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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 βIII-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.

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

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Abstract

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Introduction

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

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

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

Materials and methods

MSC isolation and growth

Institutional approval was provided for this study (University of Chester Faculty of Science and Engineering Research Ethics Committee: 060/16/CW/BS, 18 May 2016). Following owner and veterinary surgeon consent for research, canine adipose tissue-derived MSCs were isolated and cultured from surgically extracted inguinal fat pads of dogs

undergoing MSC transplantations for the treatment of joint pathology. MSCs were isolated by collagenase digestion of the tissue for 2 h at 37 ^{o}C (0.2% Type A Collagenase, Worthington Biochemical), selected through their preferential adhesion to tissue culture plastic, as reported previously (Vieira et al., 2010; Kohli et al., 2015), and cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2 with 95% air at 37 ^{o}C. Cultures were routinely passaged at 70-80% confluence using 0.25% trypsin-EDTA (Life Technologies). All experimental procedures were performed using MSC cultures at passages 3-5.

MSC characterisation

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

MSC culture-conditioned medium (MSC CM)

MSCs were seeded at a density of 1.5 x 10^6 cells in T75 tissue culture flasks in standard culture medium for 24 h to permit cell adhesion, then the medium was discarded and the cultures washed in PBS prior to feeding with 15 mL of DMEM/F12 medium supplemented with 1% ITS, 1% non-essential amino acids and 1% penicillin/streptomycin, but without any serum present. Cultures were then incubated at 37 ^{o}C in a humidified atmosphere of 5% CO_2 for 3 days, when the MSC culture-conditioned medium (MSC CM)

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

The effects of MSC CM on SH-SY5Y neuronal cells

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

The effects of MSC CM on EA.hy926 endothelial cells

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

Statistical analysis

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

Results

Characterisation of canine MSCs

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

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

Treating SH-SY5Y cells with canine MSC CM promoted their proliferation and neuronal differentiation, as determined by viable cell numbers, neurite outgrowth and immunoreactivity for βIII-tubulin (Fig. 2). There was an increase in the number of SH-SY5Y cells present, and in their extent of neurite outgrowth (Fig. 2A), which were immunopositive for βIII-tubulin (Fig. 2B) in MSC CM vs. control media. SH-SY5Y neurite length/cell (P = 0.0055) and the proportions of SH-SY5Y cells that were βIII-tubulin immunopositive (P = 0.0002) were significantly greater in MSC CM than in 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 (P < 0.01) (Fig. 2C).

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

The effects of canine MSC CM on EA.hy926 cells were examined by scratch assay, time-lapse live cell image analysis, MTT assay and tubule formation with Matrigel. 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 vs. control media (P = 0.0409) 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 significantly increased after 2 days culture in MSC CM compared with control media (P = 0.0019) (Fig. 3B). We then tested whether MSC CM could promote blood vessel formation, as delineated by endothelial tubule formation in Matrigel assays and digitised image analysis (Fig. 4). As shown (Fig. 4A), culturing EA.hy926 endothelial cells on Matrigel did not give rise to any

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

Discussion

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

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

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

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

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

Conclusions

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

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

Conflict of interest statement

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

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Appendix: Supplementary material

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

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Figure legends

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

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

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

Fig. 3. Canine mesenchymal stem cells (MSCs) secrete factors that promote EA.hy926 endothelial cell proliferation and cell migration. (A) Representative images are shown of EA.hy926 endothelial cell scratch assays. As shown, there was an increase in the extent of wound closure in canine MSC conditioned medium (MSC CM) compared with control medium after 2 days in culture. Scale bars = 200 μm. (B) Wound closure, cell division and cell migration was tracked using the Cell IQ live cell imaging platform, wherein digitised images were collected every 15 min over 2 days. Three-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were also performed to assess viable cell numbers. Top left panel: The rate of endothelial cell wound closure was significantly greater ($P = 0.0409$) in the presence of MSC CM (right line) vs. control media (blue line) over a 2-day time course. Bottom left panel: There was a significant increase in the number of dividing endothelial cells (per image) ($P = 0.0127$) in the scratch wound assays in the presence of MSC CM (red line) vs. control medium (blue line). Top right panel: There was a significant increase in total distance that endothelial cells migrated over a 2-day period in MSC CM vs. control medium ($P = 0.0001$). Bottom right panel: There were significantly 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 ±
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 $2x10^2$ 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 μm. (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 ± standard error. $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure 1

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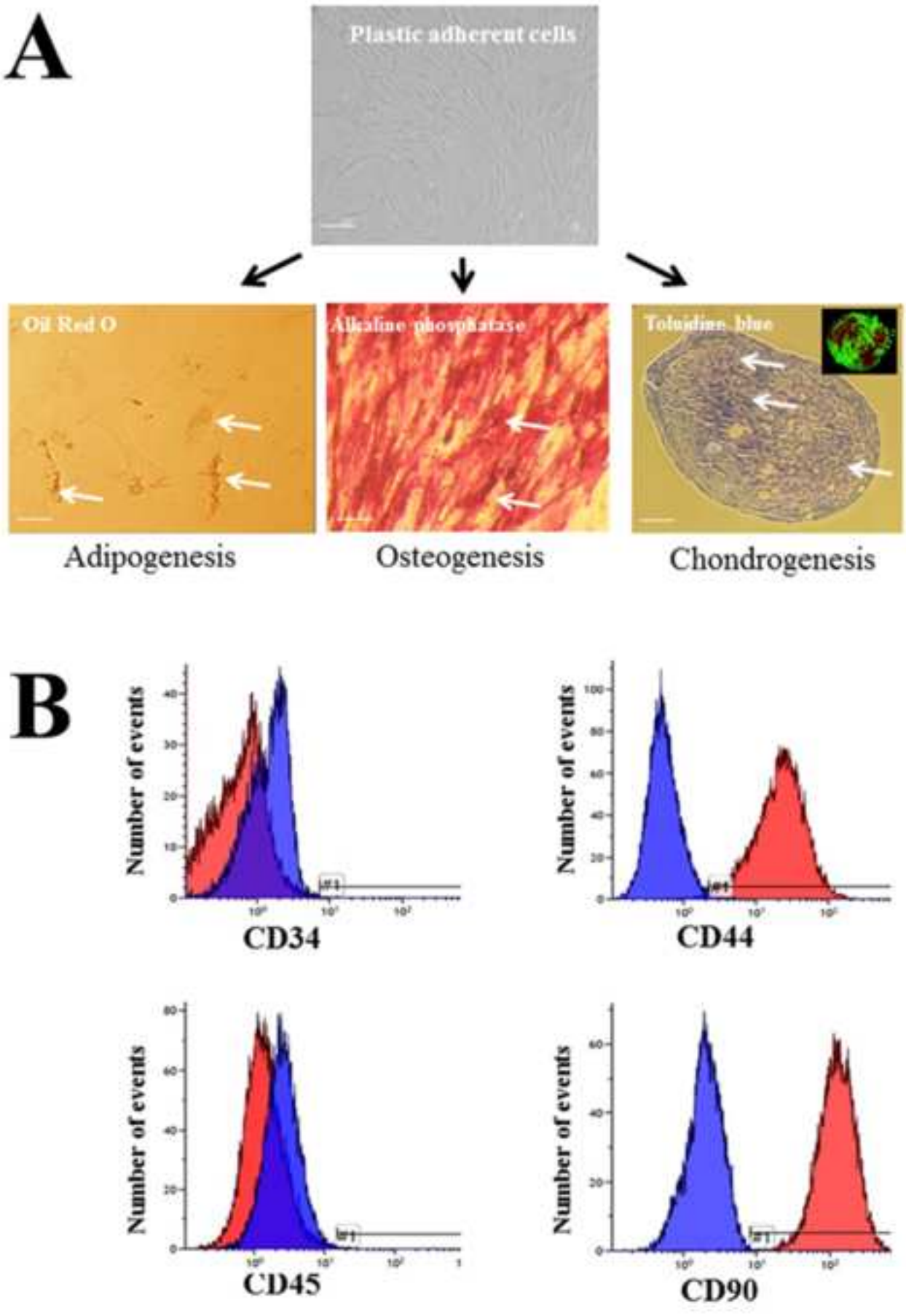

Figure 4

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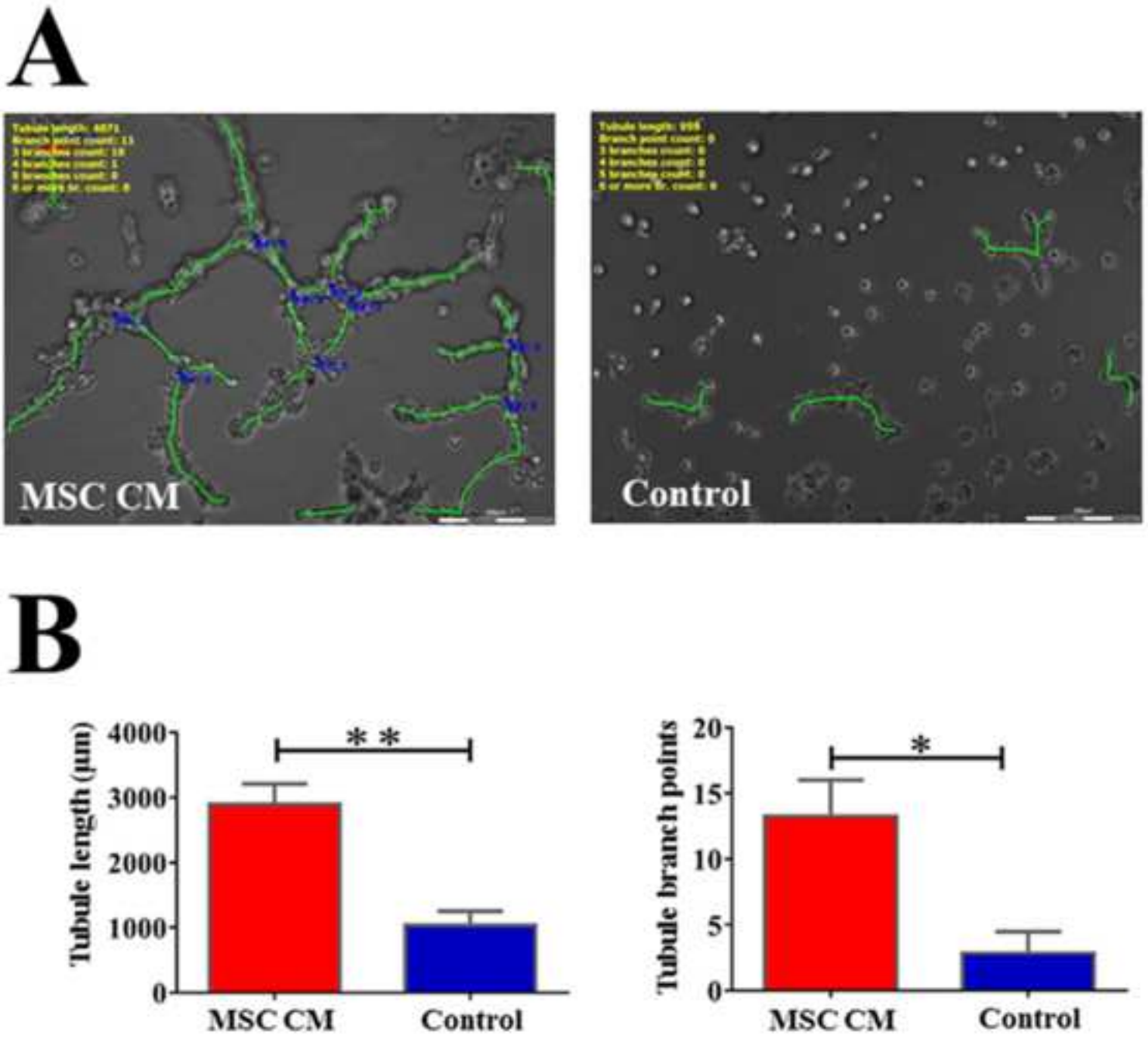

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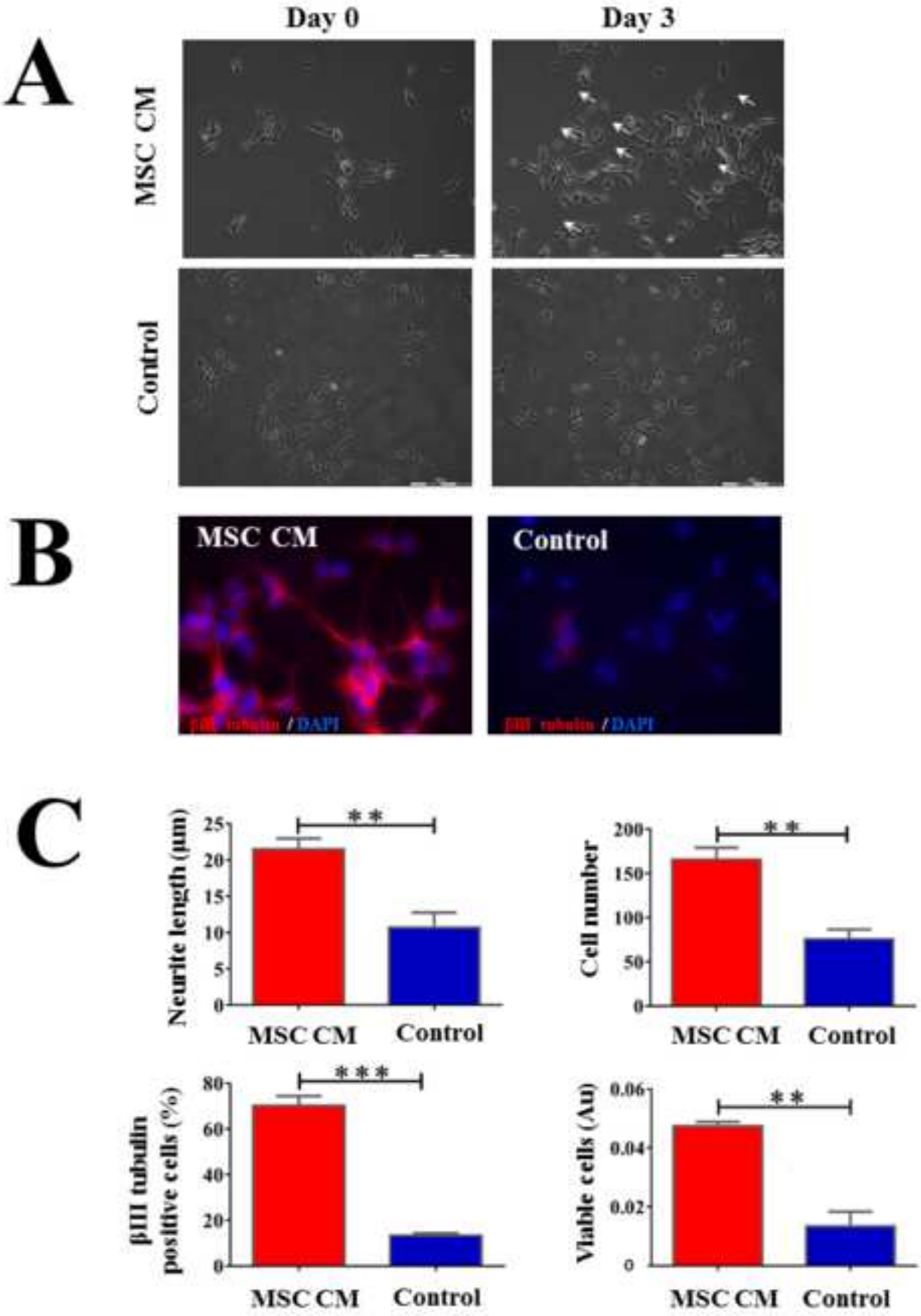

Figure 3

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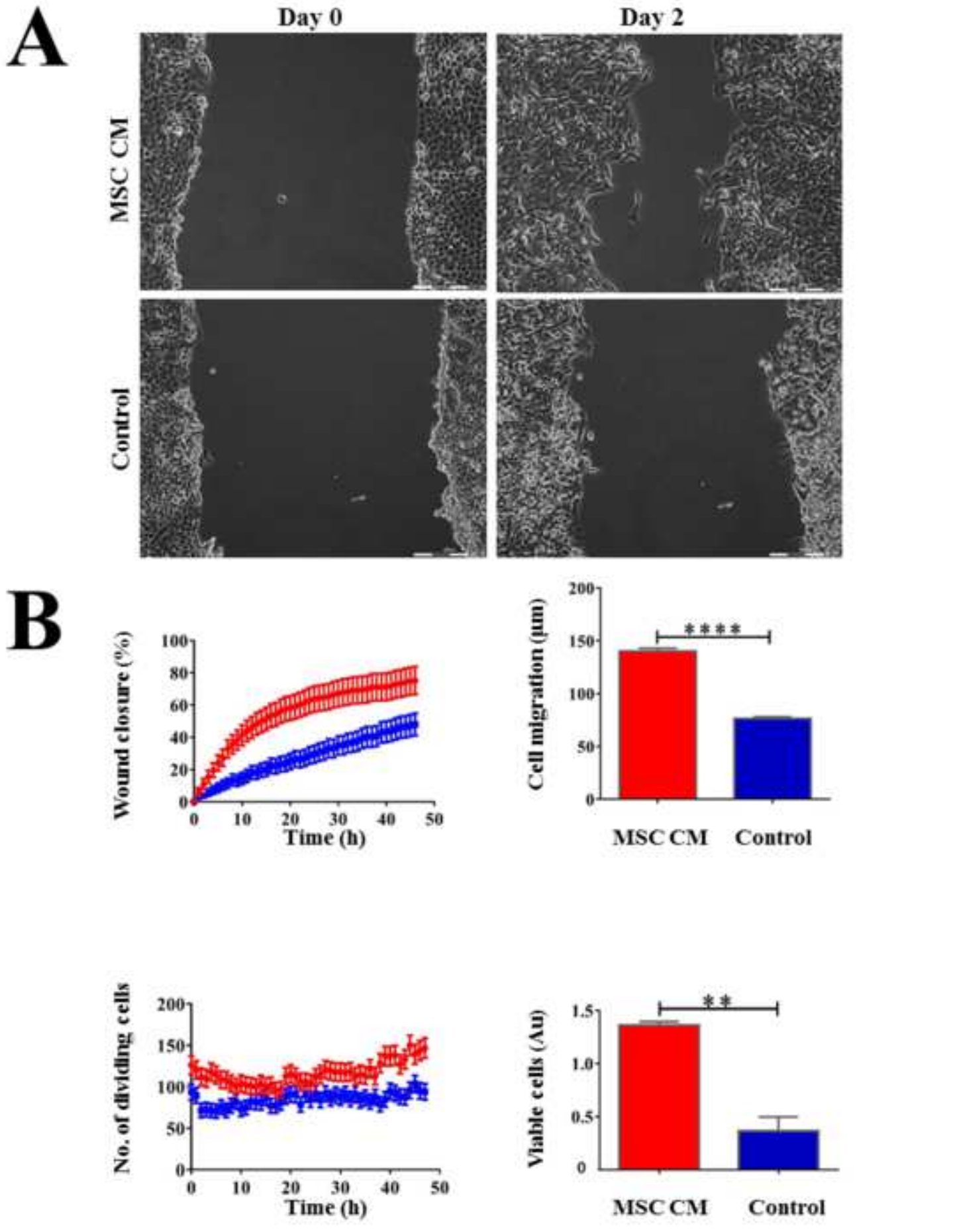

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16-00402

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.

*Revision Note

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 βIII-tubulin (Fig. 2B) in MSC CM versus control media. SH-SY5Y neurite length/cell and the proportions of SH-SY5Y cells that were βIII-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 ^{o}C” rather than 37^{o}C, “vs.” rather than versus; “min” rather than minutes, as required.