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Abstract: Valve interstitial cells (VICs) have an important role in the aetiopathogenesis of myxomatous mitral valve disease (MMVD) in the dog. Furthermore, there is evidence that valve endothelial cells (VECs) also contribute to disease development. In addition to examining native valve tissue to understand MMVD, another strategy is to separately examine VIC and VEC biology under in vitro culture conditions. The aim of this study was to isolate and characterise canine mitral VICs and VECs from normal dog valves using a combination of morphology, immunohistochemistry and reverse transcription PCR (RT-PCR).

Canine mitral VECs and VICs were isolated and cultured in vitro. The two cell populations exhibited different morphologies and growth patterns. VECs, but not VICs, expressed the endothelial markers, platelet endothelial cell adhesion molecule (PECAM-1 or CD31) and acetylated low density lipoprotein (Dil-Ac-LDL). Both VECs and VICs expressed vimentin and embryonic non-smooth muscle myosin heavy chain (SMemb), an activated mesenchymal cell marker. The myofibroblast marker, alpha smooth muscle actin (α -SMA), was detected at the mRNA level in both VEC and VIC cultures, but only at the protein level in VIC cultures. The morphological heterogeneity and expression of non-endothelial phenotypic markers in VEC cultures suggested that a mixture of cell types was present, which might be due to cell contamination and/or endothelial mesenchymal transition (EndoMT). The use of a specific endothelial culture medium for primary VEC cultures enhanced the endothelial properties of the cells and reduced α -SMA and SMemb expression.

1 **Culture and characterisation of canine mitral valve interstitial and endothelial cells**

2

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14

15

16

17 **Abstract**

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19 myxomatous mitral valve disease (MMVD) in the dog. Furthermore, there is evidence that
20 valve endothelial cells (VECs) also contribute to disease development. In addition to
21 examining native valve tissue to understand MMVD, another strategy is to separately
22 examine VIC and VEC biology under in vitro culture conditions. The aim of this study was to
23 isolate and characterise canine mitral VICs and VECs from normal dog valves using a
24 combination of morphology, immunohistochemistry and reverse transcription PCR (RT-
25 PCR).

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27 Canine mitral VECs and VICs were isolated and cultured in vitro. The two cell
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30 CD31) and acetylated low density lipoprotein (Dil-Ac-LDL). Both VECs and VICs expressed
31 vimentin and embryonic non-smooth muscle myosin heavy chain (SMemb), an activated
32 mesenchymal cell marker. The myofibroblast marker, alpha smooth muscle actin (α -SMA),
33 was detected at the mRNA level in both VEC and VIC cultures, but only at the protein level
34 in VIC cultures. The morphological heterogeneity and expression of non-endothelial
35 phenotypic markers in VEC cultures suggested that a mixture of cell types was present,
36 which might be due to cell contamination and/or endothelial mesenchymal transition
37 (EndoMT). The use of a specific endothelial culture medium for primary VEC cultures
38 enhanced the endothelial properties of the cells and reduced α -SMA and SMemb expression.

39

40 *Keywords:* Valve interstitial cell; Valve endothelial cell; Cell culture; Canine; Myxomatous
41 mitral valve disease.

42

43 **Introduction**

44 Myxomatous mitral valve disease (MMVD) is characterised by extensive changes to
45 the valve extra cellular matrix (ECM), resulting in valve distortion and mechanical instability,
46 and leading to valve insufficiency and mitral regurgitation (Black et al., 2005; Hadian et al.,
47 2007; Aupperle et al., 2009, 2010; Han et al., 2010, 2013b). The mechanisms that lead to
48 valve degeneration are not fully understood, but are presumed to involve changes in valve
49 interstitial cell (VIC) and endothelial cell (VEC) phenotype and function (Corcoran et al.,
50 2004; Disatian et al., 2008; Han et al., 2008, 2013a). Endothelial changes are characterised by
51 detachment, denuding and apoptosis with splitting of the basement membrane and increased
52 expression of basement membrane proteins, including laminin (Aupperle et al., 2009; Han et
53 al., 2013a). VICs show an activated myofibroblast phenotype and their numbers decline in
54 the overly myxomatous areas and proliferate close to the valve surface, most noticeably
55 where there has been endothelial damage (Disatian et al., 2008; Han et al., 2008, 2013a).

56

57 VICs are the cells contributing to ECM production and remodelling. Their synthetic
58 activity is driven mainly by the transforming growth factor beta (TGF- β) cytokine
59 superfamily, but is also thought to be modified by signals from the endothelial cells (Olsen et
60 al., 2003; Disatian et al., 2010; Aupperle and Disatian, 2012; Orton et al., 2012; Han et al.,
61 2013a). This VEC-VIC interaction has led to the hypothesis that VEC activation is
62 fundamental to valve matrix homeostasis and that MMVD may be driven by mechanisms
63 involving VEC damage and leading to aberrant matrix remodelling.

64

65 Future examination of MMVD will require a greater understanding of VEC-VIC
66 interactions and one approach is to do so using in vitro models. Canine mitral VICs have
67 been previously isolated and cultured using a primary explant technique which favours

68 culturing migratory cells only, but there was limited phenotypic characterisation, and there
69 are no reports of successful cultures of canine VECs (Heaney et al., 2009). The aims of this
70 study, therefore, were (1) to develop robust methods for the culture and phenotypic
71 characterisation of canine mitral VICs and VECs based on a reported protocol for porcine
72 aortic valve cell isolation (Gould and Butcher, 2010), and (2) to examine canine mitral valve
73 cell biology under the culture conditions. Furthermore, as it is recognised to be difficult to
74 maintain endothelial cell phenotype in VEC culture, different culture protocols were
75 evaluated.

76

77 **Materials and methods**

78 *Cell isolation and culture*

79 Cells were isolated from 12 healthy canine mitral valves showing no MMVD
80 evidence on gross morphological inspection. The dogs were of different breeds, aged 2-5
81 years and with equal sex distribution. All dogs were euthanased for reasons other than cardiac
82 disease. Samples were collected with full owner consent and sampling conformed to
83 institutional and national ethical guidelines.

84

85 Mitral valves were removed within minutes of euthanasia under sterile conditions,
86 rinsed in cold sterile phosphate buffered saline (PBS) and placed on ice until transferred to a
87 tissue culture laminar hood. Excess annular tissue and chordae tendineae were removed, and
88 samples were placed in 35 mm petri dishes and incubated with 5 mL pre-warmed collagenase
89 solution (600 Units/ml) in 5% CO₂ for 10 min at 37 °C. VECs were removed by gently
90 rotating a dry sterile swab over the surface of the leaflet and occasionally dabbing the swab
91 into the collagenase solution. The collagenase solution was aspirated and the suspended cells
92 pelleted by centrifuging at 1000g for 5 min at room temperature. The supernatant was

93 aspirated and the cells were re-suspended in advanced DMEM/F-12 medium (Life
94 Technologies) with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-
95 glutamine (AF-12). Cells were centrifuged, re-suspended again and seeded on culture flasks
96 or plates pre-coated with 2% gelatin and incubated at 37 °C in 5% CO₂. After VEC
97 collection, tissue was placed in a 15 mL conical tube with 10 mL collagenase solution (600
98 U/mL) and incubated for 18 h at 37 °C in 5% CO₂. After digestion, the tissue was
99 homogenized in the collagenase solution. The released cells were then seeded onto flasks and
100 cultured as described for VECs in AF-12 medium.

101

102 Media changes occurred at 2 day intervals for both populations. At confluence, cells
103 were passaged by either trypsinization (0.05% - 1% Trypsin /EDTA) or by using 1 x TrypLE
104 Express (Life Technologies). In general, VECs were expanded up to passage three and VICs
105 were cultured up to passage 8.

106

107 *Comparison of two culture media for canine mitral VECs*

108 To evaluate the effect of specific endothelial culture medium on primary VEC
109 properties, a canine endothelial basal medium kit (Cn 211k-500, Cell Application; EBM) was
110 compared with the AF-12 medium for VECs. VEC preparations (at passages 1-2) from
111 different dogs ($n = 3$) were used for the medium comparison experiment. Prior to the medium
112 switch, cells were stored in freezing medium (70% AF-12 medium/20% FBS/10%
113 dimethylsulphoxide (DMSO; Invitrogen)) and cryopreserved in -150 °C freezer or in liquid
114 nitrogen freezer. Cells were revived in a 37 °C water bath, and were washed with pre-warmed
115 AF-12 medium by centrifuging. After removal of the supernatant, the cells were re-suspended
116 in canine EBM and seeded onto culture plates pre-coated with 2% gelatin and incubated at
117 37 °C in 5% CO₂. Control cultures were maintained in AF-12 for the same VEC preparation.

118 VEC cultures in EBM and AF-12 were harvested at confluence and evaluated for endothelial
119 and mesenchymal marker expression.

120

121 *Reverse transcription-polymerase chain reaction (RT-PCR)*

122 Total RNA was isolated from both cell types using the RNeasy Mini Kit (Qiagen),
123 according to the manufacturer's protocol. Extracted RNA was treated with RNase-free DNase
124 I and concentration of RNA was measured using NanoDrop (Thermal Scientific). RNA was
125 diluted using nuclease-free water (Qiagen) and was denatured at 65 °C for 5 min, then placed
126 immediately on ice. Complementary DNA was synthesized using an Omniscript Reverse
127 Transcription Kit (Qiagen) at 40 °C for 1 h.

128

129 The following genes (Table 1) were selected to characterise the VECs and VICs:
130 *CD31* and *von Willebrand Factor (vWF)* (endothelial markers); *alpha-smooth muscle actin*
131 (*α-SMA*), *embryonic form of non-smooth muscle myosin (SMemb)* and *transgelin (SM22)*
132 (activated VIC (aVIC) markers); *vimentin* (mesenchymal origin cells). *GAPDH* was used as a
133 housekeeping gene. The primers of *vWF-1*, *α-SMA* and *GAPDH* were kind gifts (Hannah
134 Hodgkiss-Geere, the University of Edinburgh), and vWF3 primer sequence was derived from
135 Fulton et al. (2000). All other primers were originally designed using Primer 3 Input v.4.0
136 and nucleotide sequences of the above markers were obtained from the NCBI Gene Bank or
137 Ensembl databases.

138

139 PCR amplification used the Gotaq PCR Core System (Promega). Initial denaturation
140 at 95 °C for 5 min was followed by 30 cycles of 95 °C for 1 min, 58-61 °C for 1 min, 72 °C
141 for 1 min and a final extension at 72 °C for 10 min. PCR products were analysed using a

142 standard 2% agarose gel electrophoresis. Molecular Imager Gel Doc system (Bio-Rad) was
143 used to visualize the results after electrophoresis.

144

145 *Immunocytochemistry*

146 The cell suspension (200 μ L) was added to duplicate BD Falcon Culture slides, pre-
147 coated with 2% gelatin for VECs, at a density of 1×10^4 cell/well and incubated in 5% CO₂ at
148 37 °C for 24-48 h. Slides were directly fixed with acetone for 10 min at -20 °C, washed twice
149 in PBS, incubated for 1 h in blocking buffer (10% goat serum/0.1% Tween 20/PBS) and then
150 overnight in a humidity chamber at 4 °C with primary antibodies against CD31, vimentin,
151 SMemb and α -SMA (Table 2). Slides were washed twice with PBS and incubated with 100
152 μ L goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody for 1 h at room
153 temperature in a dark humid chamber. The slides were then washed three times in PBS, 4',6-
154 diamidino-2-phenylindole (DAPI) mounted (Vectashield Mounting Medium, Vector Inc), and
155 examined using a Leitz fluorescence microscope (Leica).

156

157 *DiI- acetylated-low density lipoprotein (DiI-Ac-LDL) labelling*

158 Acetylated low density lipoprotein conjugated with fluorescence dye 1, 1'-
159 dioctadecyl-3, 3, 3', 3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was used to
160 detect endothelial cells. The cell culture protocol prior to labelling was the same as that
161 described for immunocytochemistry. DiI-Ac-LDL (200 μ L; 5 μ g/mL; BT902, Biomedical
162 Technologies) reagent was added to one cell chamber and incubated for 4 h in 5% CO₂ at 37
163 °C. Slides were washed three times with PBS and were examined by fluorescence and bright
164 field microscopy.

165

166 *Image capture and processing*

167 Cell morphology was observed by light microscopy during the culture period and
168 assessed for cell morphology and confluence. Representative images were captured using a
169 camera connected Zeiss Axiovert 40 microscope. Immunofluorescence and DiI-Ac-LDL
170 fluorescent channel images were captured using a Leica Firecam (Leica). For optimal
171 visualization of the fluorescence signals, the antibody and DAPI staining images were
172 converted into grayscale with Image J software. Original antibody and DAPI channel colour
173 images were merged using Adobe Photoshop CS6 software (Adobe System). For DiI-Ac-
174 LDL labelling, bright field images were taken from the same sampled area demonstrating cell
175 distribution and were converted into grayscale images with ImageJ software.

176

177 **Results**

178 *Cell culture morphology and growth*

179 By using current collagenase digestion techniques, VEC and VIC were successfully
180 isolated from the same mitral valve ($n = 12$) and grew well in all sub-cultures. The two
181 culture types showed differences in cell morphology and growth pattern.

182

183 Endothelial colony-forming cell clusters were randomly distributed in VEC culture
184 flasks 1-2 days post primary isolation, and clustering promoted outgrowth. Cells had
185 cobblestone morphology typical of endothelial cells in culture, and most noticeably at
186 confluence (Fig.1). Confluence of passage 0 VECs was usually reached within 6-8 days in
187 one T80 flask when most cells exhibited growth contact inhibition and formed a monolayer.
188 Some cell polymorphism was also noted with spindle shapes and multiple cytoplasmic
189 extensions, which increased in numbers with increasing passages.

190

191 Cultured VICs typically had a spindle shape reminiscent of a fibroblast-like
192 morphology, although heterogeneous morphologies were also observed, and the cells
193 proliferated readily in culture (Fig.1). At confluence, the cells tended to form a whorl-like
194 pattern with multiple layers and did not exhibit growth contact inhibition (Fig. 1). Primary
195 passage 0 VICs usually reached at confluence in a T80 flask within 3 days.

196

197 *VEC phenotypic characterization*

198 Phenotypic characterization was performed on 3-4 VEC cell lines (at passages 2-3)
199 derived from different dogs. By RT-PCR, VECs expressed CD31, vimentin, as well as α -
200 SMA, SMemb and SM22 (Fig. 2). Expression of vWF was not apparent in canine mitral
201 VECs, and this finding was consistently observed with three different vWF primer pairs. All
202 vWF primer pairs were validated by probing cDNA of two endothelial nature cell lines (one
203 human umbilical vascular endothelial cell line and one canine haemangiosarcoma cell line),
204 in which the expression was clearly identified (data not show).

205

206 By immunofluorescence, CD31 was identified in VECs, particularly at cell-cell
207 junctions (Fig. 3). VECs also extensively expressed vimentin and SMemb in the cytoplasm
208 (Fig. 3), while α -SMA expression was limited and only detected in a few cells from one
209 culture. The endothelial identity of the VEC culture was also confirmed by DiI-Ac-LDL
210 uptake (Fig. 3).

211

212 *Effects of culture media on VEC culture characteristics*

213 Replacing standard culture medium (AF-12) with EBM helped to maintain a more
214 endothelial phenotype as determined by morphological assessment. Cells in both media
215 showed Dil-Ac-LDL up-take and CD31 expression, but staining was more intense in cells

216 cultured with EBM (Fig. 4). At the transcriptional level, higher CD31 expression was
217 observed in EBM cultures compared with AF-12 cultures, while α -SMA and SMemb
218 expression was reduced in the EBM cultures compared to AF-12 cultures (Fig. 5).

219 *VIC phenotypic characterization*

220 Phenotypic characterization was performed on 3-5 VIC cell lines (at passages 4-7)
221 derived from different dogs. By RT-PCR, α -SMA, vimentin, SM22 and SMemb were
222 expressed in VIC cultures in contrast to CD31 (Fig. 2). By immunofluorescence, VIC
223 cultures were also consistently negative for CD31 expression (Fig. 6). Similar to VECs, VICs
224 showed extensive cytoplasmic expression of vimentin and SMemb. α -SMA expression was
225 observed in the cytoplasm, although the expression level varied between different cultures,
226 with an estimated average of 10% cell expression. No VIC cultures showed DiI-Ac-LDL
227 uptake (Fig. 6).

228

229 **Discussion**

230 VICs have been previously cultured from dog mitral valves from normal dogs and in
231 one study from dogs with mild MMVD (Heaney et al., 2009; Waxman et al., 2012). In the
232 study by Heaney et al. (2009), cultures were initiated up to 36 h after death (Heaney et al.,
233 2009). Furthermore, their protocol favoured migratory cells, resulting in suboptimal VIC
234 isolation and culture (Taylor et al., 2000). Waxman et al. (2012) isolated mitral VICs using
235 the same collagenase digestion technique as in our study, but from a limited number of
236 laboratory Beagles that were healthy or with mild MMVD. VIC characteristics in a standard
237 tissue culture system, however, were not evaluated. To date there are no reports of culture
238 and characterisation of VECs collected from canine valves immediately after death.

239

240 In the current study, canine mitral VECs and VICs were successfully isolated from the
241 same subjects, cultured and characterized. Relatively pure cell populations were obtained and
242 there was differential expression of a range of known mitral valve cell phenotypic markers.
243 This provides fundamental information on canine mitral valve cell biology, which will
244 benefit mitral valve disease research.

245

246 VECs are known to express CD31 and to show selective uptake of DiI-Ac-LDL
247 through endocytosis. These properties allow confirmation of an endothelial origin (Cuy et al.,
248 2003; Gould and Butcher, 2010). The lack of expression of vWF was unexpected, but could
249 be due to a tissue-specific heterogeneous expression of vWF, as has been previously observed
250 in endothelium from different anatomic sites (Pusztaszeri et al., 2006). A previous report on
251 porcine mitral VECs also found vWF expression to be weak at the transcriptional level
252 (Flanagan et al., 2006). The majority of VECs exhibited typical cobblestone morphology, but
253 cellular pleomorphism was also present with spindle-shaped cells reminiscent of interstitial
254 cells and fibroblasts. This may be explained by valve-side heterogeneity (as found in aortic
255 valve VECs), VIC contamination or endothelial mesenchymal transition (EndoMT) to a VIC
256 phenotype (Simmons et al., 2005; Bischoff and Aikawa, 2011). In the current study, the
257 mRNA expression of aVIC markers (i.e. SMemb, SM22 and α -SMA) in VEC cultures
258 suggests the presence of VIC contamination and/or EndoMT in the culture system. VIC
259 contamination is a common issue in primary VEC cultures when using enzymatic digestion
260 (Butcher and Nerem, 2007; Cheung et al., 2008). However, cell contamination cannot fully
261 explain the aVIC characteristics in the VEC cultures, particularly the extensive protein
262 expression of the activated mesenchymal marker SMemb in VEC. To our knowledge, this is
263 the first study showing SMemb expression in adult VECs, which suggests that VECs in the

264 current culture system are in an activated state and have embryonic cell potential reminiscent
265 of EndoMT.

266

267 EndoMT does involve increased mesenchymal and decreased endothelial marker
268 expression (Hoerstrup et al., 1998; Cheung et al., 2008; Bischoff and Aikawa, 2011). Such
269 phenotypic transformation of VECs has been suggested to be similar to what occurs during
270 valvular development (Person et al., 2005; Butcher and Markwald, 2007; Butcher and Nerem,
271 2007; Bischoff and Aikawa, 2011). The complexity of the transition from cardiac
272 endothelium to interstitial cells involves multiple signalling pathways, but particularly
273 endothelial growth factors and member of the TGF- β superfamily (Armstrong and Bischoff,
274 2004). The possible EndoMT potential in adult valve cells has been examined previously
275 (Paruchuri et al., 2006; Paranya et al., 2001; Mahler et al., 2013). It has been hypothesized to
276 be one of the key remodelling mechanisms in heart valve diseases, where a subset of adult
277 VECs possess foetal cell characteristics similar to progenitor cells, and can replenish the VIC
278 population when needed, for example during disease or remodelling process (Paruchuri et al.,
279 2006; Liu et al., 2007; Bischoff and Aikawa, 2011).

280

281 Due to cell contamination and/or in vitro differentiation, the primary VECs were less
282 stable particularly in long term cultures. Cell purification techniques and special culture
283 medium favouring endothelial growth are potential solutions to increase VEC purity
284 (Hoerstrup et al., 1998; Cheung et al., 2008; Gould and Butcher, 2010). In the current study,
285 these two methods were evaluated separately. Trial cell sorting experiments were performed
286 on the VECs from one canine mitral valve (data not shown). By using CD146 and flow
287 cytometry (Wills et al., 2009), over 75% of positive cells were detected in the passage 1 VEC
288 culture. The separated CD146-positive cells showed stronger endothelial and decreased

289 mesenchymal properties compared to the unsorted cells in short term cultures. However,
290 myofibroblast-like morphology re-appeared later when cells reached confluence. This
291 suggests that even though cell sorting can efficiently separate VECs from a mixed population
292 culture, a standard cell culture medium (i.e. AF-12) cannot maintain the endothelial
293 properties in long term cultures. To preserve endothelial features and prevent mesenchymal
294 differentiation, VECs typically require a specific culture medium containing growth factors
295 favouring endothelial growth (Paranya et al., 2001; Wylie-Sears et al., 2011; Ci et al., 2013).
296 The use of EBM instead of AF-12 resulted in a more consistent VEC phenotype, as
297 evidenced by enhanced endothelial marker expression and decreased α -SMA and SMemb
298 expression. However, EBM did not inhibit the proliferation of contaminating cell types. A
299 combination of cell sorting and of a specific endothelial medium culture could improve the
300 purity of VECs as well as maintain them for longer culture, however, further investigations
301 are required to demonstrate it. For unsorted VECs, early passage (less than passage 3) and
302 less confluent cells should be used for studies of VECs to guarantee a reasonable culture
303 purity (Gould and Butcher, 2010).

304

305 Primary VIC cultures exhibited a fibroblast-like morphology, which is consistent with
306 previous reports (Heaney et al., 2009; Waxman et al., 2012). However, some morphological
307 variation was observed and a different response to enzymatic dissociation was observed with
308 some cells showing stronger adhesion than others. This adherence difference has been
309 associated with differential expression of α -SMA, with near 100% expression in more
310 adhesive cells (Blevins et al., 2006). The heterogeneity in native VICs has been reviewed by
311 Liu et al. (2007), who suggested that there are at least five sub-phenotypes. These sub-
312 populations respond to stimulation and environmental changes differently and have different
313 functions, but currently there is no definitive marker panel to identify each VIC sub-

314 phenotype. In general, VICs do not express VEC markers and are vimentin positive (Liu et
315 al., 2007; Heaney et al., 2009). VICs are further confirmed by the expression of SMemb,
316 SM22 and α -SMA, which are thought to indicate VIC activation (aVICs) (Della Rocca et al.,
317 2000; Rabkin et al., 2001; Wiester and Giachelli, 2003; Disatian et al., 2008; Han et al., 2008;
318 Stephens et al., 2011).

319

320 Recently aVICs have attracted some attention in the valvular research field, as they
321 were found to play a key role in valve remodelling or stromal degeneration (Rabkin et al.,
322 2001; Disatian et al., 2008; Gupta et al., 2009; Han et al., 2008). In diseased valves, there is
323 an increase of α -SMA positive cells close to the endothelium, and these aVICs are presumed
324 to contribute to valve damage and attempts at repair (Disatian et al., 2008; Han et al., 2008).
325 Moreover, in vitro studies suggest that the aVIC population is dynamic, mechanosensitive
326 and responsive to external stimulation. For example, culture conditions, such as cell density,
327 substrate types and movement, can affect the level of α -SMA expression (Engler et al., 2006;
328 Stephens et al., 2011; Xu et al., 2012). These findings may explain the discrepancy of α -SMA
329 expression observed between the current study and a previous report (Taylor et al., 2000).
330 Under culture conditions using three dimensional collagen substrates, the appearance of α -
331 SMA positive cells suggests that a cell population analogous to the disease phenotype is
332 present, but this can be reversed by mechanical treatment of the cells, reverting them to a
333 more normal fibroblast phenotype (Waxman et al., 2012). Functional studies of mitral VICs
334 are required. Cultured mitral VICs are capable of ECM synthesis and are involved in wound
335 repair (Lester et al., 1993; Durbin et al., 2005; Flanagan et al., 2006; Fayet et al., 2007; Gupta
336 et al., 2008). The effects of various conditions on canine valve cell phenotype and function
337 requires further investigation if cultured VECs and VICs are to be used for modelling mitral
338 valve disease.

339 **Conclusion**

340 The characterisation of canine mitral VECs and VICs demonstrated phenotypic
341 heterogeneity under the static culture conditions used in the current study. These findings
342 should contribute to improving the understanding of canine mitral valve cellular biology, and
343 should benefit mitral valve disease research and tissue engineering studies.

344

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348

349 **Conflict of interest statement**

350 None of the authors of this paper has a financial or personal relationship with other
351 people or organizations that could inappropriately influence or bias the content of the paper.

352

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548 heart valve interstitial cells. *Cardiovascular Pathology* 21, 65-73.
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550

551 Table 1. PCR sequences for PCR characterization of valve endothelial cells (VECs) and valve

552 interstitial cells (VICs).

Gene	Primer Sequence (5'-3')	Product size
<i>CD31</i>	AATCCCAAATTCCACGTCAG GAATGGAGCACCACAGGTTT	346 bp
<i>vWF1</i>	CTGGGAGAAGAGAGTCACGG GTGGATGGAGTACACGGCTT	235 bp
<i>vWF2</i>	GGCTGTACCTGGATGAGAGG GACAGGACAGGCTCCTTTTG	228 bp
<i>vWF3</i> (Fulton et al., 2000)	AATATAGGGCCCCGGCTCACTCAA ACATCCCCGGGCTCTTCTCATTC	512 bp
<i>SM22</i>	AAGAACGGCGTGATTCTGAG CGGTAGTGCCCATCATTCTT	269 bp
<i>α-SMA</i>	GGGGATGGGACAAAAGGACA GCCACGTAGCAGAGCTTCTCCTTGA	525 bp
<i>SMemb</i>	AGAAGCGAGCTGGAAAAGTCTG TCTTGCTCTGTCCGATTCTG	252 bp
<i>Vimentin</i>	GGAGCAGCAGAACAAGATCC AGACGTGCCAAAGAAGCATT	282 bp
<i>GAPDH</i>	CATCAACGGGAAGTCCATCT GTGGAAGCAGGGATGATGTT	428 bp

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Table 2. Details of the antibodies used for canine mitral valve endothelial (VEC) and valve interstitial cell (VIC) immunocytochemistry characterisation.

Primary antibody	Species raised	Cat Number & Supplier	Dilution
Anti-CD31	Rabbit polyclonal	Ab28364, Abcam, UK	1:200
Anti-SMembr	Rabbit polyclonal	Ab24761, Abcam, UK	1:400
Anti-vimentin	Mouse monoclonal	V6389, Sigma, USA	1:1600
Anti- α -SMA	Mouse monoclonal	A2547, Sigma, USA	1:400
Secondary antibody			
Anti-rabbit IgG (H+L) Alexa Fluor568	Goat	A11011, Invitrogen, UK	1:500
Anti-mouse IgG (H+L) Alexa Fluor488	Goat	A10667, Invitrogen, UK	1:500

559
560

561 **Figures Legends**

562

563 **Figure 1.** Canine mitral valve cell morphologies.

564 Upper Panel; primary isolated canine mitral valve endothelial cell (VEC) morphology. A. At
565 the early stage, single colony clusters randomly appeared in the cultures. B. Once the cells
566 reached confluence, the majority exhibited a cobblestone morphology and were growth-
567 contact inhibited. Scale bar = 100 μm .

568 Lower Panel; primary cultured valve interstitial cell (VIC) morphology before confluence (C)
569 and at confluence (D). Cells in VIC cultures exhibited fibroblast-like morphology, and tended
570 to arrange in a 'hill and valley' pattern in confluent cultures. Scale bar = 200 μm .

571 **Figure 2.** Canine mitral valve endothelial cells (VECs) and valve interstitial cells (VICs)
572 characterised by RT-PCR. Upper panel: VECs; the endothelial cell marker (CD31) and the
573 activated VIC markers (α -SMA, SMemb and SM22) were detected in VECs. Vimentin and
574 GAPDH were used as internal controls. Lower panel: VICs; cells were negative for the VEC
575 marker CD31, but positive for α -SMA, SMemb, SM22 and vimentin. cDNA template derived
576 from a VEC culture was used to amplify CD31 transcripts in the same experiment as a
577 positive control (data not shown). Vimentin and GAPDH were used as internal controls.

578 **Figure 3.** Canine mitral valve endothelial cells (VECs) characterised by
579 immunohistochemistry and DiI-Ac-LDL. Upper panel; DiI-Ac-LDL uptake by VECs in
580 culture (A) and cell distribution illustrated by bright field microscopy (B). Scale bar = 100
581 μm . Lower panel; characterization of canine mitral VEC culture by immunofluorescence.
582 Cell expressed CD31 (red in C), vimentin (green in G) and SMemb (red in K), but were
583 negative for α -SMA (O). DAPI (blue) stained cell nuclei. DAPI channel (A, E, I and M),
584 target protein channel (B, F, J and N) and merged images (C, G, K and O) are shown
585 respectively for each marker. The same primary cultures cell lines served as negative controls
586 (primary antibodies were omitted; D, H and L). Cells from a VIC culture showing expression
587 of α -SMA served as a positive control (P). Scale bar = 50 μm .

588 **Figure 4.** Endothelial properties of canine mitral valve endothelial cells (VECs) under
589 different culture conditions. EBM cultures (left panel) and AF-12 cultures of VECs (right
590 panel) showing uptake of DiI-Ac-LDL (upper panel) and expression of CD31 (lower panel).
591 There is a greater intensity of staining for EBM cultures. DAPI (blue) stained cell nuclei.
592 Scale bar = 100 μm

593

594 **Figure 5.** Canine mitral canine mitral valve endothelial cells (VECs) under different culture
595 conditions, characterised by semi-quantitative RT-PCR (arbitrary units compared to the
596 reference gene GAPDH). Cells in EBM demonstrated higher expression of CD31 and
597 decreased expression of α -SMA and SMemb compared to VECs and VICs cultured in AF-12.
598

599 **Figure 6.** Canine mitral valve interstitial cells (VICs) characterised by
600 immunohistochemistry and DiI-Ac-LDL. Upper panel; lack of DiI-Ac-LDL uptake by VICs
601 (A) and cell distribution illustrated by bright field microscopy (B). Scale bar = 200 μm .
602 Lower panel; characterization of canine mitral VIC culture by immunofluorescence. Cells
603 expressed vimentin (green in G), SMemb (red in K) and α -SMA (green in O), but were
604 negative for CD31 (C). DAPI (blue) stained cell nuclei. DAPI channel (A, E, I and M), target
605 protein channel (B, F, J and N) and merged images (C, G, K and O) are shown respectively
606 for each marker. The same cell lines served as negative controls (primary antibodies were

607 omitted; H, L and P). Cells from a VEC culture showing expression of α -SMA served as a
608 positive control (D). Scale bar = 50 μ m.
609

Figure1
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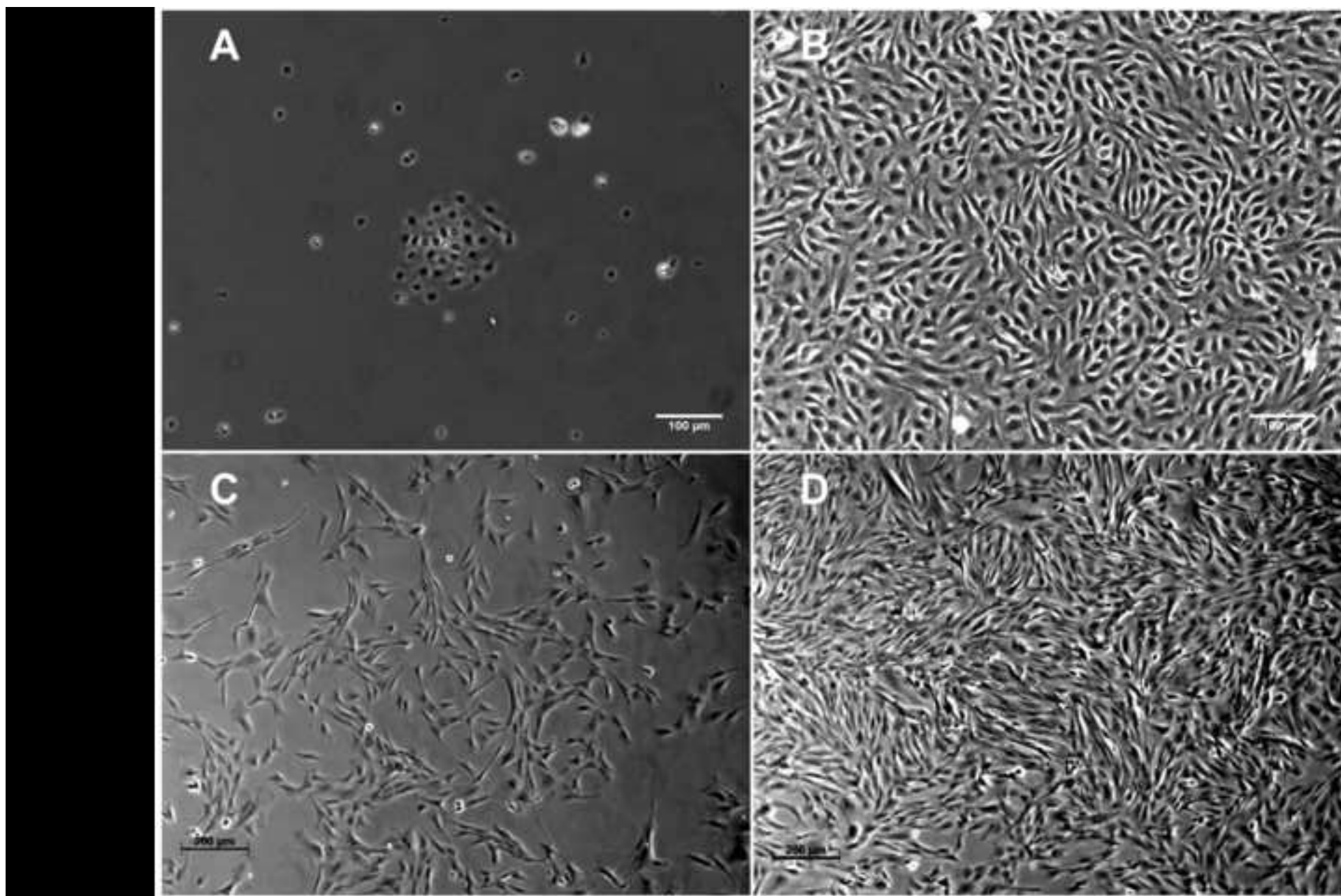


Figure2

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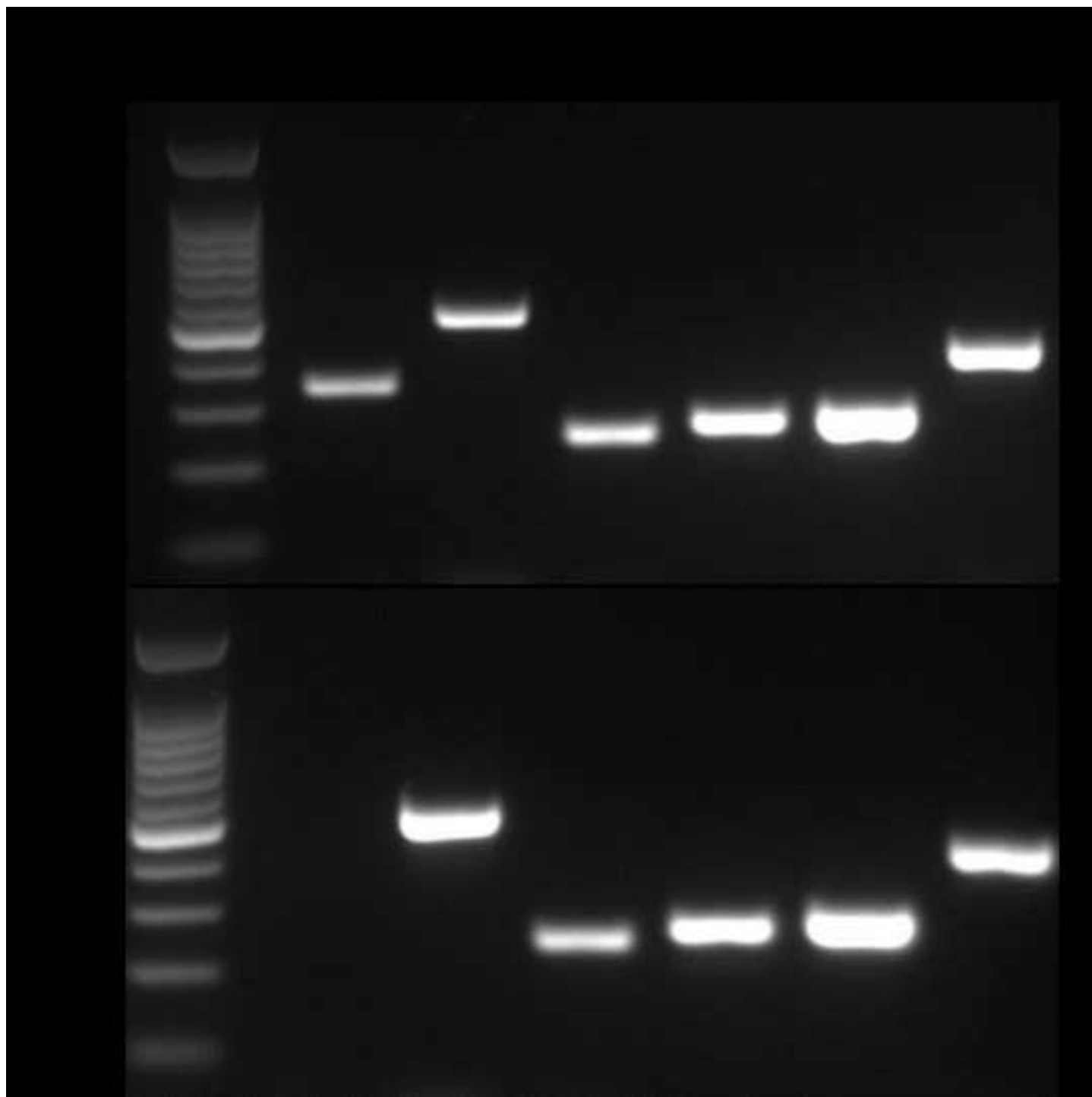


Figure3

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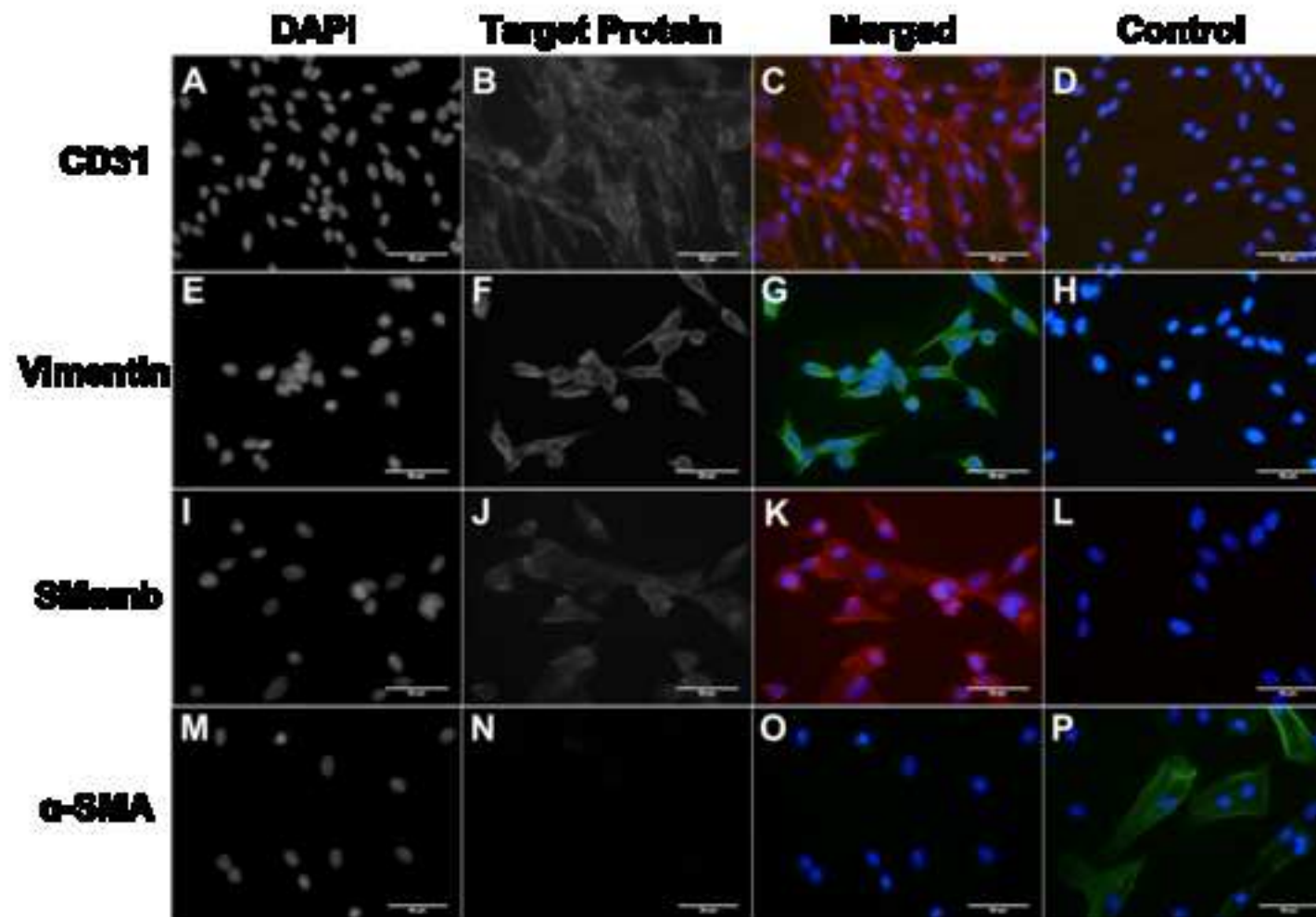
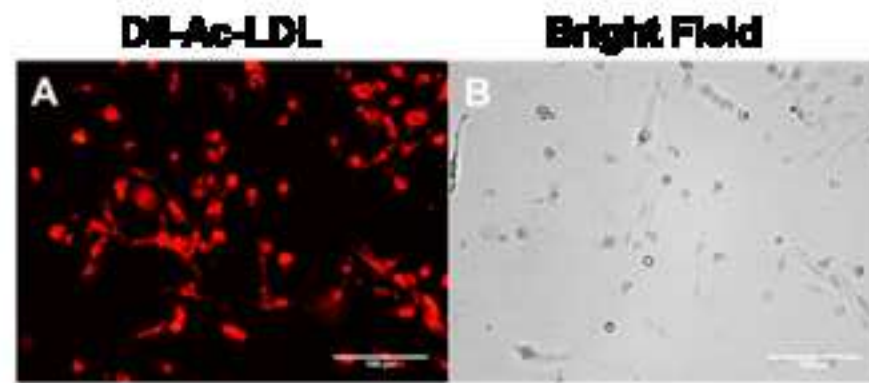


Figure4

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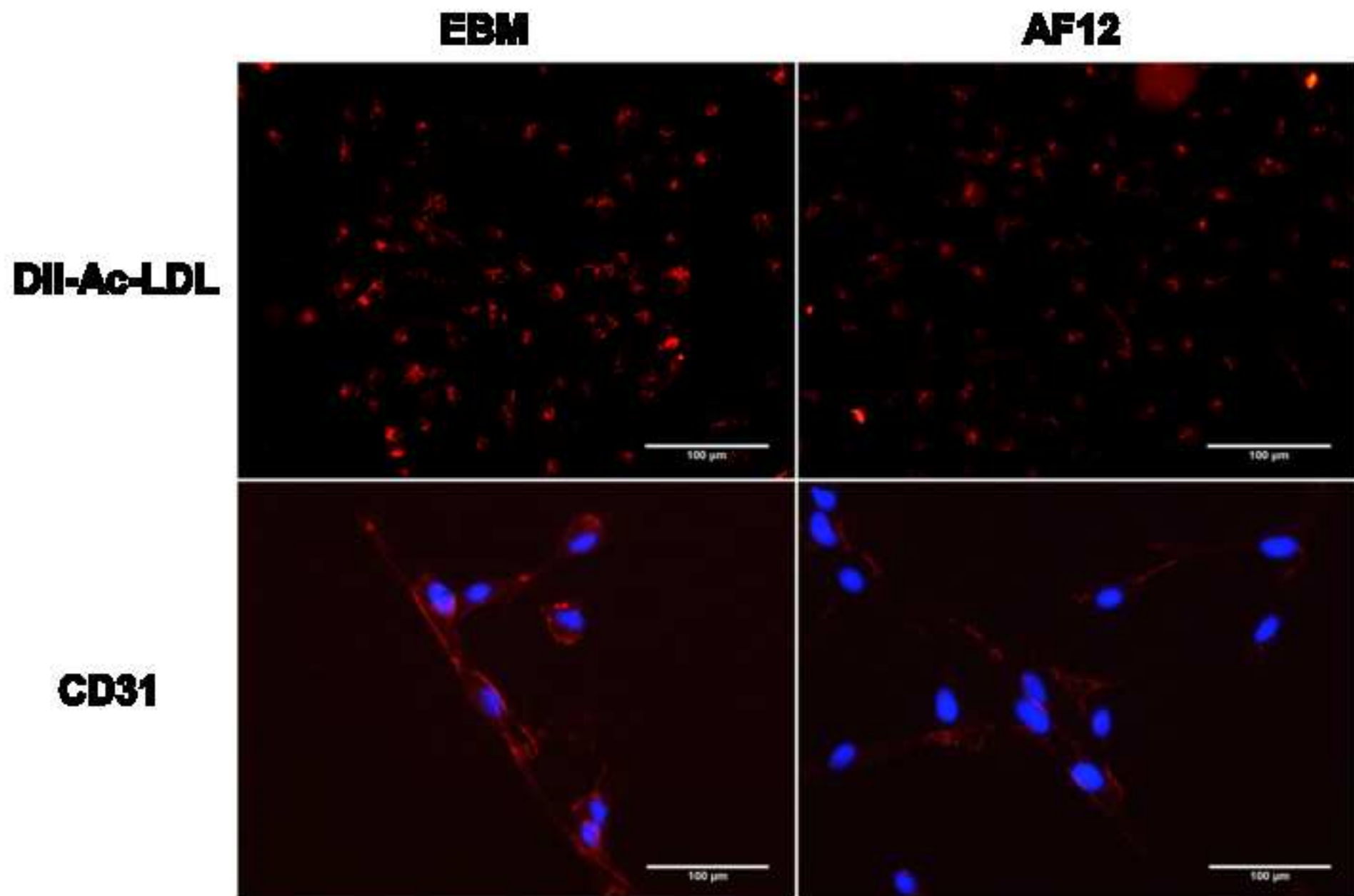


Figure5
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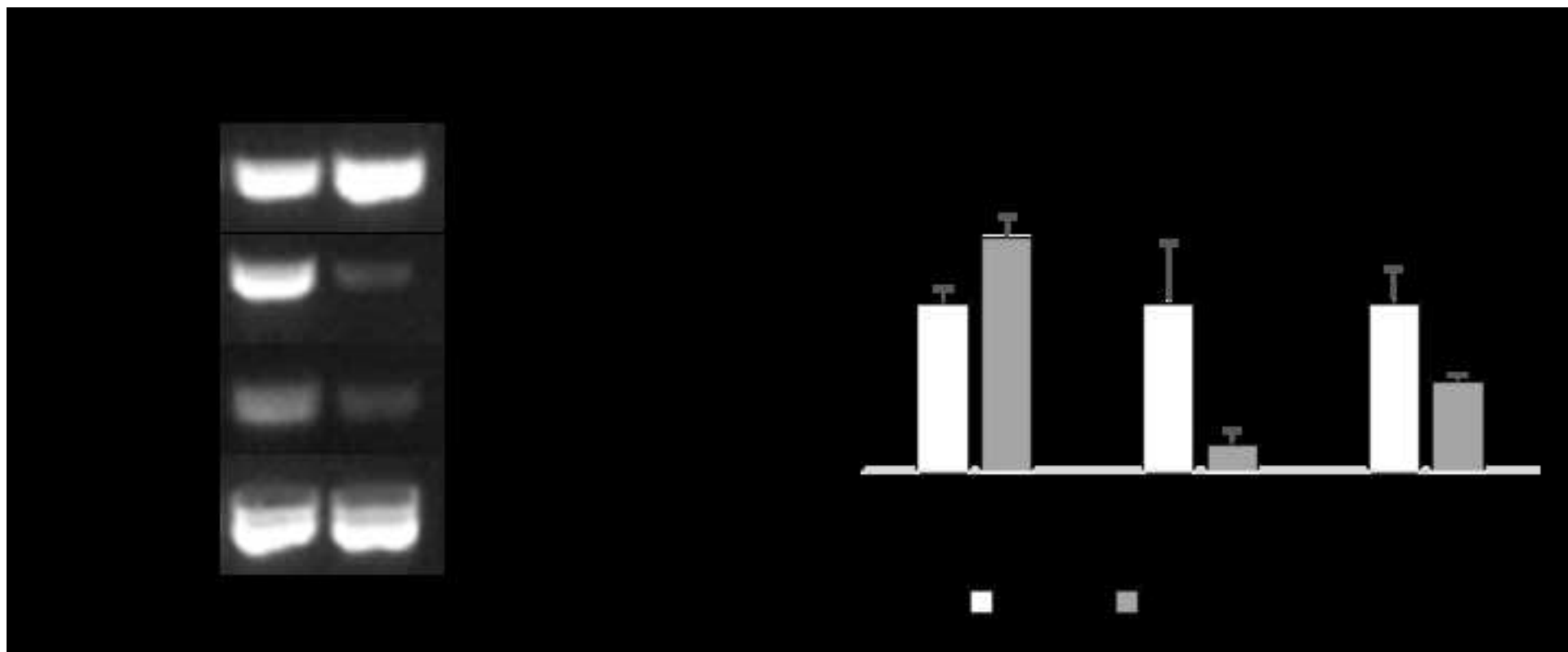


Figure6

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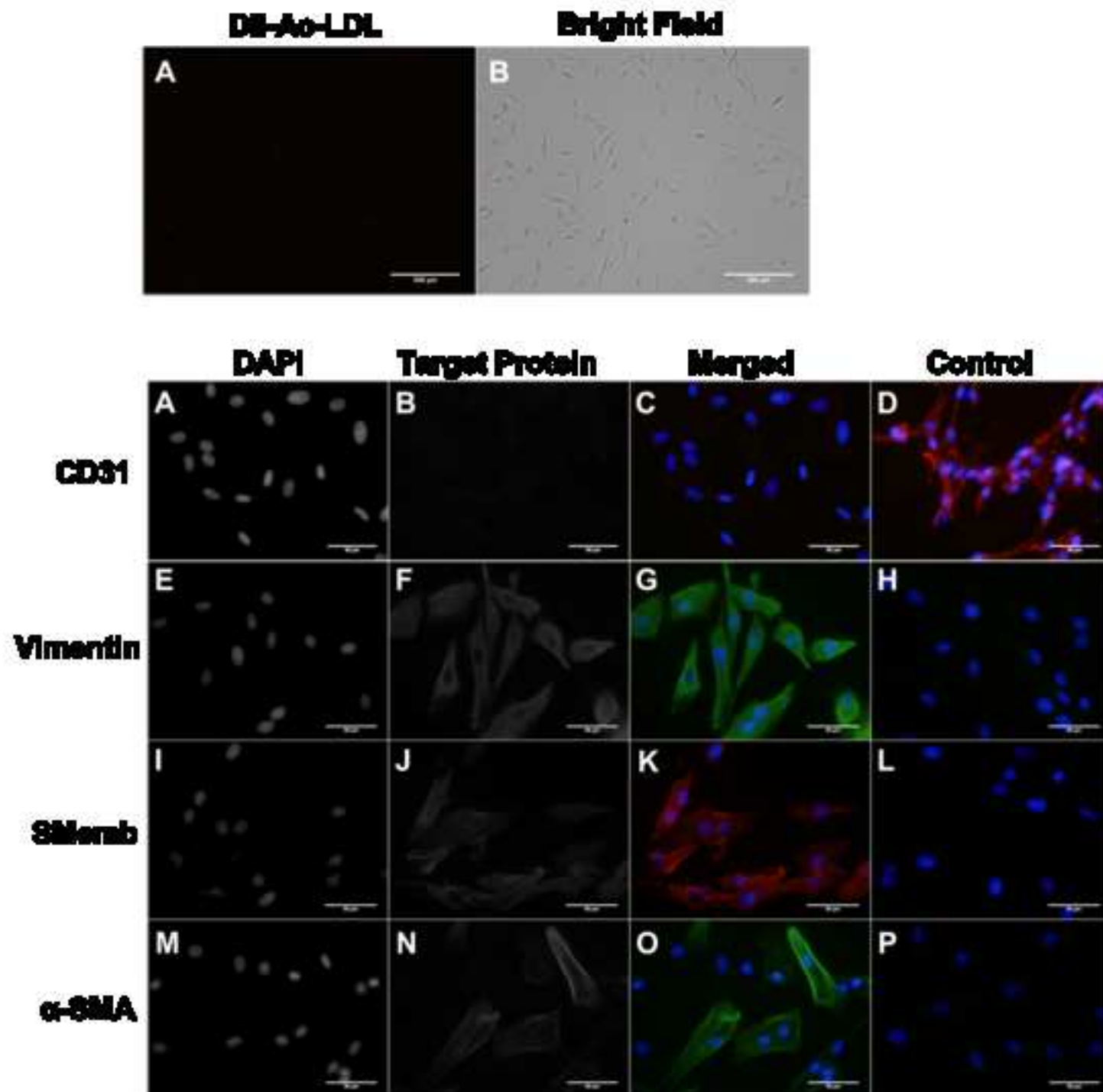


Figure 4
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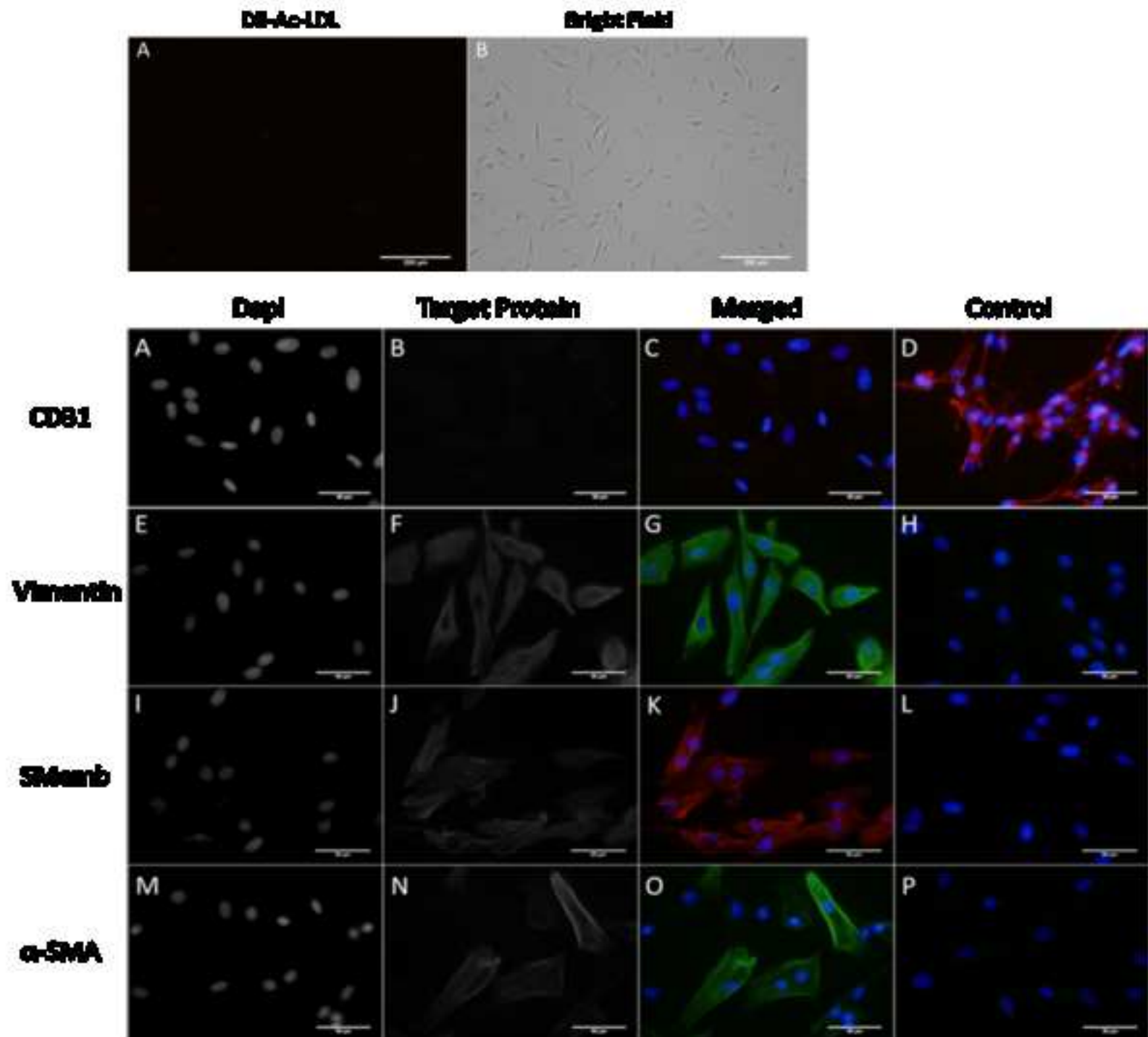
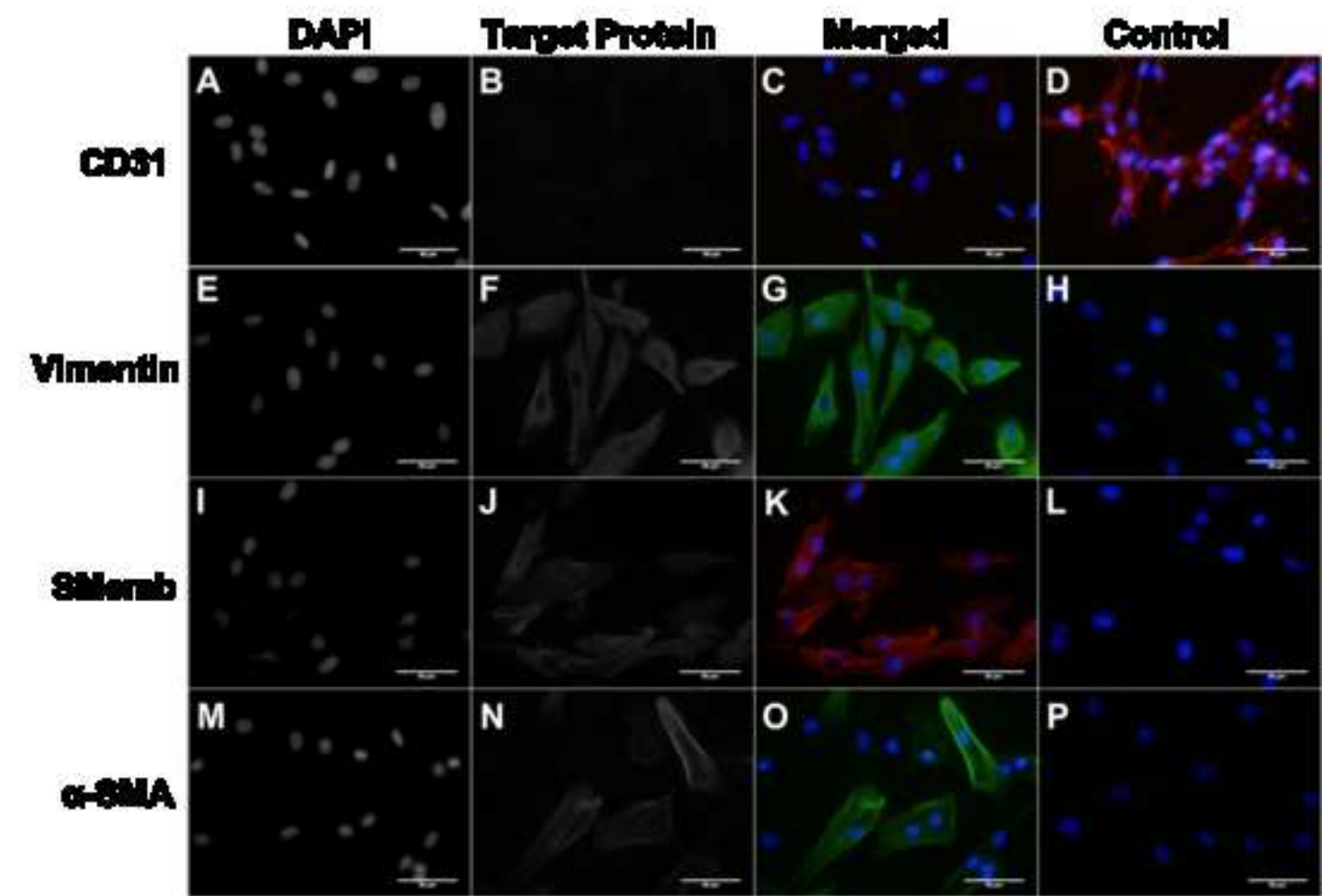
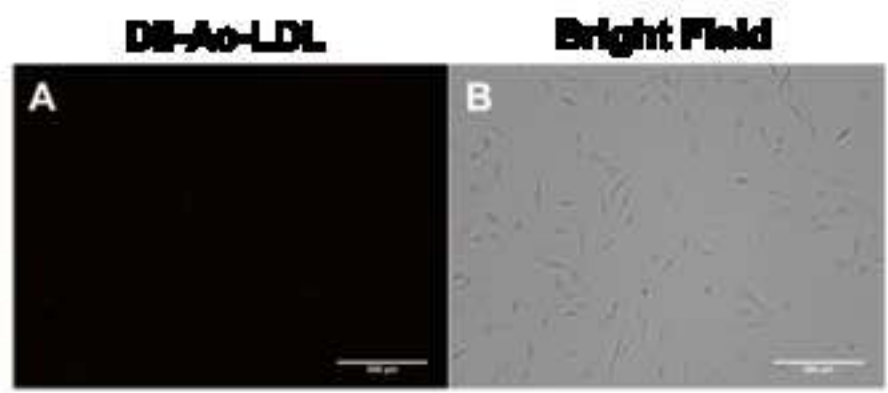


Figure 6
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Highlights:

- Successful culturing and characterising of valve interstitial and endothelial cells from the same subject.
- First reported culturing of valve interstitial cells (VICs) that did not favour a migratory phenotype.
- First reported culturing of canine valve endothelial cells (VECs).
- Assessment of the value of multiple cell markers in cell phenotyping.
- Identification of CD31 expression and acetylated low density lipoprotein (Dil-Ac-LDL) uptake as the prime markers for differentiating VECs from VICs.
- Identification of phenotypic plasticity under culture conditions and optimal passage for attaining the purest VEC population.

Highlights:

1. Successful culture and characterisation of valve interstitial and endothelial cells
2. Identification of CD31 expression and Dil-Ac-LDL uptake as the prime markers for differentiating VECs from VICs.
3. A specific endothelial culture medium for primary VEC cultures enhanced the endothelial properties of the cells

We would like to thank the reviewers for their comments. There were instances where the line numbers quoted did not match those of the current version of the manuscript, but we think we have managed to respond to each comment in turn.

Reviewer 1

Point 1: “The authors are not clear on whether they were able to culture a pure VEC population that then subsequently differentiated (e.g. via EndMT) or whether the population was heterogeneous (VEC+VIC) to start with. Their α SMA data suggests the latter, but if they combined with IHC of normal canine MV, it might support that canine VEC possess α SMA expression in vivo.”

Response: Indeed, sub-endothelial VIC contamination in primary VECs isolated by enzymatic digestion has been recognised as a common technical issue (Cheung et al., 2008; Hoerstrup et al., 1998). However, we cannot completely rule out the possibility of cell differentiation in culture. Firstly, the α -SMA expression in VECs was mainly identified at gene level and it was almost absent at protein level. It is difficult to tell whether the α -SMA gene expression was from differentiating VECs or contaminating cells or from both. Secondly, which is probably more robust evidence, is the extensive expression of activated mesenchymal marker SMemb expression in the VEC cultures. This marker has been found highly expressed in embryonic VECs during development or in activated VICs during valve disease processes, while in healthy adult heart valves, the expression has been described as absent/minimally expressed (Aikawa et al., 2006). Therefore, we propose that the canine mitral VECs we cultured in vitro may possess some embryonic differentiation potentials and we have addressed the above points in the discussion of the revised manuscript.

With regards to native valve α -SMA expression, we did look at it in normal canine MV by immunofluorescence (data not shown); the expression was very limited and mainly restricted to atrialis, and this finding is consistent with our own and others previous reports (Han et al., 2008; Disatian et al., 2008).

Point 2: “Their method is largely similar to that of Gould et al (JoVE 2012), which raises the question of whether ECM coating is essential for VEC adhesion. It would be good to know, as that can be a real problem out of the gate for others.”

Response: From our experience, the answer is no. We once accidentally cultured our VECs on polypropylene flasks without gelatin coating; the cells still adhered and proliferated, however, with a different morphology and the efficiency of adherence was not investigated (data not shown). Indeed, it has been reported that additional ECM coating to plastic culture flasks can enhance endothelial cell adherence, proliferation and affect endothelial cell morphologies (Relou et al., 1998; Schelling et al., 1988; Xia et al., 2011). Both gelatin and collagen are commonly used for valve endothelial culture in previous reports (Wylie-Sears et al., 2011; Gould and Butcher, 2010). For these reasons, we would recommend matrix coating for endothelial culture.

Point 3: “Also, it isn't clear from my reading of the manuscript whether the VEC cultured in this study ended up being suitable for follow-on experiments on VEC behavior. Are enough cells available at passage 3 without compromising homogeneity?”

Response: The answer is yes. Primary VECs P0 yields to $0.66 \times 10^6 - 3.5 \times 10^6$ depending on dog size and valve leaflet collected (anterior/posterior or both), and on average 1.5×10^6 approximately. This can further yield to approximate 1.0×10^7 at P1, and around 1×10^8 at P2. This number should be sufficient to do routine experiments such as immunocytochemistry (usually 1×10^4 cells per sample)

and RT-PCR (usually $0.5-1 \times 10^6$ cells for RNA extraction). In terms of cell purity, according to our findings, VECs cultured in AF-12 medium (without additional purification or endothelial medium replacement) showed at least 50%-60% typical endothelial cobblestone morphology within 3 passages. We didn't do endothelial phenotypic quantification on immunocytochemistry, but we presumed the actual number would be higher than morphological observation, as CD31 or DiI-Ac-LDL positive cells were heterogeneous in morphology (not limited to cobblestone shape only).

Point 4: “The authors suggest EBM culture can improve EC phenotype homogeneity at a sacrifice of proliferation, but does this approach create enough cells for an experimental assay?”

Response: Yes. When used EBM for primary P0 VECs culture, there was cell apoptosis and endothelial colony disappear during the early stages, but the cells started to proliferate rapidly in later culture periods. For those VECs experiencing EBM switch at P1 or P2, they did not show dramatically decreased proliferation compared to AF-12 culture. To avoid confusion the P0 EBM finding has been removed from the manuscript.

Point 5: “Do the authors recommend using EBM during whatever treatment would be performed, or switching to AF-12 Media?”

Response: Once starting culture the VECs in EBM we would continue with EBM rather than switching to AF-12. For experiments specifically investigating VEC behaviour and function, we would recommend to use EBM rather than AF-12, as the former is more optimal in maintaining VEC phenotype.

Point 6: “As a minor but important comment, having standard deviation bars on Figure 6 would be helpful to assess the expected variation in isolation quality and/or subsequent culture performance of the phenotypes of these cells.”

Authors' Response: We've added standard error bars on Figure 5 (original Figure 6) in the revised manuscript (n=3).

Reviewer 2

Point 1: “Not sure about numbering of subsections in M and M and in Results - what is editorial policy?”

Response: We've removed the numbering of subsections in the revised manuscript.

Point 2: “Line 88 - presumably valves showed no gross evidence of MMVD?”

Response: We've addressed this point in the revised manuscript; please see Line 84-85.

Point 3: “Line 102 - a bit more pre-amble on the media experiments would be useful, just to put them into context. Typo – passage (P1).”

Response: We've addressed this point in the revised manuscript; please see Line 109-121.

Point 4: “Line 110 - might be useful to add a third sub-heading on cell passage (see later comment).”

Response: We have addressed this point in Line 104-107 in the revised manuscript.

Point 5: “Lines 117, 135 and 149 - cells from which passage were used on these experiments - was this consistent?”

Response: We have addressed this point in Line 112, 200 and 222 in the revised manuscript.

Point 6: “Lines 137 and 149 - details of slides - chamber slides mentioned.”

Response: We have addressed this point in Line 147-148 in the revised manuscript.

Point 7: “Line 164 - was detected by RT-PCR in VEC culture?”

Response: We have addressed this point in Line 201-202 in the revised manuscript.

Point 8: “Line 177 - was detected by RT-PCR for....?”

Response: We have addressed this point in Line 223-224 in the revised manuscript.

Point 9: “Move section on effect of culture medium (lines 182-196) to before section on VIC morphology and characterization to be consistent with Methods.”

Response: We have moved this section as requested; see Line 214-220.

Point 10: “Line 194 - typo - EBM.Both...”

Response: We have addressed this point in the revised manuscript.

Point 11: “Line 201 - Waxmann reference not in references.”

Response: We have addressed this point Line 534 in the revised manuscript.

Point 12: “Line 239 - ..although it did not preserve...?”

Response: We have addressed this point in the revised manuscript.

Point 13: “Line 256 - Liu et al (2007),...”

Response: We have addressed this point in the revised manuscript.

Point 14: “Line 265 - Han 2013 - a or b?”

Response: We cannot locate this comment in the original manuscript. Effectively has been resolved

Point 15: “Several references in reference list are incorrect or incomplete - e.g. lines 381 and 386, Paranya reference has a space in it. There is variability in how journals are cited.”

Response: We have addressed these inconsistencies and errors in the reference section of the revised manuscript.

Point 16: “Images - quality of text on images is poor.”

Response: We have improved the text quality in the revised manuscript.

Point 17: “Figure 3 - top panel association of positive staining with cells shown in the bright field image is not as convincing as the other images.”

Response: We have checked the image and can confirm that the bright field image is correctly matched with the fluorescence image. However, the focusing of the bright field image was not optimal. We have tried to enhance that by adjusting brightness and contrast of the bright field image in the revised manuscript.

Point 18: “Figures 3 and 4 - for clarity legends should explain column titles - what do the target protein images show, what is the merged image.”

Response: We have addressed this point in the revised manuscript. Please see legends of Figure 3 and 6 (Figure 4 in original manuscript).

Comments E Bloome

1. The current Figure 4 is a copy of Figure 6, I think Figure 4 should be what was Figure 5 in the previous submission.

Figure identities seem correct; will up-load fresh set of figure images.

2. Cannot find Bischoff (2001) reference in the text.

No Bischoff 2001 in the text or reference list.

Comments A. Philby

1. Please check author's initials, e.g. "D.A. Argyle".

Changed to D.J.

2. Some reformatting of the Corresponding author's details will be required.

Not sure what is required.

3. Most non-standard abbreviations should be defined when first used in the Abstract and again in the Main text. Abbreviations that do not need to be defined include PCR, ELISA, DNA and RNA. In the Abstract, please change "reverse transcription polymerase chain reaction (RT-PCR)" to "reverse transcription PCR".

MS checked and corrected

4. Groups of references cited in the text should be arranged chronologically according to the idiosyncratic journal style, e.g. "Aupperle et al., 2009; Aupperle et al., 2010; Black et al., 2005; Hadian et al., 2007; Han et al., 2010; Han et al., 2013b" should be cited as "Black et al., 2005; Hadian et al., 2007; Aupperle et al., 2009, 2010; Han et al., 2010, 2013b".

Corrected; EndNote seems to think TVJ format is different!

5. Most units should be separated from values by a space, although percentages should be written without a space. Similarly, when writing temperatures, the value should be separated from the symbol for degrees Celsius by a space, e.g. "37°C" should be written as "37 °C" using the Word symbol for degrees Celsius rather than a superscripted letter "o".

MS checked and corrected

6. Some reformatting of tables and figure legends will be required.

Not sure what is required?

7. Please check spacing and punctuation throughout the manuscript.

Done.

8. Please reformat the Highlights with bullet points and define abbreviations. Each Highlight should be no more than 125 characters including spaces.

Done.



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Please find attached “Canine Mitral Valve Interstitial and Endothelial Cell Culture and Characterisation” for consideration for publication in TVJ.

The paper describes the culturing of valve interstitial and endothelial cells from canine mitral valves. This is important as there is increasing interest in using culture systems to investigate mechanisms of pathogenesis in canine mitral valve disease, not least because of the difficulties in sourcing native valves.

No previous study has reported on canine VECs, which are much harder to grow than VICs, and previous reports of VICs have used techniques that only cultured migratory phenotypes.

This study provides the basic information necessary for accurate VIC and VEC culture and the techniques required for accurate phenotyping.

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