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Title: Canine mesenchymal stem cells are neurotrophic and angiogenic: An in vitro assessment of their paracrine activity

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Abstract: Mesenchymal stem cells (MSCs) have been used in cell replacement therapies for connective tissue damage, but also can stimulate wound healing through paracrine activity. In order to further understand the potential use of MSCs to treat dogs with neurological disorders, this study examined the paracrine action of adipose-derived canine MSCs on neuronal and endothelial cell models.

The culture-expanded MSCs exhibited a MSC phenotype according to plastic adherence, cell morphology, CD profiling and differentiation potential along mesenchymal lineages. Treating the SH-SY5Y neuronal cell line with serum-free MSC culture-conditioned medium (MSC CM) significantly increased SH-SY5Y cell proliferation ( $P < 0.01$ ), neurite outgrowth ( $P = 0.0055$ ) and immunopositivity for the neuronal marker  $\beta$ III-tubulin ( $P = 0.0002$ ). Treatment of the EA.hy926 endothelial cell line with MSC CM significantly increased the rate of wound closure in endothelial cell scratch wound assays ( $P = 0.0409$ ), which was associated with significantly increased endothelial cell proliferation ( $P < 0.05$ ) and migration ( $P = 0.0001$ ). Furthermore, canine MSC CM induced endothelial tubule formation in EA.hy926 cells in a soluble basement membrane matrix. Hence, this study has demonstrated that adipose-derived canine MSC CM stimulated neuronal and endothelial cells probably through the paracrine activity of MSC-secreted factors. This supports the use of canine MSC transplants or their secreted products in the clinical treatment of dogs with neurological disorders and provides some insight into possible mechanisms of action.

1 **16-00402**

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3 **Canine mesenchymal stem cells are neurotrophic and angiogenic: An in vitro**  
4 **assessment of their paracrine activity**

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20

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31 medium (MSC CM) significantly increased SH-SY5Y cell proliferation ( $P < 0.01$ ), neurite  
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42

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44 Nerve growth; Secretome

## 45 **Introduction**

46           Mesenchymal stem cells (MSCs) were originally identified as stem/progenitor cells  
47 that differentiated to form connective tissues, e.g. as bone-forming osteoblasts and cartilage-  
48 forming chondrocytes, and the bone marrow stroma supporting haemopoiesis (Friedenstein et  
49 al., 1974; Haynsworth et al., 1992; Pittenger et al., 1999). This led to MSCs being considered  
50 important candidates for cell replacement therapies for damaged connective tissues or to  
51 support bone marrow haemopoietic stem cell transplantation (Young et al., 1998; Gupta et  
52 al., 2012; De Bari et al., 2013; Giannotti et al., 2013; Wu et al., 2013). However, it has  
53 become clear that MSCs play wider roles in tissue regeneration and wound healing as they  
54 secrete growth factors and cytokines that can stimulate endogenous cells present at wound  
55 sites (Chen et al., 2008; Park et al., 2009). This secretory function can augment tissue repair  
56 through trophic, anti-inflammatory or immunodulatory activity for various conditions,  
57 including heart disease (Gallini et al., 2015), liver damage (Berrardis et al., 2015; Owen and  
58 Newsome, 2015), skin wounds (Otero-Venas and Falanga, 2016) and central nervous system  
59 (CNS) damage (Teixeira et al., 2013). Furthermore, MSCs have been isolated from tissue  
60 sources other than bone marrow, including adipose tissues, which is an attractive source, due  
61 to its relative ease of removal (Sousa et al., 2014). The breadth of MSC activity and their  
62 ready availability has broadened the attractiveness of MSC-based therapies in regenerative  
63 medicines (Correa and Caplan, 2011).

64

65           The use of MSCs to promote wound healing after spinal cord injury (SCI) is a  
66 particular case in point. We and others have reported that human MSC secrete factors that  
67 promote neurite outgrowth (Neuhuber et al., 2005; Crigler L. et al., 2006; Wright et al., 2007,  
68 Nakano et al., 2010; Wright et al., 2010 and 2014) and endothelial cell proliferation and  
69 migration in vitro (Walter et al., 2015) and that MSC transplantation was associated with

70 decreased inflammation, increased neural survival, increased axonal regeneration and  
71 improved functional recovery after SCI in vivo (Ankeny et al., 2004; Neuhuber et al., 2005;  
72 Himes et al., 2006; Nakajima et al., 2012). This research supports MSC transplantation as a  
73 cell therapy for various conditions, including CNS damage, with a number of human trials  
74 currently underway or in development. In dogs, MSC-based cell therapies for CNS damage,  
75 particularly SCI, also have been explored. In an experimental model of SCI, MSC transplants  
76 were associated with increased neural survival and repair, increased axonal conductance  
77 velocity, reduced inflammation and increased functional recovery (Lim et al., 2007; Ryu et  
78 al., 2012). MSC transplants similarly were associated with increased function in dogs  
79 suffering from natural SCI following intervertebral disc herniation (Chung et al., 2013; Penha  
80 et al., 2014; Sarmiento et al., 2014; Besalti et al., 2015; Kim et al., 2016). Examining the  
81 efficacy of cell transplantation for naturally occurring CNS damage in dogs in this manner is  
82 an important step in the translation of experimental studies to human and animal cell  
83 therapies (Jeffery et al., 2006, 2011; Hoffman and Dow, 2016). However, for new cell  
84 therapies in dogs to be applied optimally and for this translational knowledge to human  
85 treatment to be complete, it is essential that researchers establish the mode of activity of  
86 canine MSCs. Therefore, in this study we examined whether canine MSCs isolated from  
87 adipose tissue exert a neurotrophic and angiogenic activity through their secretome.

88

## 89 **Materials and methods**

### 90 *MSC isolation and growth*

91 Institutional approval was provided for this study (University of Chester Faculty of  
92 Science and Engineering Research Ethics Committee: 060/16/CW/BS, 18 May 2016).

93 Following owner and veterinary surgeon consent for research, canine adipose tissue-derived  
94 MSCs were isolated and cultured from surgically extracted inguinal fat pads of dogs

95 undergoing MSC transplantations for the treatment of joint pathology. MSCs were isolated  
96 by collagenase digestion of the tissue for 2 h at 37 °C (0.2% Type A Collagenase,  
97 Worthington Biochemical), selected through their preferential adhesion to tissue culture  
98 plastic, as reported previously (Vieira et al., 2010; Kohli et al., 2015), and cultured in  
99 Dulbecco's modified Eagle medium/F12 (DMEM/F12) supplemented with 10% fetal bovine  
100 serum and 1% penicillin/ streptomycin (Life Technologies) in a humidified atmosphere of 5%  
101 CO<sub>2</sub> with 95% air at 37 °C. Cultures were routinely passaged at 70-80% confluence using  
102 0.25% trypsin-EDTA (Life Technologies). All experimental procedures were performed  
103 using MSC cultures at passages 3-5.

104

#### 105 *MSC characterisation*

106 MSC phenotype was examined according to the International Society for Cell  
107 Therapy (ISCT) criteria (Dominici et al., 2006), which are as follows: (1) cell adherence to  
108 tissue culture plastics; (2) adipogenic, osteogenic and chondrogenic differentiation potential;  
109 and (3) an immunoprofile for CD markers that includes immunonegativity for CD34 and  
110 CD45 and immunopositivity for CD44 and CD90, as assessed by flow cytometry (Appendix:  
111 Supplementary material).

112

#### 113 *MSC culture-conditioned medium (MSC CM)*

114 MSCs were seeded at a density of  $1.5 \times 10^6$  cells in T75 tissue culture flasks in  
115 standard culture medium for 24 h to permit cell adhesion, then the medium was discarded and  
116 the cultures washed in PBS prior to feeding with 15 mL of DMEM/F12 medium  
117 supplemented with 1% ITS, 1% non-essential amino acids and 1% penicillin/streptomycin,  
118 but without any serum present. Cultures were then incubated at 37 °C in a humidified  
119 atmosphere of 5% CO<sub>2</sub> for 3 days, when the MSC culture-conditioned medium (MSC CM)

120 was collected, filtered with a sterile filter (0.20 µm, Minisart), aliquoted and stored at -80 °C.  
121 Control medium (i.e. serum free DMEM/F12 with the same supplements, but with no cells  
122 present) was similarly incubated in T75 culture flasks for 3 days, harvested, filtered and  
123 stored. Under serum-free conditions, there was no evident loss of cell viability and greater  
124 than 98% of cells in all cultures were viable at day 3 (by trypan blue exclusion).

125

#### 126 *The effects of MSC CM on SH-SY5Y neuronal cells*

127 The human neuroblastoma cell line SH-SY5Y was used to assess neurotrophic  
128 activity of canine MSC CM, as has been performed previously with human MSC CM  
129 (Wright et al., 2010; Pires et al., 2014; Appendix: Supplementary material). This was due to a  
130 lack of available canine neuronal models, but also because we and other researchers have  
131 found similar responses to MSC secretomes in cell assays using MSCs and responder cells of  
132 the same and different species, i.e. humans, chickens and rodents (Neuhuber et al., 2005;  
133 Wright et al., 2010; Pires et al., 2014) suggesting conservation of paracrine activity.

134

#### 135 *The effects of MSC CM on EA.hy926 endothelial cells*

136 The human Ea.hy926 endothelial cell line was used as a model to examine any  
137 angiogenic activity of canine MSC CM, due to a lack of available canine endothelial cells and  
138 also because these cells have been used previously to test human MSC CM (Walter et al.,  
139 2015). EA.hy926 cell assays were performed to measure endothelial cell proliferation,  
140 endothelial cell migration and endothelial tubule formation (Appendix: Supplementary  
141 material).

142

#### 143 *Statistical analysis*

144 At least three independent experiments were performed for all analysis, i.e. using  
145 MSCs and MSC CM derived from at least three different dogs vs. at least three separate  
146 control media with 3-5 replicates for each experiment. Data were examined for normal  
147 distributions and then analysed by two-way ANOVA, independent samples Student's *t* tests  
148 or Mann Whitney *U* tests, according to whether data was distributed normally or not. All data  
149 has been presented as mean  $\pm$  standard error. *P* values  $<0.05$  was considered statistically  
150 significant. Statistical analysis was performed using GraphPad Prism7 (GraphPad Software).

151

## 152 **Results**

### 153 *Characterisation of canine MSCs*

154 At passage 3-5, cultures of canine MSCs were plastic-adherent, displayed a stromal  
155 appearance and had the capacity to undergo differentiation towards adipogenic, osteogenic  
156 and chondrogenic lineages (Fig. 1A). For adipogenic and osteogenic differentiation, there  
157 was no evident loss of cell viability, however for chondrogenic pellet cultures some cell death  
158 was apparent, but nonetheless there was also clear evidence of extracellular metachromatic  
159 staining with toluidine blue, indicative of glycosaminoglycan deposition and chondrogenic  
160 differentiation. CD immunoprofiling demonstrated that canine MSCs were largely  
161 immunonegative for CD34 ( $0.3 \pm 0.6\%$ ) and CD45 ( $0.1 \pm 0.3\%$ ) and immunopositive for  
162 CD44 ( $87.7 \pm 9.3\%$ ) and CD90 ( $94.1 \pm 9.7\%$ ; Fig. 1B). These results demonstrate that the  
163 canine cells that had been culture-expanded from adipose tissue met the necessary criteria of  
164 the ISCT (Dominici et al., 2006) to be considered MSCs.

165

166 *Canine MSC secreted factors promote SH-SY5Y cell proliferation and neuronal*  
167 *differentiation*



168 Treating SH-SY5Y cells with canine MSC CM promoted their proliferation and  
169 neuronal differentiation, as determined by viable cell numbers, neurite outgrowth and  
170 immunoreactivity for  $\beta$ III-tubulin (Fig. 2). There was an increase in the number of SH-SY5Y  
171 cells present, and in their extent of neurite outgrowth (Fig. 2A), which were immunopositive  
172 for  $\beta$ III-tubulin (Fig. 2B) in MSC CM vs. control media. SH-SY5Y neurite length/cell ( $P =$   
173 0.0055) and the proportions of SH-SY5Y cells that were  $\beta$ III-tubulin immunopositive ( $P =$   
174 0.0002) were significantly greater in MSC CM than in control medium (Fig. 2C). The  
175 increase in SH-SY5Y cell number in MSC CM vs. control medium was also significant and  
176 confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays ( $P$   
177  $< 0.01$ ) (Fig. 2C).

178

179 *Canine MSC secreted factors promote EA.hy926 endothelial cell migration, proliferation and*  
180 *tubule formation*

181 The effects of canine MSC CM on EA.hy926 cells were examined by scratch assay,  
182 time-lapse live cell image analysis, MTT assay and tubule formation with Matrigel. In  
183 EA.hy926 endothelial scratch wound assays, wound closure was markedly increased in MSC  
184 CM compared to control media (Fig. 3A). Using live cell image analysis, we found that  
185 EA.hy926 cells closed the scratch wounds significantly more quickly in MSC CM vs. control  
186 media ( $P = 0.0409$ ) by a combination of increased cell migration and cell proliferation (Fig.  
187 3B). The trophic effects of MSC CM on EA.hy926 endothelial cell proliferation were  
188 confirmed by MTT assays for the numbers of viable cells, which was significantly increased  
189 after 2 days culture in MSC CM compared with control media ( $P = 0.0019$ ) (Fig. 3B). We  
190 then tested whether MSC CM could promote blood vessel formation, as delineated by  
191 endothelial tubule formation in Matrigel assays and digitised image analysis (Fig. 4). As  
192 shown (Fig. 4A), culturing EA.hy926 endothelial cells on Matrigel did not give rise to any

193 marked tubule formation unless MSC CM was present, in which case both the total length of  
194 the endothelial tubules that formed ( $P = 0.0082$ ) and the number of endothelial tubule branch  
195 points present ( $P = 0.0307$ ) were significantly increased in MSC CM compared to control  
196 media (Fig. 4B).

197

## 198 **Discussion**

199 MSCs have been investigated for their wound healing activity, with potential  
200 applications for a wide variety for conditions including CNS damage. MSCs exert paracrine  
201 effects on cells involved in CNS repair, including neuronal cells, endothelial cells and  
202 immune cells (Neuhuber et al., 2005; Crigler et al., 2006; Wright et al., 2007; Wright et al.,  
203 2010; Nakajima et al., 2012; Wright et al., 2014; Walter et al., 2015). Furthermore, MSC  
204 transplantation in animal models of SCI was associated with enhanced neural regeneration,  
205 modulation of immune responses and improved functional outcomes (Ankeny et al., 2004;  
206 Neuhuber et al., 2005; Himes et al., 2006; Nakajima et al., 2012). Hence, MSC transplants  
207 are considered an attractive treatment option to help overcome CNS damage, particularly SCI  
208 (Wright et al., 2011).

209

210 Dogs, like humans, can suffer from SCI and paralysis, either through accidental  
211 trauma or following herniation of the intervertebral disc. Furthermore, other researchers have  
212 recently demonstrated that transplantation of autologous culture-expanded olfactory  
213 ensheathing cells (OECs) might be of benefit to dogs with SCI (Granger et al., 2012). This  
214 not only is of use to veterinary medicine, but also helps inform the development of clinical  
215 human SCI studies (Jeffery et al., 2006, 2011). Therefore, with a view to developing MSC  
216 transplants for dogs with CNS damage and to further understand the potential mechanisms of

217 action of MSC following transplantation, we investigated whether canine MSCs exerted  
218 paracrine wound healing activities similar to human and rodent MSCs.

219

220 After initially characterizing MSCs from the inguinal fat pads of dogs according to  
221 the ISCT criteria (Dominici et al., 2006), we used established in vitro assays with responder  
222 cell lines to test their paracrine activity. We report that MSC CM was trophic for SH-SY5Y  
223 neuronal cells, and stimulated neurite outgrowth and neuronal differentiation. MSC CM also  
224 was trophic for EA.hy926 endothelial cells, enhanced their migratory behaviour and  
225 stimulated endothelial tubule formation, all of which indicate angiogenic activity, although  
226 clearly further in vivo testing would help confirm this (Auerbach et al., 2003). These data  
227 support the application of MSC transplantation in dogs with SCI, as well as other CNS  
228 injuries, as enhanced neuronal survival, axonal growth and the appropriate regulation of  
229 angiogenesis are thought to represent important aspects of repair processes (Oudega et al.,  
230 2012; Quertainmont et al., 2012).

231

232 The mechanisms of action of the canine MSC secretome warrant further investigation.  
233 In other species, MSCs are known to secrete a plethora of growth factors, cytokines and  
234 extracellular matrix (ECM) components (Park et al., 2009; Walter et al., 2010, 2015). These  
235 include a variety of soluble neurotrophic factors, including nerve growth factor, brain-derived  
236 neurotrophic factor and glial-derived neurotrophic factor, as well as pleiotrophic factors that  
237 also can stimulate nerve outgrowth, such as fibroblast growth factors 1 and 2 (FGF1 and  
238 FGF2) and stromal derived factor 1 (Crigler et al., 2006; Wilkins et al., 2009; Nakano et al.,  
239 2010; Hseih et al., 2013; Kingham et al., 2014; Lin et al., 2014). Similarly, MSCs are known  
240 to secrete soluble angiogenic factors, including FGFs, hepatocyte growth factor and the  
241 highly potent vascular endothelial growth factor (Rehman et al., 2004; Cai et al., 2007). In

242 addition, at least some of the ECM components that have been identified in human MSC CM,  
243 particularly fibronectin and laminin, form a stimulatory substratum for nerve growth and also  
244 endothelial cells (Kapur and Katz, 2013; Walter et al., 2010, 2015). The identification of  
245 growth factors and ECM in the secretome of canine MSCs is somewhat hampered by a lack  
246 of canine specific antibodies, but it is highly likely that many of the factors present in the  
247 MSC secretomes of other species are similarly secreted by canine MSCs. Additionally, they  
248 could play active roles in the neurostimulatory and angiogenic effects seen in SH-SY5Y  
249 neuronal cells and EA.hy926 endothelial cells. A similar profile of growth factors and ECM  
250 components has been reported in MSC secretomes with cells cultured from a variety of tissue  
251 sources (Walter et al., 2010, 2015; Kapur and Katz, 2013; Bronckaers et al., 2014). Also,  
252 there is a high degree of conservation across species for at least some growth factors and  
253 cytokines that have been examined at the molecular level (Wen et al., 1993; Scheerlinck,  
254 1999). Nonetheless, one recent study found species-specific differences in the secretome and  
255 paracrine activity of canine and equine MSCs, particularly when cultured in serum free  
256 conditions (Clarke et al., 2016). Therefore, although it might be considered more likely that  
257 the canine MSC secretome would have even greater trophic effects on canine responder cells,  
258 a potential caveat to our study is that we tested canine MSC secretomes on human responder  
259 cell lines only. Further studies of canine neuronal and endothelial cells, as they become  
260 available, and the identification of MSC secreted factors in MSC CM, are required.

261

262 In veterinary medicine, the uptake of MSC-based therapies has been relatively low,  
263 although canine MSC transplants were used recently in the treatment of some natural injury  
264 and disease conditions, including tendon repair (Case et al., 2013), osteoarthritis (Black et al.,  
265 2007, 2008; Vilar et al., 2013, 2014; Cuevo et al., 2014), inflammatory bowel disease (Perez-  
266 Merino et al., 2015a,b) and non-infectious CNS inflammation (Zeira et al., 2015). There also

267 have been a number of studies of MSC transplants in canine SCI. In the clinical studies of  
268 SCI to date, MSC transplants have been associated with some benefits including improved  
269 gait and neurological function (Penha et al., 2014; Sarmiento et al., 2014; Besalti et al., 2015;  
270 Kim et al., 2016). However, the mechanisms of action for these reported benefits remain  
271 poorly understood. In rodent models of SCI, MSC transplants have been suggested to exert a  
272 wide variety of effects that might enhance spinal cord repair and function (Ide et al., 2010;  
273 Vaquero and Zurita 2011; Teixeira et al., 2013), including their differentiation to form  
274 replacement neural cells (Deng et al., 2014), albeit contentiously (Wright et al., 2011);  
275 immunomodulatory/anti-inflammatory activity and increased neuronal survival (Ankeny et  
276 al., 2004; Crigler et al., 2006; Nakajima et al., 2012), directing axons that bridge across the  
277 SCI lesion site (Ankeny et al., 2004); secretion of neurotrophic factors and angiogenic factors  
278 to enhance axonal regeneration (Ankeny et al., 2004; Neuhuber et al., 2005; Lu P et al., 2005;  
279 Nakajima et al., 2012); and angiogenic responses (Zeng et al., 2011; Kingham et al., 2014).  
280 Here, to our knowledge, our study has provided the first evidence that canine MSCs promote  
281 nerve growth and endothelial cell proliferation, migration and tubule formation, probably  
282 through the secretion of neurotrophic and angiogenic factors. These findings support the  
283 hypothesis that MSC transplants can promote increased neuronal function in dogs with CNS  
284 damage, including SCI, due to their paracrine activity on nerves and blood vessels in the  
285 vicinity of the wound site. Further, this study supports the concept that cell transplants in  
286 dogs with SCI, whether MSCs or OECS, provide an important natural model for the  
287 development of human cell-based therapies.

288

## 289 **Conclusions**

290 In this study, we have demonstrated for the first time that canine MSCs stimulate  
291 neuronal growth and neurite extension, endothelial cell proliferation, endothelial cell

292 migration and endothelial tubule formation in vitro. This paracrine activity has application in  
293 MSC-mediated therapies to promote tissue repair. Furthermore, the effects of the MSC  
294 secretome on neuronal and endothelial cells present at CNS lesion sites might help explain  
295 how MSC transplantation induces improved anatomical repair and functional outcomes in  
296 dogs with natural SCI.

297

### 298 **Conflict of interest statement**

299 JFI and PM are Directors of the Veterinary Tissue Bank Limited. None of the authors  
300 has any other financial or personal relationships that could inappropriately influence or bias  
301 the content of the paper.

302

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306

### 307 **Appendix: Supplementary material**

308 Supplementary data associated with this article can be found, in the online version, at  
309 doi: ...

310

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625 **Figure legends**

626

627 Fig. 1. Characterisation of canine mesenchymal stem cells (MSCs). (A) Representative  
628 images are shown of plastic adherent, fibroblastic cells under phase contrast microscopy prior  
629 to treatment with inducers of differentiation (top panel) and after inductions to become oil red  
630 O-positive adipocytic cells, alkaline phosphatase-positive osteoblastic cells,, and toluidine  
631 blue-stained cartilaginous extracellular matrix and cells, as indicated (positivity arrowed,  
632 bottom panels). Cell viability in all two-dimensional cultures was > 95%, but there was a loss  
633 of cell viability during the chondrogenic differentiation of MSCs in pellet cultures (visualised  
634 following Live/Dead staining and confocal microscopy; inset, bottom left panel). Scale bars =  
635 20  $\mu$ m. (B) Representative histograms of flow cytometric analysis of canine MSCs following  
636 immunocytochemical staining for CD34, CD44, CD45 and CD90. Immunoreactivity with  
637 irrelevant isotype-matched control antibodies is shown in blue, while immunoreactivity for  
638 each of the CD markers is shown in red.

639

640 Fig. 2. Canine mesenchymal stem cells (MSCs) secrete factors that promote SH-SY5Y  
641 neuronal cell proliferation, neurite outgrowth and neuronal differentiation. (A) Representative  
642 images are shown of SH-SY5Y neuronal cells following culture for 3 days in the presence of  
643 canine MSC conditioned medium (MSC CM) or in control medium. As shown, there was  
644 clear evidence of increased cell numbers and neurite outgrowth (arrowed) in MSC CM  
645 compared with control cultures. Scale bars = 200  $\mu$ m. (B) Representative images of SH-  
646 SY5Y cells after 3 days of culture in the presence of canine MSC CM or in control medium  
647 and following immunocytochemical staining for the neuronal marker,  $\beta$ III-tubulin. As shown,  
648  $\beta$ III-tubulin positive cells were seen in MSC CM to a much greater extent than under control  
649 conditions. (C) The Cell IQ imaging platform was used to quantify SH-SY5Y cell numbers

650 and neurite outgrowth. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)  
651 assays were also performed to assess viable cell numbers, and the proportion of cells  
652 immunopositive for  $\beta$ III-tubulin was scored. There were significant increases in viable  
653 neuronal cell numbers ( $P < 0.01$ ), the length of neurite outgrowth ( $P = 0.0055$ ) and the  
654 proportions of  $\beta$ III-tubulin immunopositive cells ( $P = 0.0002$ ) in canine MSC CM compared  
655 to the control medium. Data has been presented as mean  $\pm$  standard error.  $**P < 0.01$ ,  $*** P$   
656  $< 0.001$ .

657

658 Fig. 3. Canine mesenchymal stem cells (MSCs) secrete factors that promote EA.hy926  
659 endothelial cell proliferation and cell migration. (A) Representative images are shown of  
660 EA.hy926 endothelial cell scratch assays. As shown, there was an increase in the extent of  
661 wound closure in canine MSC conditioned medium (MSC CM) compared with control  
662 medium after 2 days in culture. Scale bars = 200  $\mu$ m. (B) Wound closure, cell division and  
663 cell migration was tracked using the Cell IQ live cell imaging platform, wherein digitised  
664 images were collected every 15 min over 2 days. Three-(4,5-dimethylthiazol-2-yl)-2,5-  
665 diphenyltetrazolium bromide (MTT) assays were also performed to assess viable cell  
666 numbers. Top left panel: The rate of endothelial cell wound closure was significantly greater  
667 ( $P = 0.0409$ ) in the presence of MSC CM (right line) vs. control media (blue line) over a 2-  
668 day time course. Bottom left panel: There was a significant increase in the number of  
669 dividing endothelial cells (per image) ( $P = 0.0127$ ) in the scratch wound assays in the  
670 presence of MSC CM (red line) vs. control medium (blue line). Top right panel: There was a  
671 significant increase in total distance that endothelial cells migrated over a 2-day period in  
672 MSC CM vs. control medium ( $P = 0.0001$ ). Bottom right panel: There were significantly  
673 more viable endothelial cells present after 2 days in culture in canine MSC CM vs. control

674 medium, as determined by MTT assay ( $P = 0.0019$ ). Data has been presented as mean  $\pm$   
675 standard error.  $**P < 0.01$ ,  $****P < 0.0001$ .

676

677 Fig. 4. Canine mesenchymal stem cells (MSCs) secrete factors that promote EA.hy926  
678 endothelial tubule formation. EA.hy926 endothelial cells were seeded at  $2 \times 10^2$  cells/well in  
679 96-well plates coated previously with Matrigel reduced growth factor and treated with canine  
680 MSC conditioned medium (MSC CM) or control medium. (A) Representative images are  
681 shown of the growth pattern of EA.hy926 endothelial cells after 24 h in canine MSC CM vs.  
682 control medium. As shown, the EA.hy926 cells in canine MSC CM formed aggregates and  
683 tubes, which was not evident in control medium. Scale bars = 200  $\mu\text{m}$ . (B) Image analysis  
684 demonstrated that there were significant increases in both the total tubule length ( $P = 0.0082$ )  
685 and total numbers of branch points ( $P = 0.0307$ ) in canine MSC CM compared to control  
686 medium. Data has been shown as mean  $\pm$  standard error.  $*P < 0.05$ ,  $**P < 0.01$ .



Figure 1  
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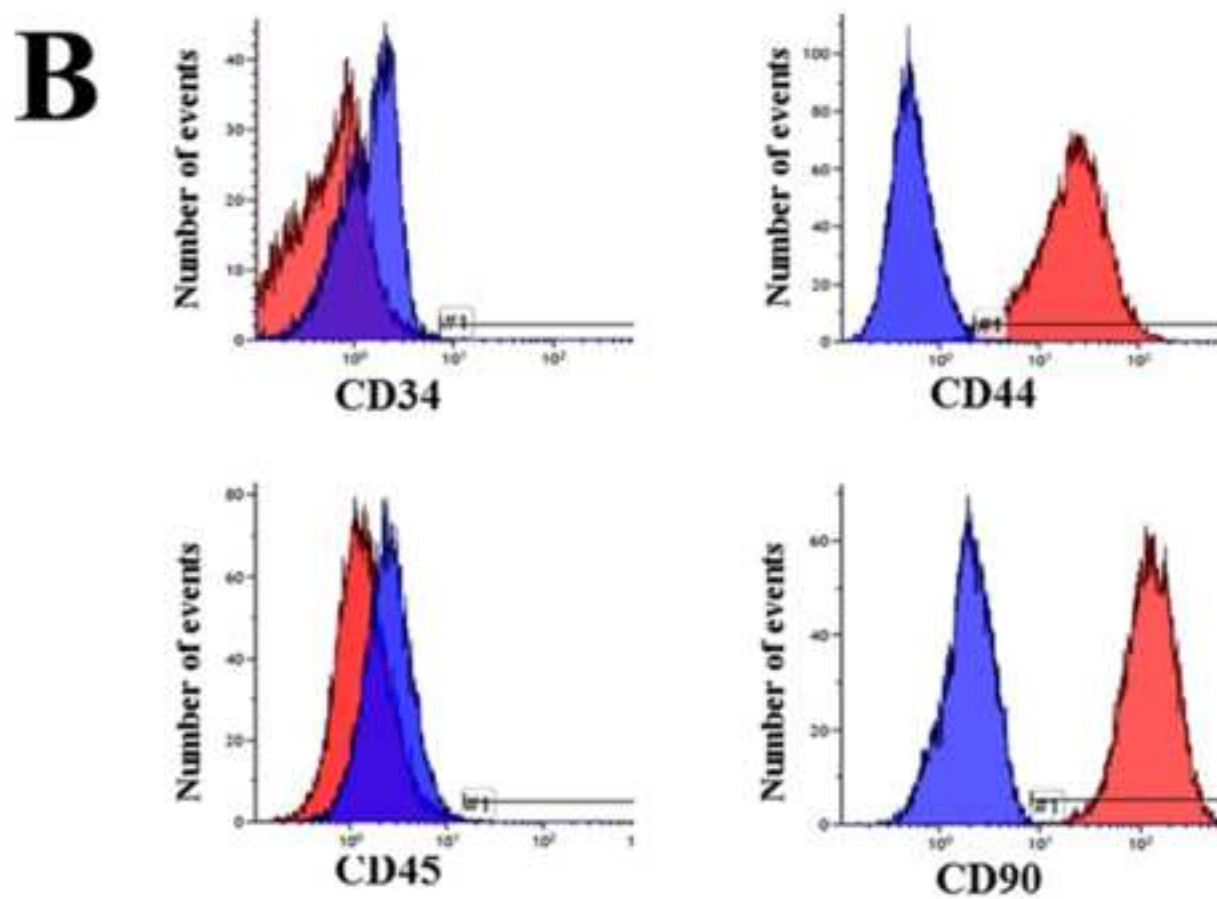
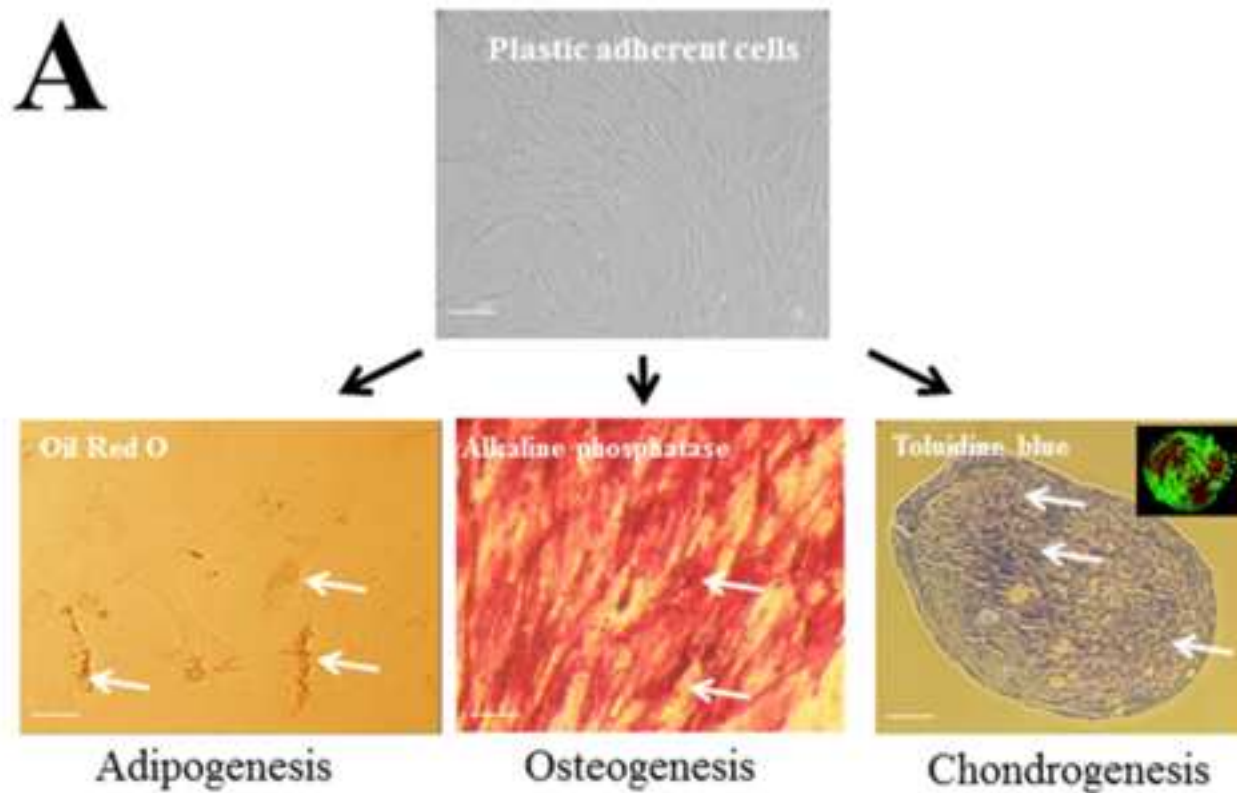
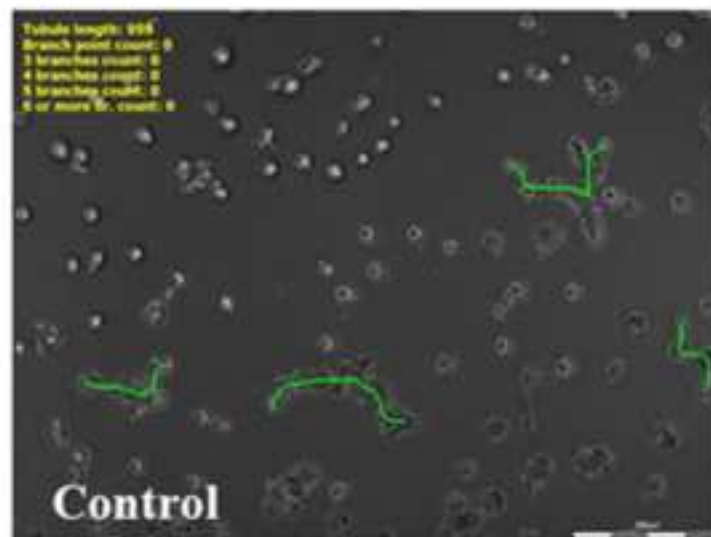
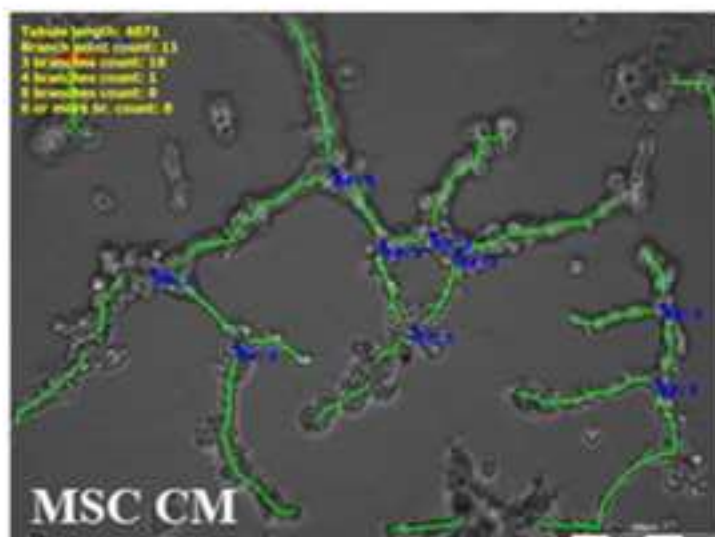


Figure 4  
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# A



# B

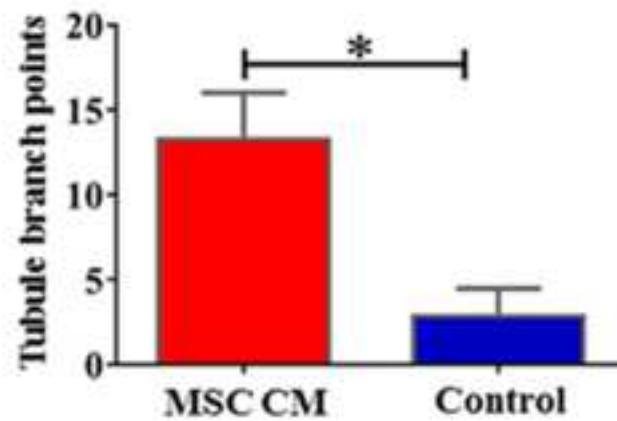
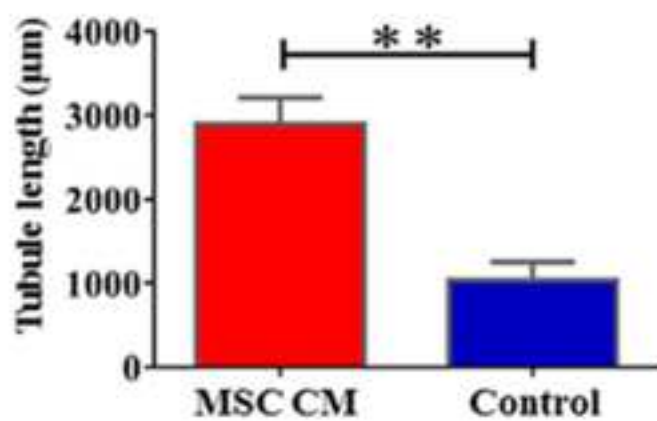


Figure 2  
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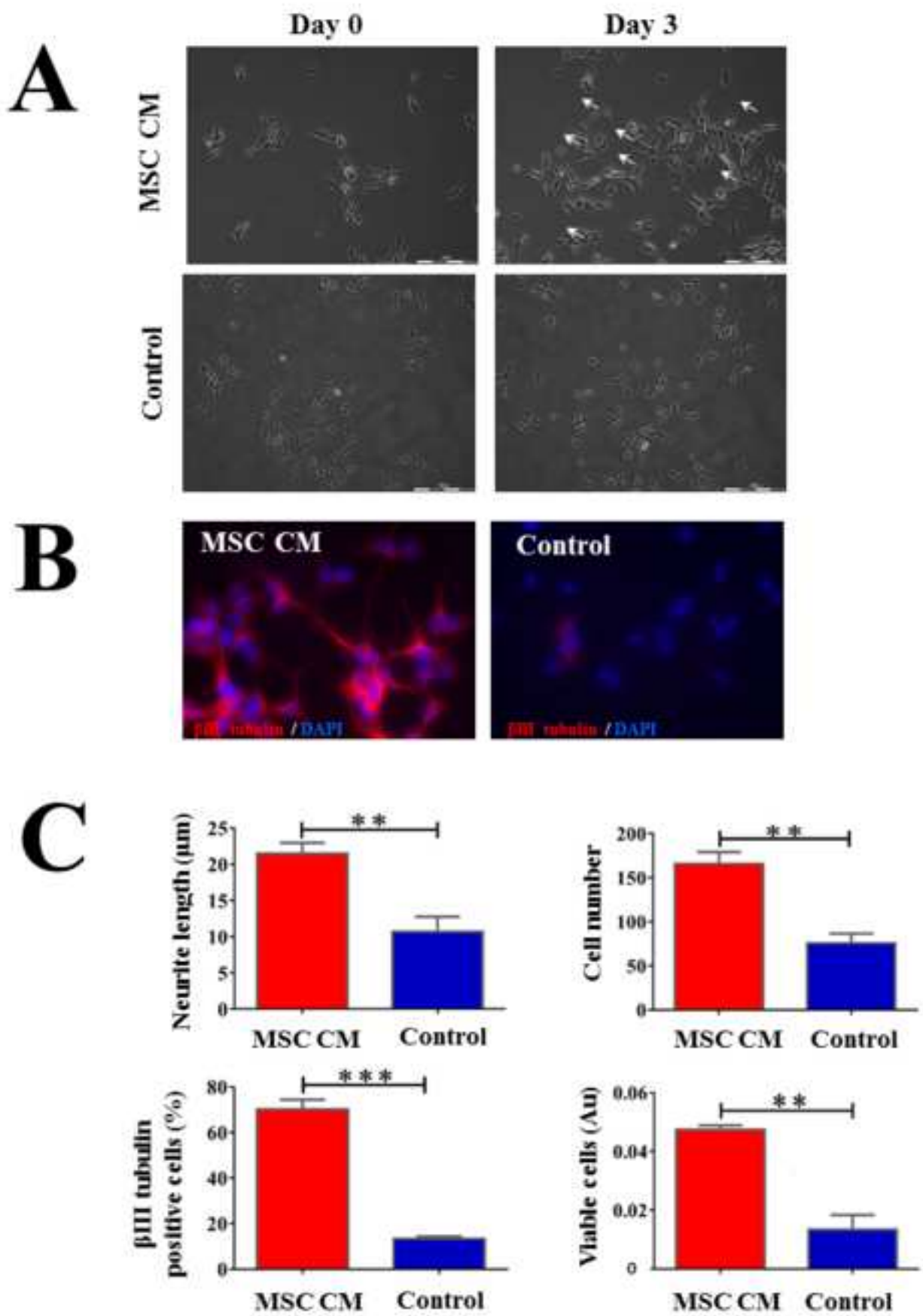
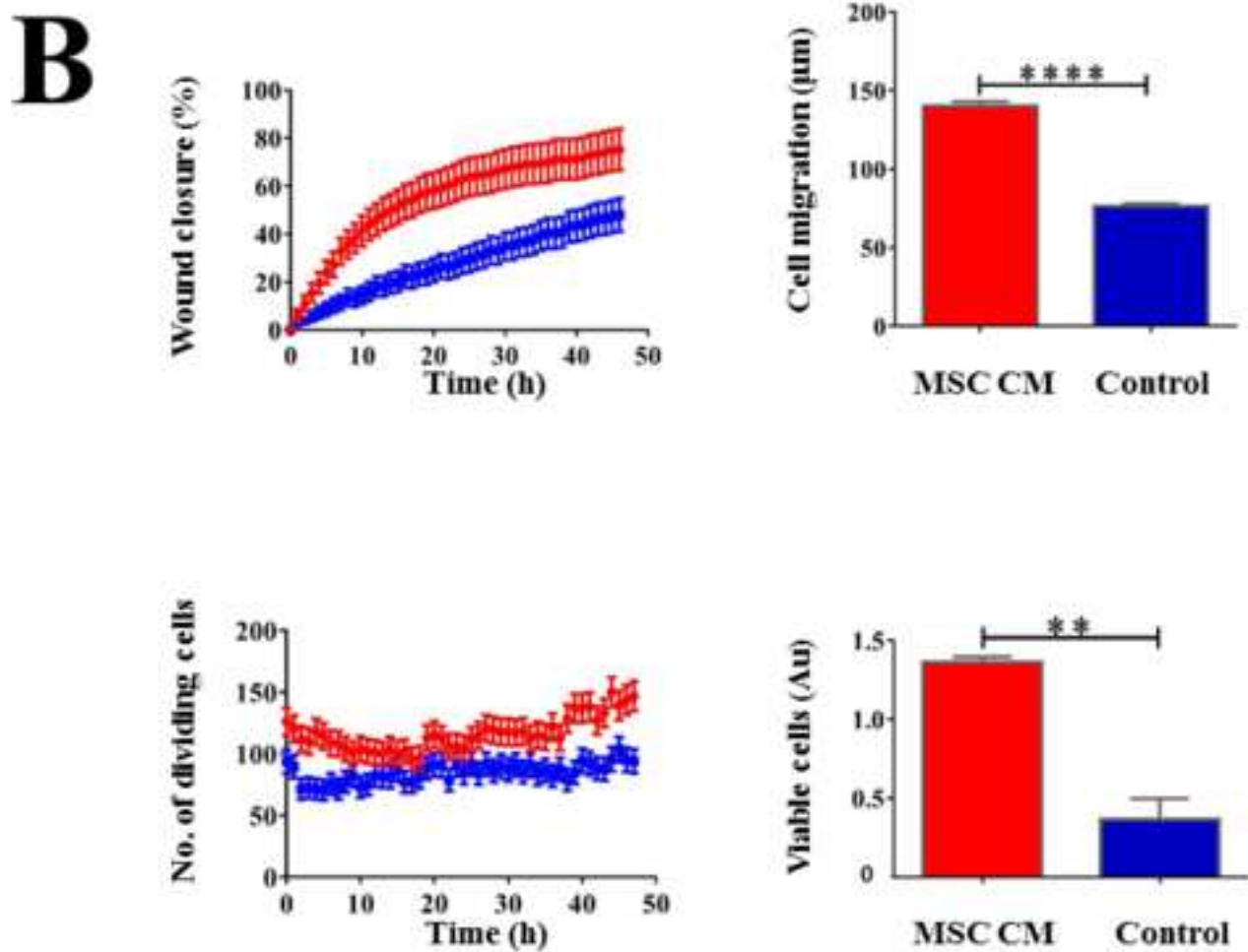
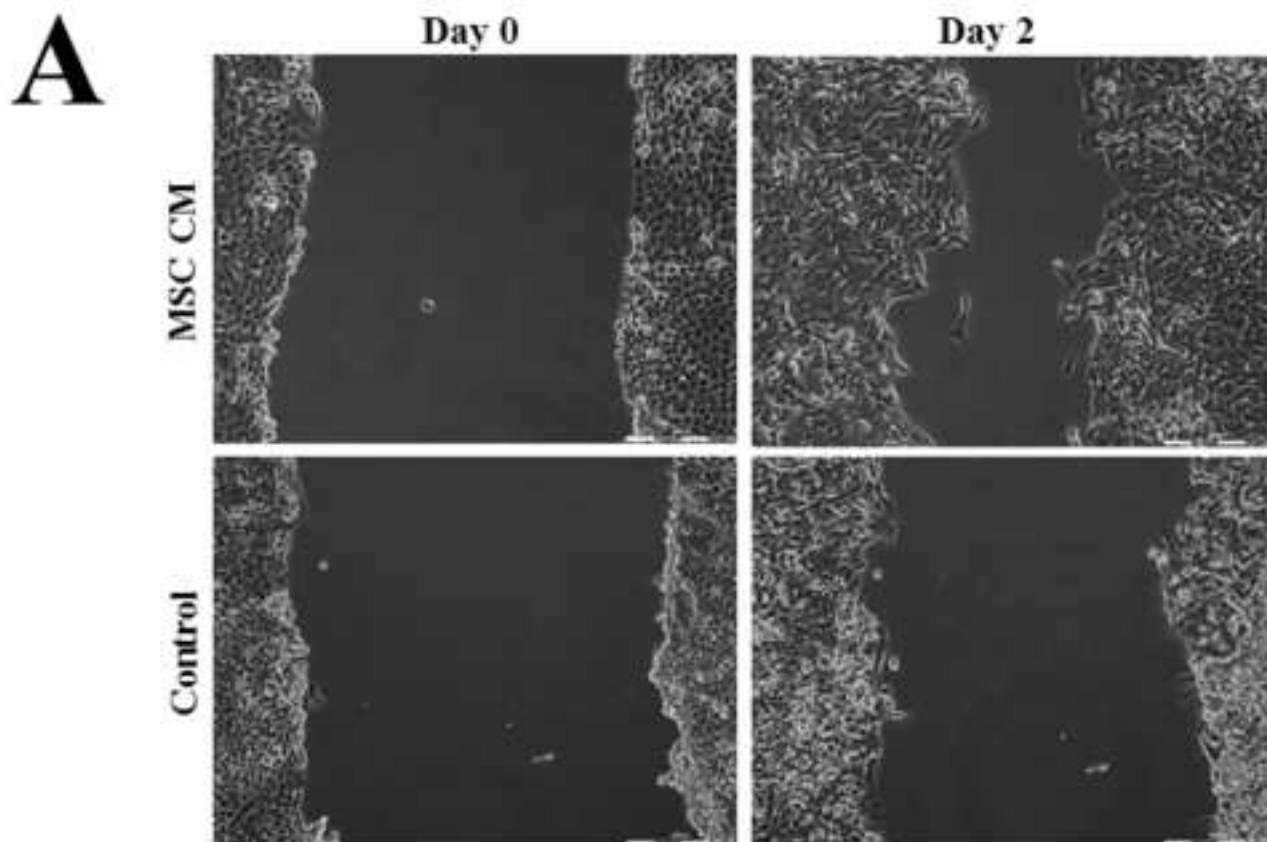


Figure 3  
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## **16-00402**

### **Highlights**

- Mesenchymal stem cells (MSCs) can be isolated and cultured from adipose tissue biopsies from dogs
- Canine MSCs secrete factors to stimulate neuronal outgrowth and endothelial proliferation, migration and tubule formation
- Canine MSCs and secretomes may promote wound repair following transplants in dogs with natural spinal cord injury

### **Revision note**

Thank you for the advice re: including *P* values where we have indicated significance in the manuscript. These values have now been added, with required additions to the abstract, results, and figure legends. Where cell proliferation was discussed in a single phrase, e.g. “there was a significant increased in SH-SY5Y cell proliferation”, which was assessed by numbers of dividing cells and MTT assays, the *P* value was indicated as being  $P < 0.01$ , for simplicity, rather than giving each *P* value for the number of dividing cells and MTT. Where cell division or MTT results are described individually, we have given the appropriate *P* value.

Dear Editors,

Thank you for your further consideration of our manuscript for publication in The Veterinary Journal and for the positive outcome. We are very happy that the reviewers are satisfied with the revised version and grateful to the Scientific Editor, Makoto Bonkobara, for his further editorial input in preparing the manuscript for publication. We have made the required changes suggested and these are incorporated into the two updated files now re-submitted:

1. 16-00402R2 edited MB 180816 R3.docx  
The manuscript text.

2. e-only supplementary materialR3.docx  
The revised Supplementary material

The detailed response to the required editorial changes is listed below, in red text. I hope that the paper is now ready for publication in TVJ.

Best regards,  
Eustace

Ms. No. YTVJL-D-16-00402R2

Canine mesenchymal stem cells are neurotrophic and angiogenic: an in vitro assessment of their paracrine activity for application in spinal cord repair  
The Veterinary Journal

Editorial Comments:

Dear Dr. William Eustace Johnson,

Thank you for resubmitting this manuscript to be considered further for publication in The Veterinary Journal. Your paper has been re-examined by two previous referees and both reviewers are satisfied with your revision. I have now edited the manuscript.

Please proof-read the manuscript 16-00402R2 edited MB 180816 (which should be downloaded from the File Inventory on the Elsevier Electronic System, EES) to ensure that editorial changes have not altered the meaning.

**We have proof read the manuscript and agree to all changes.**

Please modify the following points.

- Please shorten the background part of the abstract.

**We have shortened the background information such that the Abstract is reduced from 246 words to 206 words.**

**Specifically the text below has been removed:**

**“Mesenchymal stem cells (MSCs) differentiate into adipocytes, chondrocytes and**



osteoblasts and have been used to repair connective tissue damage in cell replacement therapies. However, MSC-mediated tissue repair/regeneration is also associated with their secretion of factors that can act in a paracrine fashion to stimulate endogenous cells at wound sites. In order to further understand the potential use of MSCs to treat dogs with neurological disorders, such as spinal cord injury (SCI), this study has examined the paracrine activity of canine MSCs isolated and cultured from inguinal fat pads on neuronal and endothelial cell models.”  
(94 WORDS)

And this has been replaced with the following:

“Mesenchymal stem cells (MSCs) have been used in cell replacement therapies for connective tissue damage, but also can stimulate wound healing through paracrine activity. In order to further understand the potential use of MSCs to treat dogs with neurological disorders, this study has examined the paracrine action of adipose-derived canine MSCs on neuronal and endothelial cell models.”  
(57 WORDS)

- Institutional approval of the experiment: Please state the date of approval along with the reference number (e.g. 060/16/CW/BS, 18 August 2016).

We have now added the date of Institutional approval to the Methods section.

- Main text, results section: Fig. 2, A to C should appear in alphabetical order (A, B, C not A, C, B). Fig. 3 is also the same.

The Results text has been edited to address each figure in alphabetical order. Specifically the following text has been included:

For Fig 2:

“There was an evident increase in the number of SH-SY5Y cells present, and in their extent of neurite outgrowth (Fig. 2A), which were immunopositive for  $\beta$ III-tubulin (Fig. 2B) in MSC CM versus control media. SH-SY5Y neurite length/cell and the proportions of SH-SY5Y cells that were  $\beta$ III-tubulin immunopositive were significantly greater in MSC CM compared to control medium (Fig. 2C). The increase in SH-SY5Y cell number in MSC CM vs. control medium was also significant and confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Fig. 2C).”

For Fig 3:

“In EA.hy926 endothelial scratch wound assays, wound closure was markedly increased in MSC CM compared to control media (Fig. 3A). Using live cell image analysis, we found that EA.hy926 cells closed the scratch wounds significantly more quickly in MSC CM versus control media by a combination of increased cell migration and cell proliferation (Fig. 3B). The trophic effects of MSC CM on EA.hy926 endothelial cell proliferation were confirmed by MTT assays for the numbers of viable cells, which was shown to be significantly increased after two days culture in MSC CM compared with control media (Fig. 3B).”

- Please define all abbreviations used in each figure legend.

All abbreviations have now been defined.

- Supplementary material: Please use 12 point Times font throughout the text. Please ensure that the text is formatted according to the style of The Veterinary Journal.

The Supplementary material has been amended so that the text is 12 point Times font throughout. We have also amended the text to the style of TVJ by changing various needed aspects of the nomenclature, units, and abbreviations, e.g. “mL” rather than ml, “37 °C” rather than 37°C, “vs.” rather than versus; “min” rather than minutes, as required.