Tricupsid Valve                       | 6                 | Adult (2 years)                               |
| Pulmonary Valve                       | 6                 | Adult (2 years)                               |
| Left Ventricle                        | 6                 | Adult (2 years)                               |
| Interventricular septum               | 6                 | Adult (2 years)                               |
| Aortic Base                           | 6                 | Adult (2 years)                               |
| Aortic Arch                           | 6                 | Adult (2 years)                               |
| Descending Thoracic Aorta             | 6                 | Adult (2 years)                               |
| Abdominal Aorta                       | 6                 | Adult (2 years)                               |
| Pulmonary Artery                      | 6                 | Adult (2 years)                               |

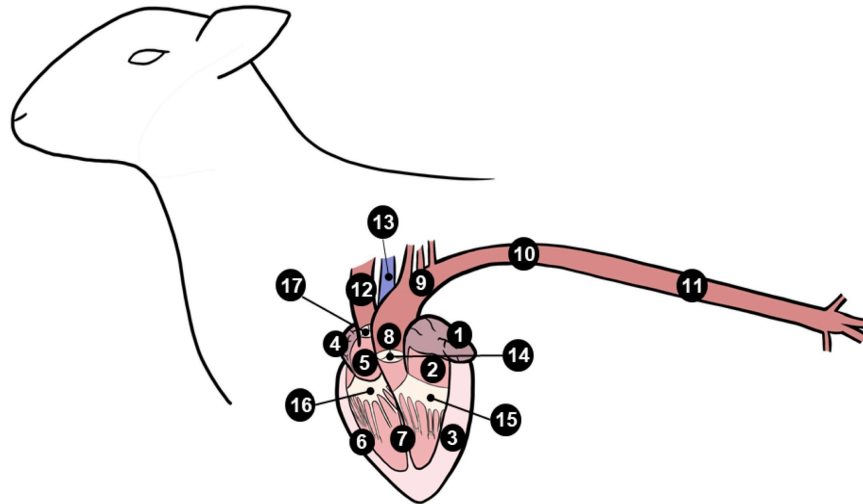
1060 Table 2. Summary of expression profiles of ECM and calcification genes during pre- to post- natal  
 1061 development in the sheep cardiovascular system. Colour key for myocardial tissue only (no foetal  
 1062 samples were available for arterial tissues): Red - Overall changed expression from foetal to adult;  
 1063 Green - Changed expression from pre- to post- natal stages; Blue - Changed expression during post-  
 1064 natal stages.

| Tissue                  | Genes that increased in expression through development | Genes that decreased in expression through development | Gene(s) with highest expression through development | Gene(s) with lowest expression through development |
|-------------------------|--|--|---|--|
| <i>Myocardium</i>       |  |  |   |  |
| Left ventricle          | <i>ANKH</i>  | <i>COL1A1</i>  | <i>MMP2</i>   | <i>TNFRSF11B</i>                                   |
|                         | <i>RUNX2</i>   | <i>FBN2</i>  |   |  |
|                         |  | <i>MMP2</i>  |   |  |
|                         |  | <i>SPP1</i>  |   |  |
|                         |  | <i>FBN1</i>  |   |  |
|                         |  | <i>BGN</i>   |   |  |
|                         |  | <i>TIMP1</i>   |   |  |
|                         |  | <i>TNFRSF11B</i>                                       |   |  |
| Interventricular septum | <i>ANKH</i>  | <i>BGN</i>   | <i>BGN</i>  | <i>TNFRSF11B</i>                                   |
|                         | <i>MGP</i>   | <i>FBN2</i>  | <i>FBN1</i>   |  |
|                         |  | <i>SPP1</i>  | <i>MGP</i>  |  |
|                         |  | <i>KCNK3</i>   |   |  |
|                         |  | <i>FBN1</i>  |   |  |
|                         |  | <i>COL1A1</i>  |   |  |
| <i>Arteries</i>         |  |  |   |  |
| Pulmonary artery        | <i>TIMP1</i>   | <i>FBN2</i>  | <i>MGP</i>  | <i>RUNX2</i>                                       |
|                         | <i>ENPP1</i>   | <i>SPP1</i>  |   |  |
|                         | <i>RUNX2</i>   |  |   |  |
| Aortic root             | -  | <i>COL1A1</i>  | <i>MGP</i>  | <i>RUNX2</i>                                       |
|                         |  | <i>BGN</i>   |   | <i>SPP1</i>  |
|                         |  | <i>FBN1</i>  |   | <i>ANKH</i>  |
|                         |  | <i>FBN2</i>  |   |  |
|                         |  | <i>MMP2</i>  |   |  |
|                         |  | <i>ENPP1</i>   |   |  |
|                         |  | <i>SPP1</i>  |   |  |
|                         |  | <i>RUNX2</i>   |   |  |
|                         |  | <i>KCNK3</i>   |   |  |
| Aortic arch             | <i>TNFRSF11B</i>                                       | <i>FBN2</i>  | <i>MGP</i>  | <i>RUNX2</i>                                       |
|                         | <i>MGP</i>   | <i>SPP1</i>  |   |  |
|                         | <i>RUNX2</i>   |  |   |  |
| Abdominal aorta         | <i>COL1A1</i>  | <i>FBN2</i>  | <i>MMP2</i>   | <i>RUNX2</i>                                       |
|                         | <i>FBN1</i>  | <i>SPP1</i>  | <i>MGP</i>  |  |
|                         | <i>TIMP1</i>   | <i>RUNX2</i>   | <i>BGN</i>  |  |
|                         | <i>ANKH</i>  |  |   |  |
|                         | <i>TNFRSF11B</i>                                       |  |   |  |

1065 Table 3. Summary of 5 clusters from the sheep cardiovascular transcriptome dataset. Gene Ontology  
 1066 (GO) term analysis was performed using DAVID Functional Annotation tool (<https://david.ncifcrf.gov/>).  
 1067 BP = Biological Processes; CC: Cellular component; MF: Molecular Function. Full gene lists and  
 1068 cardiovascular expression data are presented in Supplemental Dataset 1. \*Up to 3000 genes  
 1069 maximum can be input into DAVID, thus two runs were performed with the <sup>a</sup>top 3000 genes and then  
 1070 <sup>b</sup>bottom 3000 genes. EASE score = modified Fisher Exact. > indicates decreasing expression.  
 1071

| Cluster | Number of genes | Number of un-annotated genes | Expression profile description                                 | Functional class                   | GO term   | EASE score (p-value)                            | EASE score (p-value; Benjamini corrected)                           | Genes included (Gene symbols)  |
|---------|-----------------|------------------------------|--|------------------------------------|---|---|---|--|
| 1       | 3543            | 529                          | Cardiac valves   | Various, Housekeeping              | * (BP) mRNA 3'-end processing; RNA export from nucleus  | <sup>a,b</sup> 8.1E-6;<br><sup>a,b</sup> 1.5E-4 | <sup>a,b</sup> 0.0064;<br><sup>a</sup> 0.063,<br><sup>b</sup> 0.055 | COL1A1, COL3A1, MMP2, TIMP1  |
| 3       | 192             | 42                           | Cardiac valves (highest in aortic valve)                       | ECM organisation, bone development | (BP) extracellular matrix organization; skeletal system development; osteoblast differentiation   | 6.02E-4;<br>0.00336;<br>1.12E-5                 | 0.124;<br>0.31;<br>0.0099   | BGN, COL1A2, SPARC; BGLAP, COL1A2, GDF10; BMP4, BGLAP, SNAI1-2, SOX8 |
| 22      | 27              | 5                            | Cardiac valves   | Housekeeping, Immune               | (BP) immune response<br><br>(MF) integral component of membrane   | 0.067;<br>0.061                                 | 0.999;<br>0.928   | CCR6, ENPP1, NFIL3; CD47, ENPP1, IL6ST, SMAD2                        |
| 24      | 25              | 3                            | Cardiac valves (Aortic valve > Mitral valve > Tricuspid valve) | ECM                                | (BP) positive regulation of transcription from RNA polymerase II promoter; transcription from RNA polymerase II promoter<br><br>(CC) extracellular matrix | 0.108;<br>0.123;<br>0.045                       | 0.999;<br>0.999;<br>0.943   | MEOX1, TRPS1, RBPJ, NLRP3; FMOD, FBN1, COL6A1                        |
| 36      | 20              | 5                            | Auricles (Left > Right)  | Muscle contraction                 | (BP) potassium ion transport;<br><br>(MF) calcium ion binding;<br><br>(CC) extracellular space  | 0.002;<br>0.06;<br>0.221                        | 0.16;<br>0.921;<br>0.999  | KCNQ3, KCNJ3, KCNK3; MYL7, PAM, MYL4; DKK3, NPPA, PAM                |

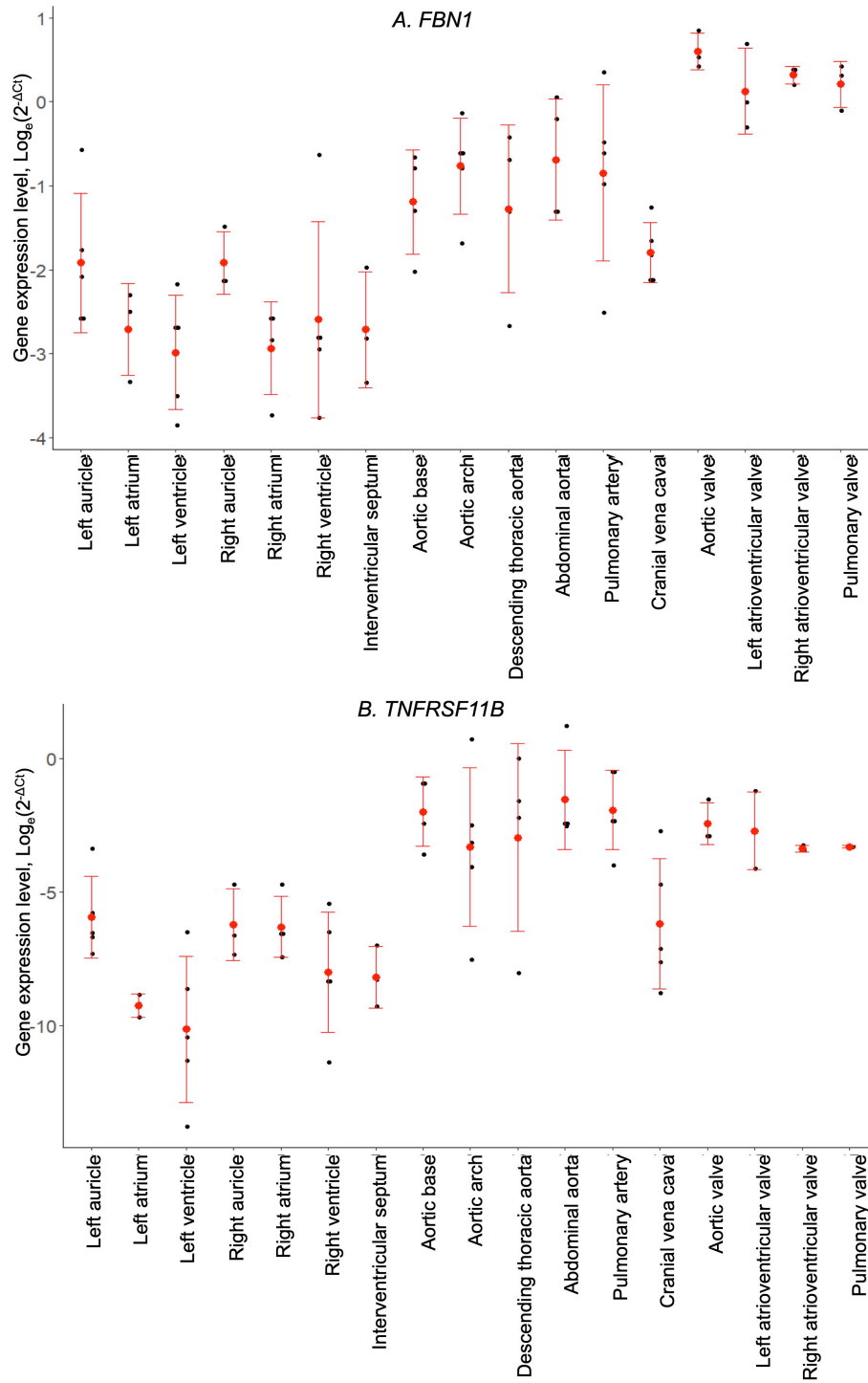
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| Number | Tissue        | Region                    | Mineralisation inhibitors |       |      |     |      | Markers   |       |      |
|--------|---------------|---------------------------|---------------------------|-------|------|-----|------|-----------|-------|------|
|        |               |                           | ANKH                      | ENPP1 | FBN1 | MGP | NT5E | TNFRSF11B | RUNX2 | SPP1 |
| 1      | Myocardium    | Left auricle              |                           |       |      |     |      |           |       |      |
| 2      |               | Left atrium               |                           |       |      |     |      |           |       |      |
| 3      |               | Left ventricle            |                           |       |      |     |      |           |       |      |
| 4      |               | Right auricle             |                           |       |      |     |      |           |       |      |
| 5      |               | Right atrium              |                           |       |      |     |      |           |       |      |
| 6      |               | Right ventricle           |                           |       |      |     |      |           |       |      |
| 7      |               | Interventricular septum   |                           |       |      |     |      |           |       |      |
| 8      | Artery        | Aortic root               |                           |       |      |     |      |           |       |      |
| 9      |               | Aortic arch               |                           |       |      |     |      |           |       |      |
| 10     |               | Descending thoracic aorta |                           |       |      |     |      |           |       |      |
| 11     |               | Abdominal aorta           |                           |       |      |     |      |           |       |      |
| 12     |               | Pulmonary artery          |                           |       |      |     |      |           |       |      |
| 13     | Vein          | Cranial vena cava         |                           |       |      |     |      |           |       |      |
| 14     | Cardiac valve | Aortic valve              |                           |       |      |     |      |           |       |      |
| 15     |               | Mitral valve              |                           |       |      |     |      |           |       |      |
| 16     |               | Tricuspid valve           |                           |       |      |     |      |           |       |      |
| 17     |               | Pulmonary valve           |                           |       |      |     |      |           |       |      |



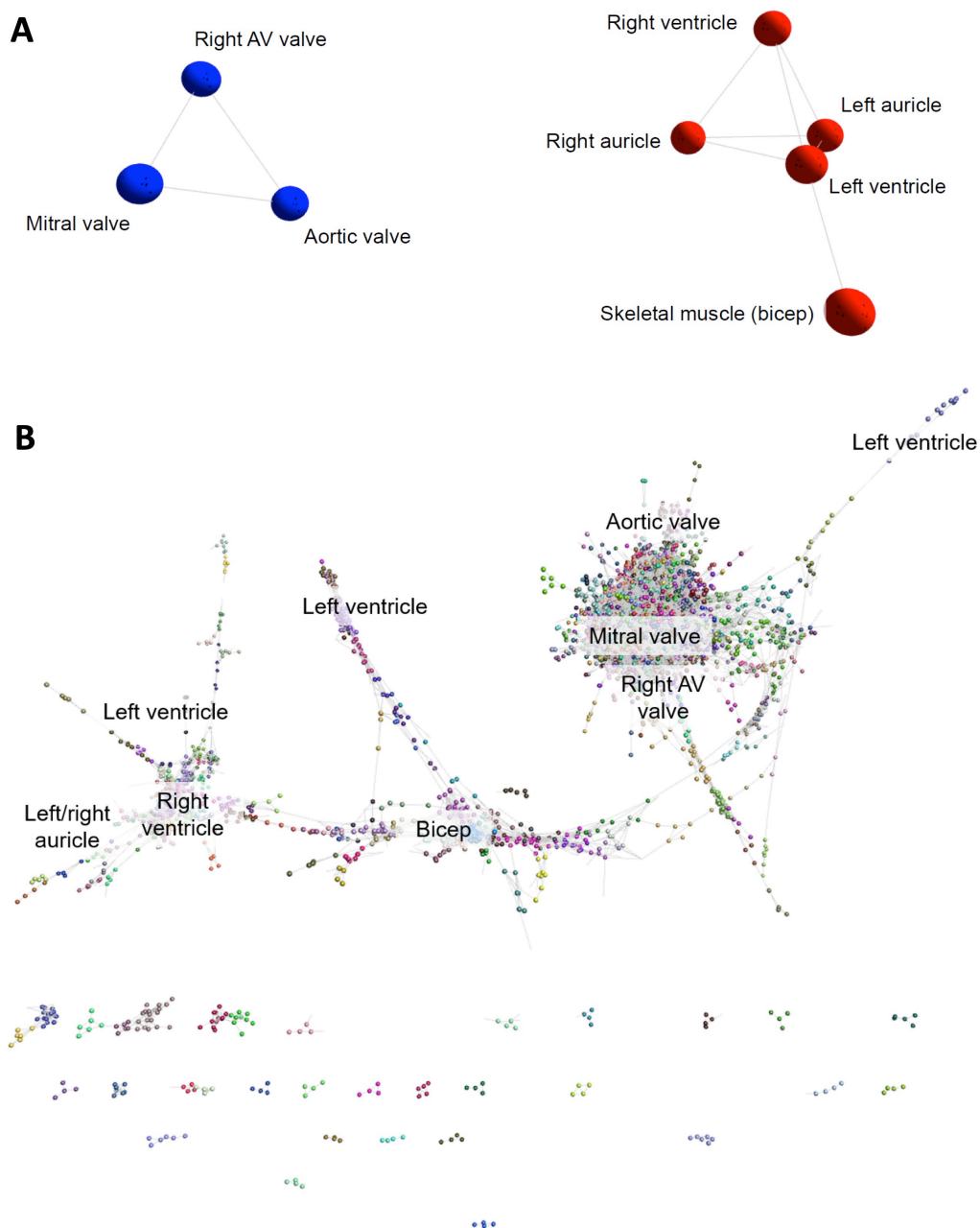
1075 Figure 1: Summary of mRNA expression profiles of key vascular calcification genes in the sheep  
1076 cardiovascular system. Blue blocks indicate where genes were found to be most highly expressed in  
1077 this study. AV = atrioventricular.  
1078



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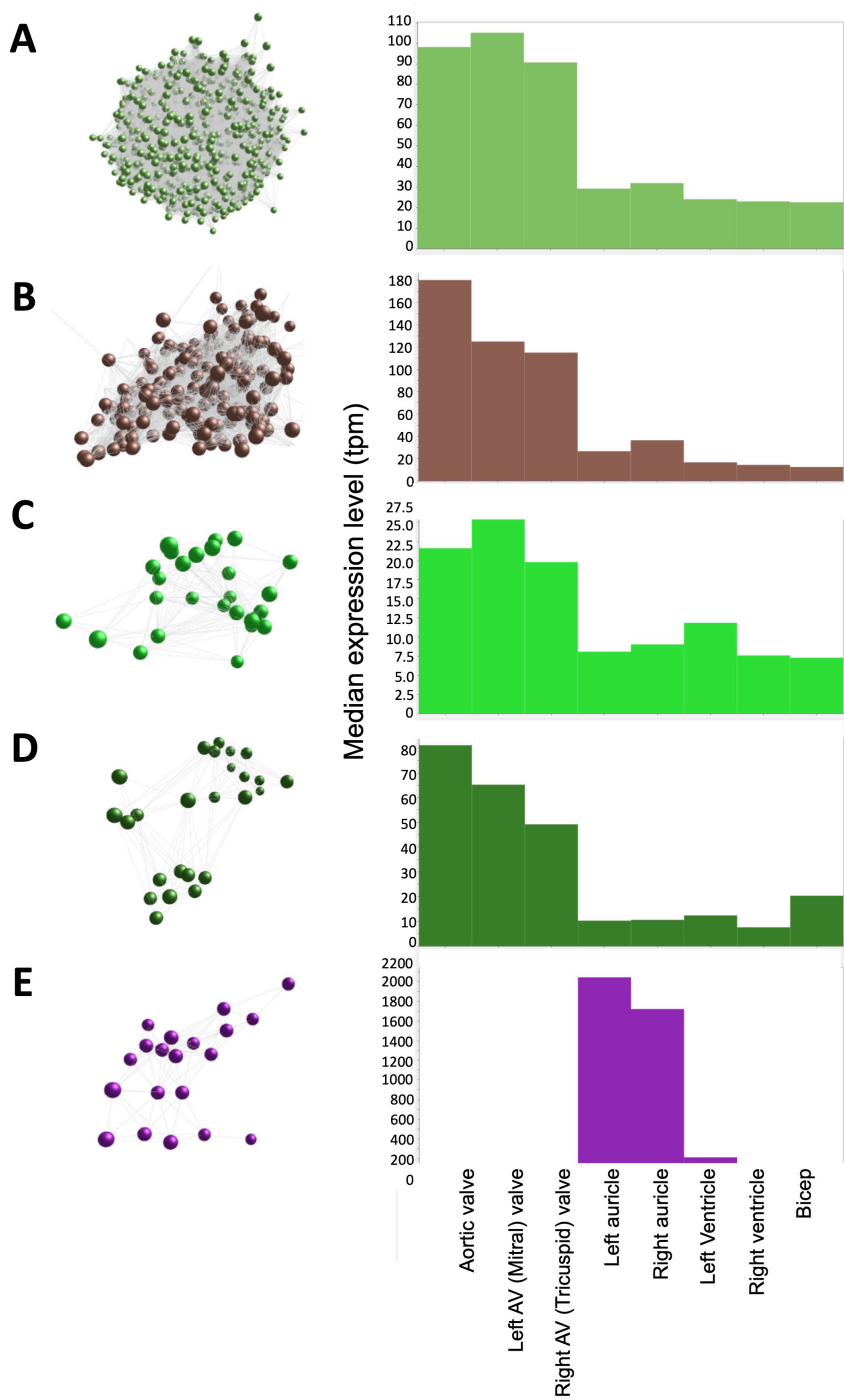
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1081 Figure 2: mRNA expression levels for individual animals, determined by RT-qPCR. (A) fibrillin-1  
1082 (*FBN1*) and (B) osteoprotegerin (*TNFRSF11B*). Gene expression levels were normalised to the  
1083 geomean of *GAPDH* and *YWHAZ*. Dot plots show individual data points (black dot), the mean  
1084 expression for each tissue (red dot) and standard deviation (red error bars).  
1085



1086  
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1088 Figure 3. Cardiovascular gene co-expression networks. (A) Sample-to-sample analysis. Network  
1089 layout of 8 tissue types revealed two distinct components: the first containing cardiac valve samples  
1090 (blue), and the second with myocardium/skeletal muscle tissues (red). Pearson correlation co-efficient  
1091  $r \geq 0.91$ . (B) Gene-to-gene analysis. Gene clusters are distinguished by colour, and the labels indicate  
1092 clusters of genes most highly expressed in the different tissues. With a Pearson correlation coefficient  
1093 of  $r \geq 0.99$ , the gene-to-gene network was comprised of 11,341 nodes (genes) with 938,652  
1094 connections. Markov clustering algorithm (MCL) clustering of the graph (inflation value 2.2) resulted in  
1095 555 clusters (containing >3 genes).  
1096



1097  
1098

1099 Figure 4. Average expression of genes within clusters. X axis shows the samples; Y axis shows the  
1100 average expression for the cluster in tpm. Spheres (nodes) in co-expression clusters (left) denote  
1101 individual genes; lines represent connections between genes. The histogram (right) shows median  
1102 expression levels in transcripts per million (TPM) in the different tissues. AV = atrioventricular. (A)  
1103 Cluster 1, the largest cluster, contained 3543 genes, with 529 unannotated genes. A co-expression  
1104 cluster highly expressed in the sheep cardiac valves compared to the myocardium and bicep. (B)  
1105 Cluster 3 contained 192 genes, with 40 unannotated genes. A co-expression cluster highly expressed  
1106 in the sheep cardiac valves, particularly in the aortic valves, compared to the myocardium and bicep.  
1107 (C) Cluster 22 contained 27 genes, with 5 unannotated genes. A co-expression cluster highly  
1108 expressed in the sheep cardiac valves compared to the myocardium and bicep. (D) Cluster 24  
1109 contained 25 genes, with 3 unannotated genes. A co-expression cluster highly expressed in the  
1110 sheep cardiac valves compared to the myocardium and bicep. (E) Cluster 36 contained 20 genes,  
1111 with 5 unannotated genes. A co-expression cluster highly expressed in the sheep auricles compared  
1112 to the cardiac valves, the ventricles and the bicep.  
1113

1114 **Supplementary Tables**

1115

1116 Supplementary Table 1. Sheep primers for RT-qPCR. Primers in black were designed using Primer3  
1117 (<http://primer3.ut.ee/>) to span exon-exon junctions, and obtained from Invitrogen (Paisley, UK).  
1118 Primers in blue were obtained from Primerdesign Ltd (Eastleigh, UK).

1119

1120 Supplementary Table 2. Details of tissues sequenced to generate the RNA-seq dataset for the  
1121 cardiovascular gene expression atlas. Skeletal muscle (bicep) was also included, as an example of  
1122 another muscle tissue, for comparative analysis. All libraries were Illumina 125 bp paired end  
1123 stranded libraries.

1124

1125 **Supplementary Figures**

1126

1127 Supplementary Figure 1. Gene expression profiles during development in the left ventricle. Genes  
1128 include: (A) collagen type I alpha 1, *COL1A1*, (B) biglycan, *BGN*, (C) matrix metalloproteinase 2,  
1129 *MMP2*, (D) TIMP metalloproteinase inhibitor 1, *TIMP1*, (E) fibrillin 1, *FBN1*, (F) fibrillin 2, *FBN2*, (G)  
1130 secreted phosphoprotein1/osteopontin, *SPP1*, (H) progressive ankylosis protein, *ANKH*, (I)  
1131 osteoprotegerin, *TNFRSF11B*, and (J) Runt-related transcription factor 2, *RUNX2*. Black dots show  
1132 gene expression from individual animals (n = 3-5) and red dot and error bars show the mean ±  
1133 standard deviation (SD) per tissue. Gene expression levels were normalised to the geomean of  
1134 *GAPDH* and *YWHAZ*. In blue, asterisk (\*) denotes significant differences compared to foetal d100  
1135 sheep, triangle (Δ) compared to newborn sheep and circle (o) compared to 8 week old sheep, where  
1136 1 symbol = 0.01 < p < 0.05, 2 symbols = 0.001 < p < 0.01 and 3 symbols = p < 0.001.

1137

1138 Supplementary Figure 2. Gene expression profiles during development in the interventricular septum.  
1139 Genes include: (A) collagen type I alpha 1, *COL1A1*, (B) biglycan, *BGN*, (C) matrix metalloproteinase  
1140 2, *MMP2*, (D) fibrillin 1, *FBN1*, (E) fibrillin 2, *FBN2*, (F) secreted phosphoprotein1/osteopontin, *SPP1*,  
1141 (G) progressive ankylosis protein, *ANKH*, (H) matrix Gla protein, *MGP*, and (I) Potassium two pore  
1142 domain channel subfamily K member 3, *KCNK3*. Black dots show gene expression from individual  
1143 animals (n = 3-5) and red dot and error bars show the mean ± standard deviation (SD) per tissue.  
1144 Gene expression levels were normalised to the geomean of *GAPDH* and *YWHAZ*. In blue, asterisk (\*)  
1145 denotes significant differences compared to foetal d100 sheep, triangle (Δ) compared to newborn  
1146 sheep, circle (o) compared to 1 week old sheep and square (□) compared to 8 week old sheep, where  
1147 1 symbol = 0.01 < p < 0.05, 2 symbols = 0.001 < p < 0.01 and 3 symbols = p < 0.001.

1148

1149 Supplementary Figure 3. Gene expression profiles during development in the pulmonary artery.  
1150 Genes include: (A) TIMP metalloproteinase inhibitor 1, *TIMP1*, (B) fibrillin 2, *FBN2*, (C) ectonucleotide  
1151 pyrophosphatase/ phosphodiesterase 1, *ENPP1*, (D) secreted phosphoprotein1/osteopontin, *SPP1*,  
1152 and (E) Runt-related transcription factor 2, *RUNX2*. Black dots show gene expression from individual  
1153 animals (n = 3-5) and red dot and error bars show the mean ± standard deviation (SD) per tissue.



1154 Gene expression levels were normalised to the geomean of *GAPDH* and *YWHAZ*. In blue, asterisk (\*)  
1155 denotes significant differences compared to newborn sheep, triangle ( $\Delta$ ) compared to 8 week old  
1156 sheep, where 1 symbol =  $0.01 < p < 0.05$ , 2 symbols =  $0.001 < p < 0.01$  and 3 symbols =  $p < 0.001$ .

1157

1158 Supplementary Figure 4. Gene expression profiles during development in the aortic root. Genes  
1159 include: (A) collagen type I alpha 1, *COL1A1*, (B) biglycan, *BGN*, (C) matrix metalloproteinase 2,  
1160 *MMP2*, (D) fibrillin 1, *FBN1*, (E) fibrillin 2, *FBN2*, (F) ectonucleotide pyrophosphatase/  
1161 phosphodiesterase 1, *ENPP1*, (G) secreted phosphoprotein1/osteopontin, *SPP1*, and (H) Potassium  
1162 two pore domain channel subfamily K member 3, *KCNK3*. Black dots show gene expression from  
1163 individual animals ( $n = 3-5$ ) and red dot and error bars show the mean  $\pm$  standard deviation (SD) per  
1164 tissue. Gene expression levels were normalised to the geomean of *GAPDH* and *YWHAZ*. In blue,  
1165 asterisk (\*) denotes significant differences compared to newborn sheep, triangle ( $\Delta$ ) compared to 1  
1166 week old sheep and circle (o) compared to 8 week old sheep, where 1 symbol =  $0.01 < p < 0.05$ , 2  
1167 symbols =  $0.001 < p < 0.01$  and 3 symbols =  $p < 0.001$ .

1168

1169 Supplementary Figure 5. Gene expression profiles during development in the aortic arch. Genes  
1170 include: (A) fibrillin 2, *FBN2*, (B) secreted phosphoprotein1/osteopontin, *SPP1*, (C) matrix Gla protein,  
1171 *MGP*, (D) osteoprotegerin, *TNFRSF11B*, and (E) Runt-related transcription factor 2, *RUNX2*. Black  
1172 dots show gene expression from individual animals ( $n = 3-5$ ) and red dot and error bars show the  
1173 mean  $\pm$  standard deviation (SD) per tissue. Gene expression levels were normalised to the geomean  
1174 of *GAPDH* and *YWHAZ*. In blue, asterisk (\*) denotes significant differences compared to newborn  
1175 sheep and triangle ( $\Delta$ ) compared to 1 week old sheep, where 1 symbol =  $0.01 < p < 0.05$ , 2 symbols =  
1176  $0.001 < p < 0.01$  and 3 symbols =  $p < 0.001$ .

1177

1178 Supplementary Figure 6. Gene expression profiles during development in the abdominal aorta. Genes  
1179 include: (A) collagen type I alpha 1, *COL1A1*, (B) TIMP metalloproteinase inhibitor 1, *TIMP1*, (C)  
1180 fibrillin 1, *FBN1*, (D) fibrillin 2, *FBN2*, (E) secreted phosphoprotein1/osteopontin, *SPP1*, (F)  
1181 progressive ankylosis protein, *ANKH*, (G) osteoprotegerin, *TNFRSF11B*, and (H) Runt-related  
1182 transcription factor 2, *RUNX2*. Black dots show gene expression from individual animals ( $n = 3-5$ ) and  
1183 red dot and error bars show the mean  $\pm$  standard deviation (SD) per tissue. Gene expression levels  
1184 were normalised to the geomean of *GAPDH* and *YWHAZ*. In blue, asterisk (\*) denotes significant  
1185 differences compared to newborn sheep and triangle ( $\Delta$ ) compared to 8 week old sheep, where 1  
1186 symbol =  $0.01 < p < 0.05$ , 2 symbols =  $0.001 < p < 0.01$  and 3 symbols =  $p < 0.001$ .

1187

1188 Supplementary Figure 7. mRNA expression profile for (A) matrix Gla protein (*MGP*), (B) progressive  
1189 ankylosis protein homologue (*ANKH*). Gene expression levels were normalised to the geomean of  
1190 *GAPDH* and *YWHAZ*. Dot plots show individual data points (black dot), the mean expression for each  
1191 tissue (red dot) and standard deviation (red error bars).

1192

1193 Supplementary Figure 8. mRNA expression profile for (A) ecto-5'-nucleotidase (*NT5E*) and (B) Runt-  
1194 related transcription factor 2 (*RUNX2*). Gene expression levels were normalised to the geomean of  
1195 *GAPDH* and *YWHAZ*. Dot plots show individual data points (black dot), the mean expression for each  
1196 tissue (red dot) and standard deviation (red error bars).

1197

1198 Supplementary Figure 9. mRNA expression profile for (A) ectonucleotide  
1199 pyrophosphatase/phosphodiesterase 1 (*ENPP1*) and (B) secreted phosphoprotein1/osteopontin  
1200 (*SPP1*). Gene expression levels were normalised to the geomean of *GAPDH* and *YWHAZ*. Dot plots  
1201 show individual data points (black dot), the mean expression for each tissue (red dot) and standard  
1202 deviation (red error bars).

1203

1204 Supplementary Figure 10. RNA-seq expression profiles of selected genes. Expression levels were  
1205 measured using RNA-seq and shown as median expression levels in transcripts per million (TPM; n =  
1206 4-6). Y axis shows normalised median TPM (Bush et al. 2017). (A-D) Cluster 1 gene expression  
1207 profiles. Genes include collagen type I alpha 1 (*COL1A1*), collagen type III alpha 1 (*COL3A1*), matrix  
1208 metalloproteinase 2 (*MMP2*) and tissue inhibitor of metalloproteinases 1 (*TIMP1*). (E-G) Cluster 3  
1209 gene expression profiles. Genes include collagen type I alpha 2 (*COL1A2*), bone gamma-  
1210 carboxyglutamate protein (*BGLAP*) and biglycan (*BGN*). (H-J) Cluster 22 gene expression profiles.  
1211 Genes include ectonucleotide pyrophosphate/phosphodiesterase 1 (*ENPP1*), ADAM metalloproteinase  
1212 with thrombospondin type 1 motif 6 (*ADAMTS6*) and SMAD family member 2 (*SMAD2*). (K-L) Cluster  
1213 24 gene expression profiles. Genes include fibrillin 1 (*FBN1*) and fibromodulin (*FMOD*). (M-O) Cluster  
1214 36 gene expression profiles. Gene include potassium two pore domain channel subfamily K member  
1215 3 (*KCNK3*), natriuretic peptide A (*NPPA*) and Dickkopf WNT signalling pathway inhibitor 3 (*DKK3*).

1216

### 1217 **Supplemental Datasets**

1218

1219 Supplemental Dataset 1: Gene expression estimates as transcripts per million (TPM) for seven  
1220 cardiovascular tissues and skeletal muscle bicep generated for the sheep gene expression atlas using  
1221 Kallisto.

1222

1223 Supplemental Dataset 2: Genes contained within each cluster from the gene to gene network analysis  
1224 presented in Figure 1 (B) and Figure 2. Pearson correlation coefficient  $r \geq 0.99$ , MCL (inflation = 2.2).

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1226