1 # *In vitro* culture and characterization of equine peripheral blood

2 # mesenchymal stromal cells

3

4 **Jan H Spaas[a], Catharina De Schauwer[b], Pieter Cornillie[c], Evelyne Meyer[d], Ann Van**

5 **Soom[b], Gerlinde R Van de Walle[a*]**

6

7 [a]Department of Comparative Physiology and Biometrics, Faculty of Veterinary Medicine, Ghent University B-

8 9820 Merelbeke, Belgium

9 [b]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University B-

10 9820 Merelbeke, Belgium

11 [c]Department of Morphology, Faculty of Veterinary Medicine, Ghent University B-9820 Merelbeke, Belgium

12 [d]Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University

13 B-9820 Merelbeke, Belgium

14

15 [*]Corresponding author: Tel. +32 9 264 74 76

16 *Email address:* Gerlinde.VandeWalle@ugent.be (G.R. Van de Walle)

17

18 Running title: Equine peripheral blood MSC characterization

19

20

Abstract

The use of mesenchymal stromal cells (MSC) for the treatment of orthopedic injuries in horses has been described in several studies. In contrast to human MSC, no official guidelines have been proposed to classify a particular cell as an equine MSC. For peripheral blood (PB)-derived MSC in specific, only a limited characterization has been reported to date. In the present study, we have increased the currently available data on PB-derived MSC. To this end, MSC were isolated from equine PB samples and colony forming unit (CFU) assays as well as population doubling time (PDT) calculations from P_0 to P_{10} were performed. Hereby, two types of colonies, fingerprinted and dispersed, could be observed based on macroscopic as well as microscopic features. Moreover, after an initial lag phase as indicated by a negative PDT at P_0 to P_1, the MSC divided rapidly as shown by a positive PDT at all further passages. Furthermore, an immunophenotypic characterization was performed with trypsin- as well as accutase-detached MSC, to evaluate a potential trypsin-sensitive destruction of epitopes of certain antigens. Hereby, it was found that the isolated MSC were positive for CD29, CD44, CD90 and CD105, and negative for CD45, CD79α, MHC II and a monocyte/macrophage marker, irrespective of the cell detaching agent used. Finally, a trilineage differentiation towards osteoblasts, chondroblasts and adipocytes was confirmed using different histological staining methods.

Keywords: Peripheral blood; Horse; Mesenchymal stromal cells

46 **Introduction**

47 Stem cells are defined as cells displaying a self-renewal capacity either with or without
48 differentiation, depending on the symmetry of the division (Horvitz and Herskowitz, 1992).
49 More specifically, mesenchymal stromal cells (MSC) are adult stem cells derived from the
50 mesodermal germ layer. In 2006, the International Society for Cellular Therapy (ISCT) has
51 carefully determined the qualities human cells must possess in order to be defined as MSC
52 (Dominici et al., 2006). Hereby, human MSC have to be (i) plastic-adherent; (ii) positive for
53 the markers CD73, CD90 and CD105 and negative for the markers CD14 (or CD11b), CD34,
54 CD45, CD79a (or CD19) and MHC II; and (iii) able to differentiate into different cell types of
55 the mesodermal germ layer such as osteoblasts, chondroblasts and adipocytes. The use of
56 other human MSC markers such as CD29 and CD44 was also reported (Majumdar et al.,
57 2003; Pittenger et al., 1999).

58

59 For equine MSC, no such guidelines have been described up to now, although this would
60 greatly benefit researchers working in this field (De Schauwer et al., 2011b). Sources of
61 equine MSC reported are bone marrow (BM), adipose tissue (AT), umbilical cord, amniotic
62 fluid, umbilical cord blood (UCB), peripheral blood (PB), gingiva and periodontal ligament
63 (Ahern et al., 2011; Carrade et al., 2011; Koch et al., 2007; Koerner et al., 2006; Mensing et
64 al., 2011; Park et al., 2011). For MSC isolated from equine BM, AT and UCB, the use of
65 several MSC markers and a successful trilineage differentiation have been described (Guest et
66 al., 2008; Hoynowski et al., 2007; Koch et al., 2009; Radcliffe et al., 2010). In contrast, only a
67 limited characterization of equine PB-derived MSC has been reported to date, despite the
68 existence of several papers describing their isolation. Hereby, only one group has described a
69 more extended immunophenotypic characterization of PB-derived MSC using two of the
70 proposed positive markers, namely CD44 and CD90, and two of the proposed negative

71 markers, CD34 and CD45 (Martinello et al., 2010). Nevertheless, for the negative markers
72 used in this study, no information was provided on the positive controls used to confirm
73 cross-reactivity with equine cells and a potential influence of the detachment product on
74 epitope expression was not evaluated. The latter might be of importance, since a recent paper
75 by Hackett *et al.* describes a destructive effect of trypsin on the CD14 epitope of equine BM-
76 derived cells, indicating that attentiveness is needed when evaluating negative stem cell
77 markers on trypsin-detached cells (Hackett et al., 2011). Moreover, and aside from the
78 immunophenotypic characterization, the results of different studies on the differentiation of
79 equine PB MSC into cartilage are contradictory (Giovannini et al., 2008; Koerner et al.,
80 2006). All this indicates the need for more characterization of PB-derived equine MSC.
81
82 Current clinical regenerative therapies with MSC in horses mainly use BM-derived MSC
83 for the treatment of tendinopathies (Crovace et al., 2007; Smith, 2008; Smith et al., 2003) and
84 BM-or AT- derived MSC for the treatment of osteoarthritis (Frisbie et al., 2009). The most
85 obvious disadvantages of BM and AT are the difficulty and invasiveness of the harvesting
86 procedure. An excellent alternative would be blood, such as UCB collected at birth or PB
87 from an adult horse. Despite the fact that sampling UCB is non-invasive, the most important
88 disadvantages are that autologous UCB is not always available and a sterile collection of UCB
89 is only possible under highly hygienic circumstances. Hence, horse-owners have to be very
90 motivated in order to obtain an UCB sample suitable for MSC isolation. These disadvantages
91 inherent to UCB-derived MSC can be avoided using PB as a source for MSC. Peripheral
92 blood can be easily taken in a sterile manner, making this a readily accessible source of
93 autologous MSC when injuries occur and hence, indicates the potential of PB as a source of
94 equine MSC for regenerative therapies. Moreover, the first clinical applications of an
95 heterogenous population of PB-derived stem cells have been recently described for the

96 treatment of ophthalmologic pathologies in horses (Marfe et al., 2011; Spaas et al., 2011). In
97 order to standardize the promising results of this regenerative therapy, it is indispensable to
98 use a well-characterized and homogenous stem cell population.
99
100 Therefore, the goal of the current study was to broaden the knowledge on the
101 characterization of equine PB-derived MSC by (i) determining the growth efficiency and
102 proliferation rate of the cells, (ii) using a more wide-ranging set of complementary markers
103 for their immunophenotyping and (iii) performing trilineage differentiation experiments.
104

105 **Materials and Methods**

106 *Isolation of putative peripheral blood (PB)-derived mesenchymal stromal cells (MSC)*

107 Ten mL of blood from the *vena jugularis* of four adult Warmblood horses was collected into
108 EDTA tubes and transported at 4°C to the laboratory within 4 hours after sampling. The blood
109 was centrifuged at 1000 xg for 20 minutes at room temperature (RT). The buffy coat fraction
110 was collected and diluted 1:1 with phosphate buffered saline (PBS). Subsequently, the cell
111 suspension was gently layered on a Percoll gradient (density 1.080 g/mL; GE Healthcare) and
112 centrifuged at 600 xg for 15 minutes at RT, as previously described (De Schauwer et al.,
113 2011a). The interphase was collected, washed three times with PBS by centrifuging at 200 xg
114 for 10 minutes, and cells were planted at $16x10^4$ cells/cm^2 in a T_{75} flask in culture medium
115 consisting of low glucose (LG) Dulbecco's modified Eagle medium (DMEM) (Invitrogen),
116 supplemented with 30% fetal calf serum (FCS) (GIBCO), 10^{-7} M low dexamethasone, 50
117 µg/mL gentamicin, 10 µl/mL antibiotic-antimycotic solution, 250 ng/mL fungizone (all from
118 Sigma) and 2 mM ultraglutamine (Invitrogen). The medium was refreshed twice a week and
119 the putative MSC were maintained at 37°C and 5% CO_2. At 70% confluency, cells were
120 trypsinized with 0.25% trypsin-EDTA (P_0) and were further cultured for 10 subsequent

121 passages (P_1 to P_{10}) in expansion medium, with the latter being identical to the culture
122 medium but without dexamethasone.
123
124 *Colony forming unit (CFU) assay*
125 Ten, 50 and 100 MSC were plated per 94 mm plate and fixed 8 days later at -20°C for 10
126 minutes using 90% ethanol. Crystal violet stainings were performed to visualize the CFUs
127 macroscopically and the total number of CFUs per plate were counted. These experiments
128 were done in triplicate for all samples.
129
130 *Population Doubling Time (PDT) determination*
131 Cell doubling time (CDT) was calculated from P_0 to P_{10} exactly as previously described
132 (Hoynowski et al., 2007), using the following formula: $CDT= \ln(N_f/N_i)/\ln 2$ with N_f as the
133 final number of cells and N_i the initial number of cells. For the population doubling time
134 (PDT), the cell culture time (in days) was divided by the CDT (Hoynowski et al., 2007).
135
136 *Flow cytometric immunophenotyping*
137 To characterize the undifferentiated equine MSC immunophenotypically, the expression of
138 several MSC markers was evaluated simultaneously by flow cytometry. Cells were detached
139 using either trypsin (Invitrogen) or accutase (Innovative Cell Technologies). Per series, $2x10^5$
140 cells were used and labeled with the following panel of primary antibodies: CD29-Alexa^{488}
141 (Biolegend, clone TS2/16), CD44-APC (BD, clone IM7), CD45-Alexa^{488} (Serotec, clone F10-
142 89-4), CD79α-Alexa^{647} (Serotec, clone HM57), CD90 (VMRD, clone DH24A), CD105-PE
143 (Abcam, clone SN6), MHC II (Serotec, clone CVS20) and a monocyte/macrophage marker-
144 Alexa^{488} (Serotec, clone MAC387). For the detection of the CD79α and
145 monocyte/macrophage marker, fixation and permeabilization pretreatment was carried out

146 with commercially available reagents (Invitrogen). In general, cells were incubated for 15
147 minutes on ice in the dark with the primary antibodies and washed twice in LG DMEM with
148 1% BSA. Secondary Alexa^{647}-linked and PE-linked antibodies (Invitrogen), again incubated
149 for 15 minutes on ice in the dark, were used to label the CD90 and MHC II positive cells,
150 respectively. In addition, viability assessment with the nucleic acid stain 7-amino-actinomycin
151 D (7-AAD, Sigma) was performed on the non-fixed cells. At least 10,000 cells were acquired
152 using a FACS Canto flow cytometer (Becton Dickinson Immunocytometry systems) equipped
153 with a 488 nm solid state and a 633 nm HeNe laser, and these data were subsequently
154 analysed with the FACS Diva software. To assess cross-reactivity of the differentiated blood
155 cell markers, for which stem cells should be negative, proper positive equine control cells
156 consisting of equine peripheral blood mononuclear cells (PBMC) were used. In addition, cells
157 were incubated with or without (autofluorescence) isotype-specific IgG's (mouse IgG_1, mouse
158 IgM and rat IgG_{2b}) in parallel to establish the background signal.
159
160 *Trilineage cell differentiation*
161 For the osteogenic differentiation, $3x10^3$ cells/cm^2 were planted in a 4-well plate and
162 incubated in expansion medium until cells were 70% confluent. At that point, osteogenic
163 differentiation medium was added and refreshed twice a week. This medium consisted of LG
164 DMEM (Invitrogen) supplemented with 10% FCS (GIBCO), 0.2 mM L-ascorbic acid-2-
165 phosphate (Fluka), 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/mL
166 gentamycin and 10 µl/mL antibiotic-antimycotic solution (all from Sigma) (De Schauwer et
167 al., 2011a; Koch et al., 2007). Three weeks later, differentiation was evaluated using Alkaline
168 Phosphatase (Millipore detection kit) and Alizarin Red S staining in order to evaluate calcium
169 phosphate deposition. For chondrogenic differentiation, $2.5x10^5$ cells/5mL were brought in a
170 three-dimensional culture system, centrifuged at 150 xg for 5 minutes at RT and resuspended

in 0.5 mL chondrogenic inducing medium which was refreshed twice a week. This medium was based on the basal differentiation medium (Lonza), supplemented with 10 ng/mL transforming growth factor-β_3 (Sigma). Differentiation was daily evaluated macroscopically and after 3 weeks of incubation, an Alcian Blue staining was performed on 8 µm histological sections after paraffin embedding of the chondrospheres. For adipogenic differentiation, 2.1×10^4 cells/cm^2 were planted in a 4-well plate in expansion medium until the cells were 70% confluent and adipogenic inducing medium was added subsequently. After 3 days, this medium was replaced with adipogenic maintenance medium for 1 day. This cycle was repeated four more times after which the cells were refreshed twice with adipogenic maintenance medium. The adipogenic inducing medium consisted of LG DMEM (Invitrogen) supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 µg/mL recombinant human-insuline, 0.2 mM indomethacin, 15% rabbit serum, 50 µg/mL gentamycin and 10 µl/mL antibiotic-antimycotic solution (all from Sigma) (De Schauwer et al., 2011a; Koch et al., 2007). The adipogenic maintenance medium was identical but without dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine. Differentiation was evaluated after 3 weeks of cultivation using Oil Red O staining. As a control for the trilineage differentiation, MSC were cultivated for 3 weeks in expansion medium at the same concentrations and in the same culture vessels, and all stainings were performed identically.

Results

Putative peripheral blood (PB)-derived equine MSC are plastic adherent

The first plastic adherent colonies were noticed starting from 16-18 days after culturing of the isolated peripheral blood (PB) mononuclear fractions and around 21 days post seeding, cells became confluent with the formation of a monolayer (Fig. 1A).

196 *Putative PB-derived equine MSC have self-renewal growth properties*

197 Mesenchymal stromal cells (MSC) can be evaluated using the well-characterized colony
198 forming unit (CFU) assays. Hereby, a limited number of cells (10, 50 and 100) were seeded
199 on a large surface and cultured for 8 days. At that time point, colonies in two different stages
200 could be observed macroscopically. First, there were dispersed CFUs identified by a spotted,
201 vague macroscopic morphology and as rather distant cells microscopically (Fig. 1B). Second,
202 darker and more packed CFUs were found with a microscopic fingerprint pattern (Fig. 1B). In
203 general, more dispersed compared to fingerprint CFUs were observed for all three seeding
204 concentrations (Table 1).

205 To determine the growth efficiency and proliferation rate of the putative MSC, population
206 doubling times (PDT) in days were calculated from P_0 up to P_{10}. After an initial lag phase,
207 indicated by a negative PDT at $P_{0\rightarrow1}$, the putative MSC divided rapidly as shown by a positive
208 PDT at all further passages tested (Table 2).

209

210 *Putative PB-derived equine MSC are positive for MSC markers and negative for*
211 *differentiated blood cell markers*

212 Flow cytometry, comprising a wide-ranging set of cellular markers, was used for the
213 immunophenotypical characterization of the putative equine MSC. Hereby, it was found that
214 the cells were positive for the stem cell markers CD29, CD44, CD90 and CD105 (Fig. 2). In
215 addition, the putative MSC were negative for the panleukocyte marker CD45, the B-
216 lymphocyte marker CD79α, the monocyte/macrophage marker and a marker for MHC II,
217 which is present on antigen presenting cells (Fig. 3). Moreover, the negative results with the
218 differentiated blood cell markers were due to the actual absence of these antigens on the PB-
219 derived equine MSC since (i) these markers stained positive on the equine PBMC control
220 cells, demonstrating cross-reactivity with the equine antigens (data not shown) and (ii) the

221 results were virtually identical when using accutase-detached MSC (Fig. 3). In addition, no
222 signal was detected with relevant isotype controls for all cell markers used (Fig. 2 & 3).
223
224 *Putative PB-derived equine MSC are capable of differentiating in vitro towards osteoblasts,*
225 *chondroblasts and adipocytes*
226 The putative MSC were further subjected to a functional characterization by differentiation
227 experiments using selective media. After 3 weeks of culture in osteogenic medium, the
228 morphology of almost all cultured cells changed from spindle-shaped to stellate and irregular
229 (Fig. 4B). Differentiated cells formed multiple individual clusters with a clear calcium
230 deposition in the extracellular matrix and the presence of intracellular phosphatase as
231 determined by Alizarin Red S (Fig. 4A) and Alkaline Phosphatase (Fig. 4B) stainings,
232 respectively. The control MSC on the other hand, maintained their spindle-shaped
233 morphology with the formation of a monolayer and without any positivity for both staining
234 methods (Fig. 4A & B).
235 For the chondrogenic differentiation, putative equine MSC were grown in a three-
236 dimensional culture system with chondrogenic medium and within 3 days of culture, spherical
237 colonies, identified as chondrospheres, were already noted macroscopically (Fig. 5A). The
238 chondrospheres increased visually in size during the differentiation period and after staining
239 with Alcian Blue, the presence of cartilage lacunes surrounded by sulphated acid
240 mucopolysaccharides was clearly observed (Fig. 5B). The size of the control pellet (Fig. 5A)
241 on the other hand, decreased gradually with packed cells without cartilage lacunes (Fig. 5B).
242 In addition, the control pellet stained negative for Alcian Blue which confirmed the absence
243 of mucopolysaccharides (Fig. 5B).
244 The differentiation towards adipocytes was performed using adipogenic inducing and
245 maintenance media. The cell morphology changed from a spindle shaped towards a more

246 round morphology during the differentiation process. Moreover, the production of lipid
247 droplets was visualized using an Oil Red O staining (Fig. 4C). Controls of non-differentiated
248 MSC subjected to the same detection method maintained their spindle-shaped morphology
249 with the formation of a monolayer and stained negative (Fig. 4 & 5).

250

251 **Discussion**

252 Recently, the use of peripheral blood (PB) stem cells has been reported as a valuable tool
253 in equine regenerative medicine (Marfe et al., 2011; Spaas et al., 2011). However, in order to
254 correctly evaluate their potential, an extensive characterization is warranted. Whereas in 1997
255 Lazarus *et al.* described that human mesenchymal stem cells (MSC) could not be recovered
256 from peripheral blood, Zvaifler *et al.* reported in 2000 the first isolation of mesenchymal
257 precursor cells from human blood based on morphological features, cell proliferation assays,
258 positivity for the MSC marker CD105 and osteogenic differentiation (Lazarus et al., 1997;
259 Zvaifler et al., 2000). For horses, the isolation of PB-derived MSC was described for the first
260 time in 2006 and was based on morphology combined with a differentiation towards
261 osteocytes and adipocytes (Koerner et al., 2006). However, no immunophenotypic
262 characterization of the cells was performed and the differentiation towards cartilage was
263 unsuccessful. In 2008, another research group managed to produce chondroblasts from equine
264 PB-derived MSC, although this was only achieved after 9 weeks of differentiation and also in
265 that study, no immunophenotypic characterization of the cells was carried out (Giovannini et
266 al., 2008). More recently, CD44 and CD90 have been used as positive immunophenotypic
267 markers for equine PB-derived MSC, but these cells were also positive for the hematopoietic
268 stem cell marker CD117 and no differentiation towards chondrocytes was reported
269 (Martinello et al., 2010). Also other researchers identified a MSC population in the peripheral
270 blood using CD105 and CD90 as positive markers, but no further characterization was

performed (Marfe et al., 2011). In the present study, we therefore aimed to perform a more extended characterization of PB-derived MSC, both immunophenotypically as well as functionally.

In order to produce valid data for an immunophenotypic characterization, it is necessary to use proper isotype controls to exclude aspecific antibody reaction and to use positive control cells for the confirmation of cross reactivity in equines, since only about 4% of human antibodies reacts with the equivalent equine proteins (Ibrahim et al., 2007). Another important feature to take into consideration when performing flow cytometric analyses is the fact that some epitopes can be destroyed by trypsin, resulting in a false negative result (Hackett et al., 2011). Since MSC are not only phenotyped by the presence of stem cell markers but also by the absence of several differentiated cell markers, trypsin-sensitivity might be a concern. Indeed, recently it has been described that CD14, present on e.g. macrophages, neutrophils and dendritic cells and which is used as a negative marker for human MSC, appears to be actually present on equine bone marrow (BM)-derived MSC, but is absent when using trypsinized cells, indicating that this protein contains a tryspin-sensitive epitope (Hackett et al., 2011). So in order to evaluate whether the negative MSC markers which we used in the present study are truly absent and not just merely destroyed by trypsinization, MSC samples were detached using the cell detaching agent accutase and the expression of the negative cell markers were compared to trypsin-detached MSC from the same horse. Since we did not observe any difference when using both cell-detaching agents, we conclude that the negative cell markers we tested in our study recognize trypsin-insensitive epitopes and hence, the PB-derived equine cells fulfill all qualities to be immunophenotyped as MSC.

295 The expression of different markers on equine BM-derived MSC has been tested at
296 different time points after harvesting and hereby, it was found that expression of MSC
297 markers can vary during cultivation but stabilizes after 2 to 3 weeks post isolation (Radcliffe
298 et al., 2010). Due to the late appearance of MSC after seeding PB mononuclear fractions, we
299 were unable to immunophenotype the PB-derived MSC earlier than 3 weeks post isolation in
300 order to evaluate if a similar variation in expression also occurs. However, the levels of
301 expression of the cell markers of BM-derived MSC at 3 weeks (positive as well as negative
302 markers) were similar to the expression levels we found in the current study with equine PB-
303 derived MSC, indicating that the source of MSC most likely does not influence the level of
304 cell marker expression on these equine cells.

305

306 In contrast to previous studies, where the differentiation towards chondroblasts was
307 unsuccessful or only accomplished after 9 weeks of culturing in chondrogenic medium
308 (Giovannini et al., 2008; Koerner et al., 2006), we could confirm in the present study the
309 differentiation of PB-derived MSC towards chondrocytes as early as 3 weeks post
310 differentiation. First, we observed chondrospheres macroscopically in the 3-dimensional
311 cultures as early as 3 days and secondly, a positive Alcian Blue staining at 3 weeks post
312 culture confirmed the differentiation towards chondroblasts. A possible explanation for this
313 discrepancy could be the use of commercially prepared chondrogenic differentiation medium,
314 supplemented with transforming growth factor-β_3 in the present study, whereas previous
315 studies used an in-house prepared culture medium. Furthermore, we supplemented our culture
316 medium with dexamethasone already at passage 0 (P_0), which is in line with descriptions for
317 culturing MSC from equine umbilical cord blood (UCB) (De Schauwer et al., 2011a; Koch et
318 al., 2007). Since dexamethasone is known to be essential for differentiation, adding this

319 potent synthetic glucocorticoid at the time of isolation might potentially have primed the
320 equine MSC for proper chondrogenic differentiation later on.
321

322 **Conclusion**

323 In conclusion, this study provides additional insights into the characterization of equine
324 PB-derived MSC which can prove to be valuable not only for future research on equine PB-
325 derived MSC in specific, but also on equine mesenchymal stromal cells in general.
326

327 **Conflict of interest statement**

328 The authors declare that they have no competing interests.
329

330 **Acknowledgements**

331 This study was supported by a grant to J. S. from the agency for innovation by science and
332 technology (IWT). The authors wish to thank Isabel Lemahieu, Petra Van Damme, Jurgen
333 Decraene and Kristel Demeyere for their technical assistance.
334

335 **References**

336 Ahern, B.J., Schaer, T.P., Terkhorn, S.P., Jackson, K.V., Mason, N.J., Hankenson, K.D.,
337 2011. Evaluation of equine peripheral blood apheresis product, bone marrow, and adipose
338 tissue as sources of mesenchymal stem cells and their differentation potential. American
339 journal of veterinary research 72, 127-133.

340 Carrade, D.D., Owens, S.D., Galuppo, L.D., Vidal, M.A., Ferraro, G.L., Librach, F.,
341 Buerchler, S., Friedman, M.S., Walker, N.J., Borjesson, D.L., 2011. Clinicopathologic
342 findings following intra-articular injection of autologous and allogeneic placentally derived
343 equine mesenchymal stem cells in horses. Cytotherapy 13, 419-430.

344 Crovace, A., Lacitignola, L., De Siena, R., Rossi, G., Francioso, E., 2007. Cell therapy for
345 tendon repair in horses: an experimental study. Veterinary Research Communications 31
346 Suppl 1, 281-283.

347 De Schauwer, C., Meyer, E., Cornillie, P., De Vliegher, S., Van de Walle, G.R., Hoogewijs,
348 M., Declercq, H., Govaere, J., Demeyere, K., Cornelissen, M., Van Soom, A., 2011a.
349 Optimization of the isolation, culture and characterization of equine umbilical cord blood
350 mesenchymal stromal cells. Tissue Engineering. Part C, Methods. Epub ahead of print.

351 De Schauwer, C., Meyer, E., Van de Walle, G.R., Van Soom, A., 2011b. Markers of stemness
352 in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 75, 1431-1443.

353 Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans,
354 R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent
355 mesenchymal stromal cells. The International Society for Cellular Therapy position
356 statement. Cytotherapy 8, 315-317.

357 Frisbie, D.D., Kisiday, J.D., Kawcak, C.E., Werpy, N.M., McIlwraith, C.W., 2009. Evaluation
358 of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem
359 cells for treatment of osteoarthritis. Journal of orthopaedic research : official publication of
360 the Orthopaedic Research Society 27, 1675-1680.

361 Giovannini, S., Brehm, W., Mainil-Varlet, P., Nesic, D., 2008. Multilineage differentiation
362 potential of equine blood-derived fibroblast-like cells. Differentiation; research in
363 biological diversity 76, 118-129.

364 Guest, D.J., Ousey, J.C., Smith, M.R.W., 2008. Defining the expression of marker genes in
365 equine mesenchymal stromal cells. Stem Cells and Cloning: Advances and Applications 1,
366 1-9.

367 Hackett, C.H., Flaminio, M.J., Fortier, L.A., 2011. Analysis of CD14 expression levels in
368 putative mesenchymal progenitor cells isolated from equine bone marrow. Stem cells and
369 development 20, 721-735.

370 Horvitz, H.R., Herskowitz, I., 1992. Mechanisms of asymmetric cell division: two Bs or not
371 two Bs, that is the question. Cell 68, 237-255.

372 Hoynowski, S.M., Fry, M.M., Gardner, B.M., Leming, M.T., Tucker, J.R., Black, L., Sand,
373 T., Mitchell, K.E., 2007. Characterization and differentiation of equine umbilical cord-
374 derived matrix cells. Biochemical and Biophysical Research Communications 362, 347-
375 353.

376 Ibrahim, S., Saunders, K., Kydd, J.H., Lunn, D.P., Steinbach, F., 2007. Screening of anti-
377 human leukocyte monoclonal antibodies for reactivity with equine leukocytes. Veterinary
378 immunology and immunopathology 119, 63-80.

379 Koch, T.G., Heerkens, T., Thomsen, P.D., Betts, D.H., 2007. Isolation of mesenchymal stem
380 cells from equine umbilical cord blood. BMC Biotechnology 7, 26.

381 Koch, T.G., Thomsen, P.D., Betts, D.H., 2009. Improved isolation protocol for equine cord
382 blood-derived mesenchymal stromal cells. Cytotherapy 11, 443-447.

383 Koerner, J., Nesic, D., Romero, J.D., Brehm, W., Mainil-Varlet, P., Grogan, S.P., 2006.
384 Equine peripheral blood-derived progenitors in comparison to bone marrow-derived
385 mesenchymal stem cells. Stem Cells 24, 1613-1619.

386 Lazarus, H.M., Haynesworth, S.E., Gerson, S.L., Caplan, A.I., 1997. Human bone marrow-
387 derived mesenchymal (stromal) progenitor cells (MPCs) cannot be recovered from
388 peripheral blood progenitor cell collections. Journal of Hematotherapy 6, 447-455.

389 Majumdar, M.K., Keane-Moore, M., Buyaner, D., Hardy, W.B., Moorman, M.A., McIntosh,
390 K.R., Mosca, J.D., 2003. Characterization and functionality of cell surface molecules on
391 human mesenchymal stem cells. Journal of biomedical science 10, 228-241.

392 Marfe, G., Massaro-Giordano, M., Ranalli, M., Cozzoli, E., Di Stefano, C., Malafoglia, V.,
393 Polettini, M., Gambacurta, A., 2011. Blood Derived Stem Cells: An Ameliorative Therapy
394 in Veterinary Ophthalmology. Journal of cellular physiology DOI 10.1002/jcp.22953.

395 Martinello, T., Bronzini, I., Maccatrozzo, L., Iacopetti, I., Sampaolesi, M., Mascarello, F.,
396 Patruno, M., 2010. Cryopreservation does not affect the stem characteristics of multipotent
397 cells isolated from equine peripheral blood. Tissue Engineering. Part C, Methods 16, 771-
398 781.

399 Mensing, N., Gasse, H., Hambruch, N., Haeger, J.D., Pfarrer, C., Staszyk, C., 2011. Isolation
400 and characterization of multipotent mesenchymal stromal cells from the gingiva and the
401 periodontal ligament of the horse. BMC veterinary research 7, 42.

402 Park, S.B., Seo, M.S., Kang, J.G., Chae, J.S., Kang, K.S., 2011. Isolation and characterization
403 of equine amniotic fluid-derived multipotent stem cells. Cytotherapy 13, 341-349.

404 Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D.,
405 Moorman, M.A., Simonetti, D.W., Craig, S., Marshak, D.R., 1999. Multilineage potential
406 of adult human mesenchymal stem cells. Science 284, 143-147.

407 Radcliffe, C.H., Flaminio, M.J., Fortier, L.A., 2010. Temporal analysis of equine bone
408 marrow aspirate during establishment of putative mesenchymal progenitor cell
409 populations. Stem cells and development 19, 269-282.

410 Smith, R.K., 2008. Mesenchymal stem cell therapy for equine tendinopathy. Disability and
411 rehabilitation 30, 1752-1758.

412 Smith, R.K., Korda, M., Blunn, G.W., Goodship, A.E., 2003. Isolation and implantation of
413 autologous equine mesenchymal stem cells from bone marrow into the superficial digital
414 flexor tendon as a potential novel treatment. Equine veterinary journal 35, 99-102.

415 Spaas, J.H., Gambacurta, A., Polettini, M., Broeckx, S., Van Hoeck, F., De Schauwer, C.,
416 Van de Walle, G.R., Van Soom, A., 2011. Purification and expansion of stem cells from
417 equine peripheral blood, with clinical applications. Vlaams Diergeneeskundig Tijdschrift
418 80, 129-135.

419 Zvaifler, N.J., Marinova-Mutafchieva, L., Adams, G., Edwards, C.J., Moss, J., Burger, J.A.,
420 Maini, R.N., 2000. Mesenchymal precursor cells in the blood of normal individuals.
421 Arthritis Research 2, 477-488.

422

423

424

425 **Table 1.**

426 **Colony forming unit (CFU) assays of putative peripheral blood (PB)-derived equine**
427 **mesenchymal stromal cells (MSC) of four horses (H_1 to H_4). Data represent the means ±**
428 **standard deviations.**

Number of seeded cells	Isolation	Fingerprint colonies	Dispersed colonies	Total colonies
CFU_{10}	**H_1**	7±2	17±5	24±7
	H_2	6±1	11±4	17±5
	H_3	6±2	9±1	16±3
	H_4	9±5	10±4	19±9
CFU_{50}	**H_1**	20±4	47±5	68±4
	H_2	17±3	38±3	55±6
	H_3	17±3	38±6	55±5
	H_4	26±3	47±6	73±3
CFU_{100}	**H_1**	25±13	75±13	100±26
	H_2	32±2	82±6	114±6
	H_3	32±3	70±10	101±10
	H_4	32±9	65±11	99±12

429

430

431 **Table 2.**

432 **Population doubling time (PDT) in days of the putative peripheral blood (PB)-derived**

433 **equine mesenchymal stromal cells (MSC) of four horses (H_1 to H_4).**

Passage (P)	PDT H_1	PDT H_2	PDT H_3	PDT H_4
$P_{0\rightarrow1}$	-5.46	-6.25	-3.29	-3.72
$P_{1\rightarrow2}$	0.70	1.27	0.77	0.82
$P_{2\rightarrow3}$	1.21	0.98	0.75	1.17
$P_{3\rightarrow4}$	1.03	1.14	1.12	1.52
$P_{4\rightarrow5}$	1.49	1.02	0.98	0.92
$P_{5\rightarrow6}$	0.90	1.22	1.47	1.21
$P_{6\rightarrow7}$	1.35	1.00	1.23	1.41
$P_{7\rightarrow8}$	1.27	1.15	1.13	1.06
$P_{8\rightarrow9}$	0.74	1.02	1.01	1.92
$P_{9\rightarrow10}$	0.79	1.18	1.02	1.01

434

435

436 **Figure Legends**

437 **Fig. 1:** Adherent putative equine mesenchymal stromal cells (MSC). (**A**) Representative
438 pictures of putative single MSC and a MSC monolayer. (**B**) Representative macroscopic and
439 microscopic images of dispersed colony forming units (CFUs) and fingerprint CFUs after
440 crystal violet staining. Scale bars represent 50µm.

441

442 **Fig. 2:** Immunophenotypic characterization with positive markers for equine mesenchymal
443 stromal cells (MSC). Two laser flow cytometry was performed with a set of 4 MSC positive
444 markers: CD29, CD44, CD90 and CD105. Representative histograms show relative numbers
445 of cells versus mean fluorescence intensity. The light and dark grey histograms represent the
446 relevant isotype control staining and marker antibody staining, respectively with the
447 corresponding mean percentage of positive cells ± standard deviation.

448

449 **Fig. 3:** Expression of negative cell markers on trypsin- and accutase-detached putative equine
450 mesenchymal stromal cells (MSC). Two laser flow cytometry was performed with a set of 4
451 MSC negative markers: CD45, CD79α, MHC II and a monocyte/macrophage marker.
452 Representative histograms show relative numbers of cells versus mean fluorescence intensity
453 after trypsinization (histograms on the left) or detachment with accutase (histograms on the
454 right). The light and dark grey histograms represent the relevant isotype control staining and
455 marker antibody staining, respectively with the corresponding mean percentage of positive
456 cells ± standard deviation.

457

458 **Fig. 4:** Osteogenic and adipogenic differentiation of putative equine mesenchymal stromal
459 cells (MSC). Representative microscopic images of the Alizarin Red S (**A**) and Alkaline
460 Phosphatase staining (**B**) in order to confirm osteogenesis. The production of lipid droplets is

461 illustrated using Oil Red O staining (**C**). Also the negative control cells are presented. Scale

462 bars represent 50µm.

463

464 **Fig. 5:** Chondrogenic differentiation of putative equine mesenchymal stromal cells (MSC). A

465 representative macroscopic image of an encircled chondrosphere and a control pellet,

466 indicated with an arrow, at 2 weeks after cultivation (**A**). Alcian Blue staining indicated

467 cartilage lacunes surrounded by sulphated acid mucopolysaccharides only in the differentiated

468 chondrosphere pellets (**B**). Scale bars represent 50µm.

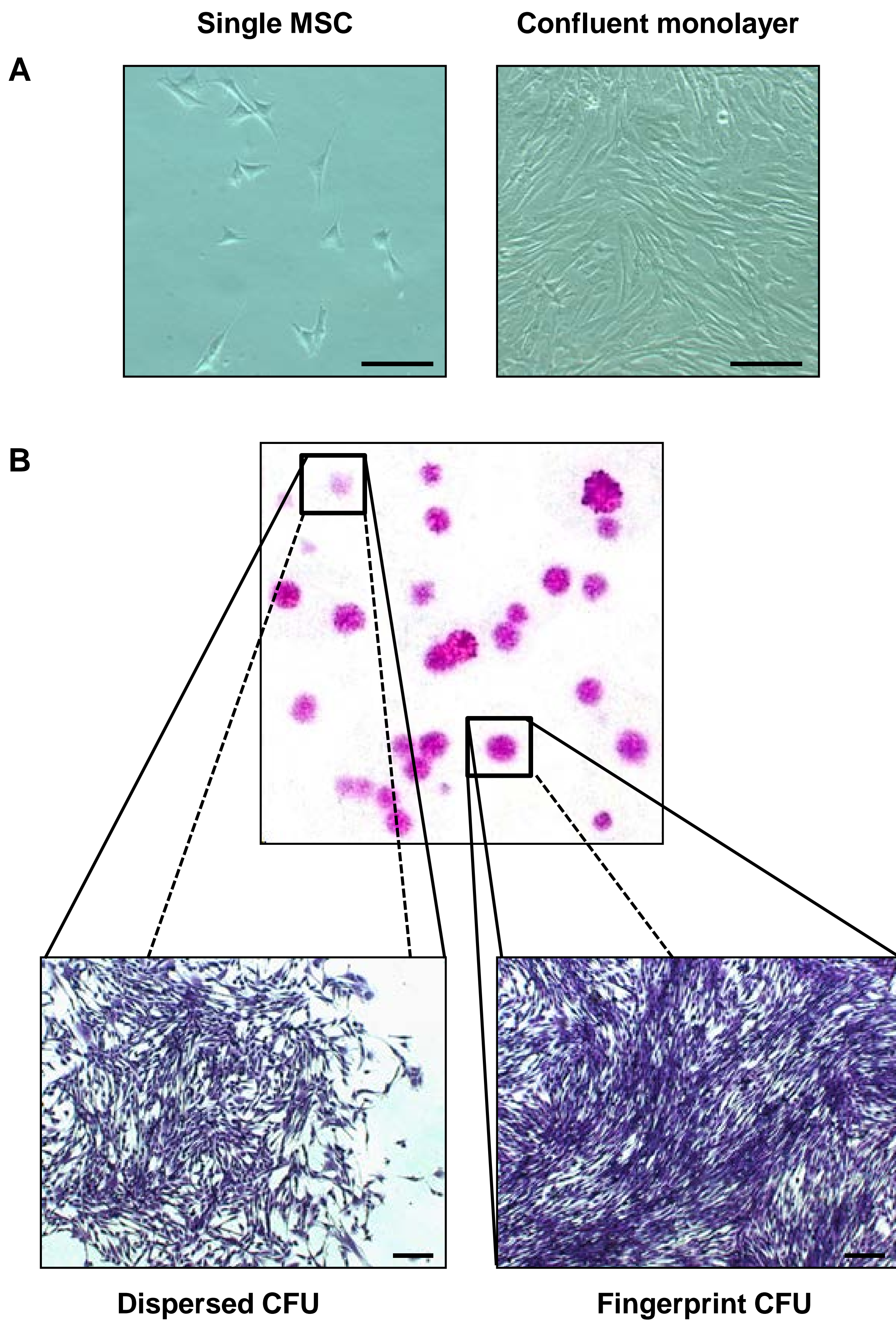
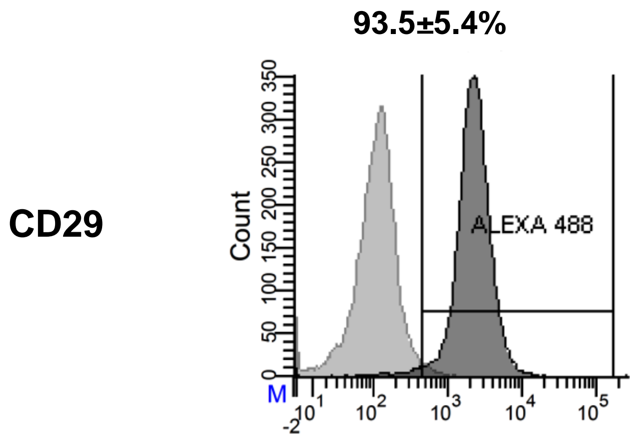
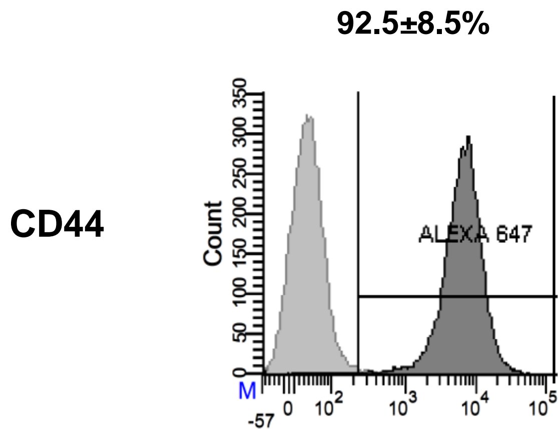
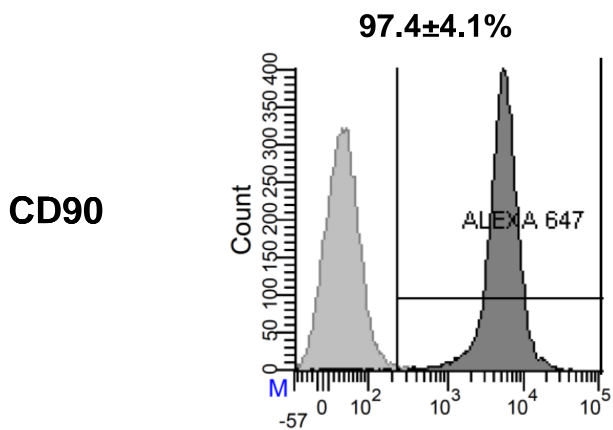


Figure 1 (Spaas *et al.*)







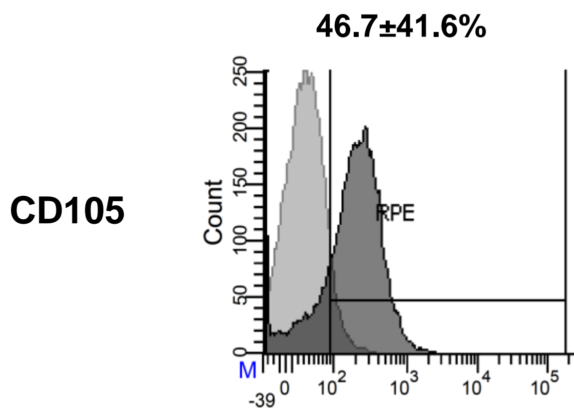
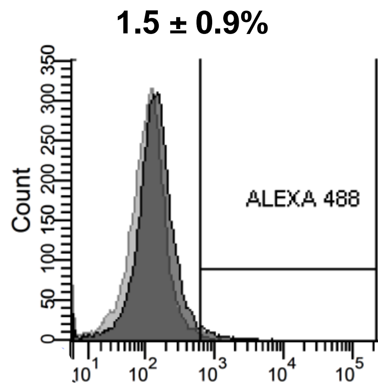
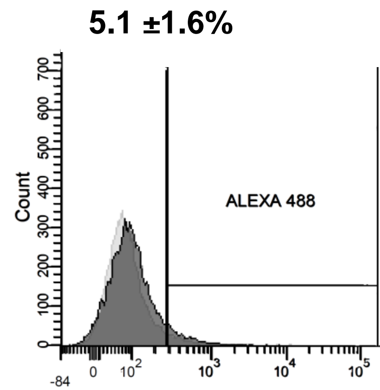
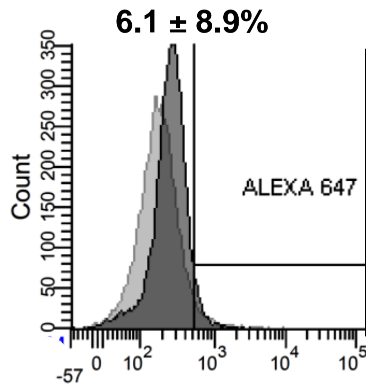


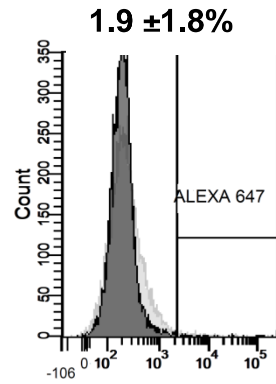
Figure 2 (Spaas *et al.*)

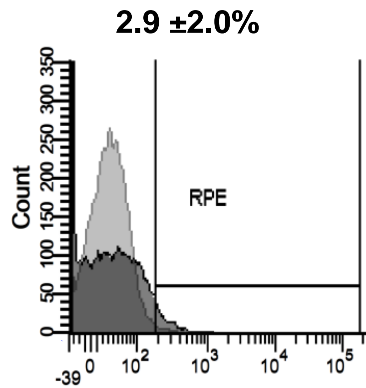
	Trypsin	Accutase
CD45	1.5 ± 0.9%	5.1 ±1.6%
CD79α	6.1 ± 8.9%	1.9 ±1.8%
MHC II	2.9 ±2.0%	2.8 ±3.1%
Monocyte/ Macrophage	5.6 ± 5.1%	5.5 ±1.8%

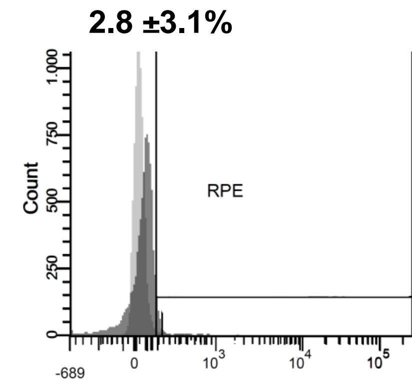


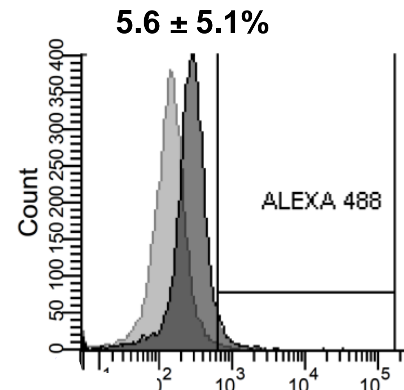












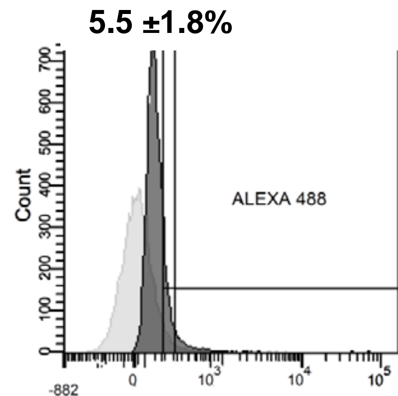


Figure 3 (Spaas *et al.*)

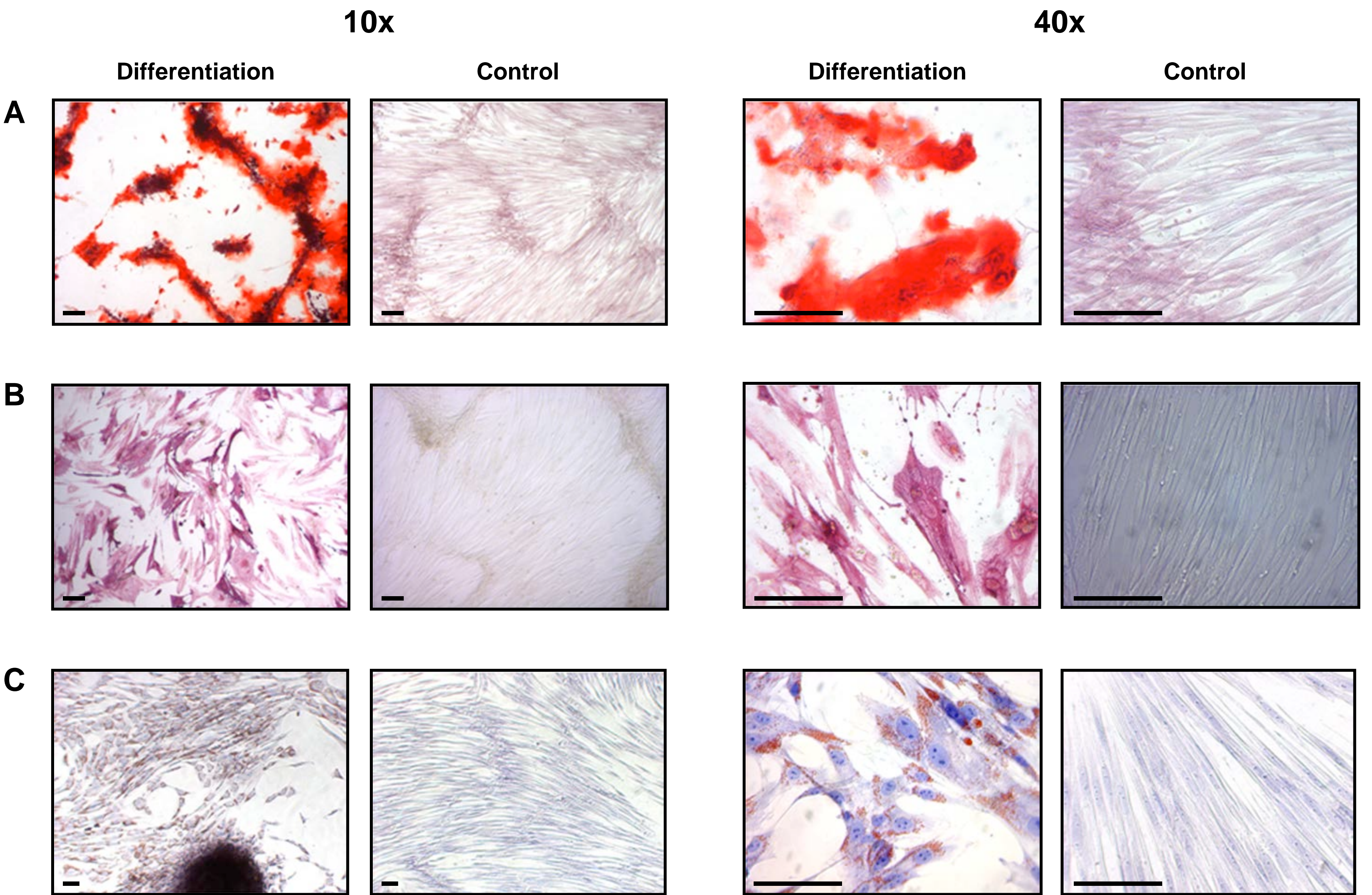
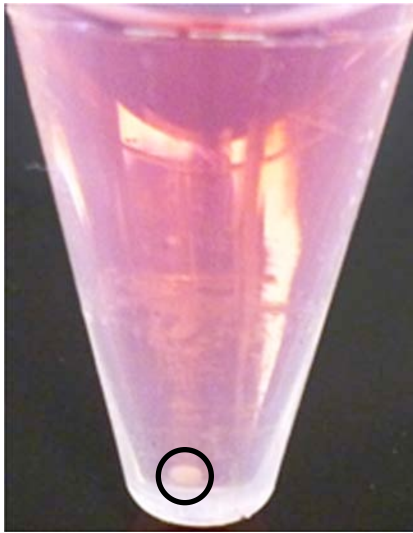


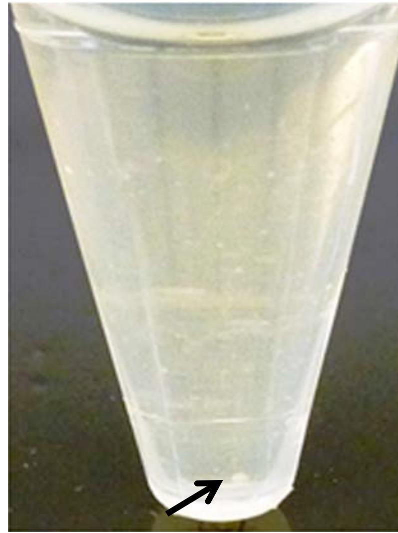
Figure 4 (Spaas *et al.*)

A

Chondropshere

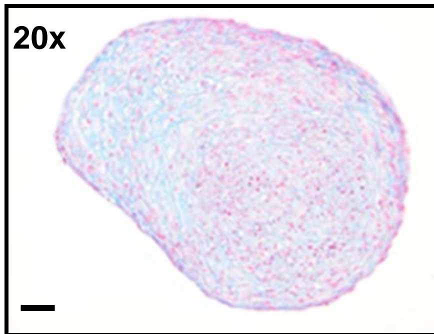


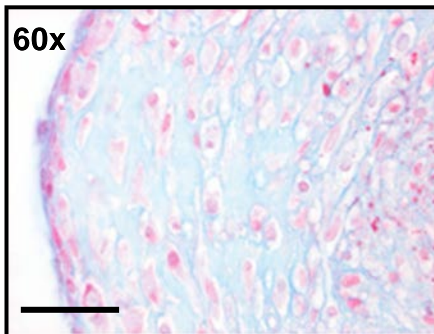
Control pellet

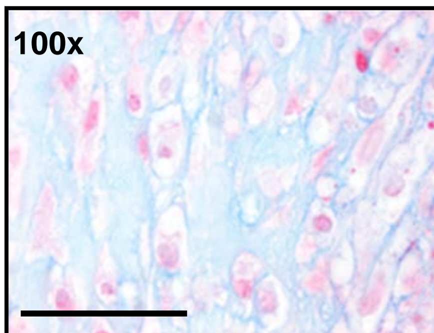


B

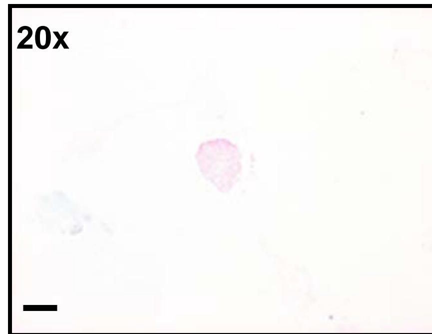
Differentiation

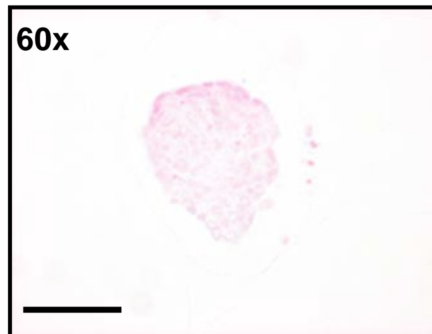






Control





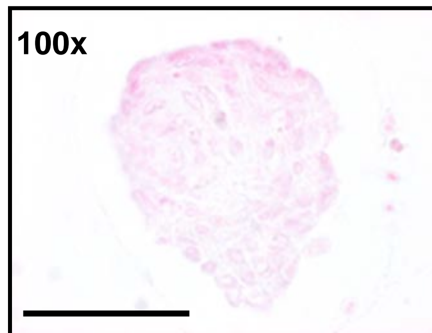


Figure 5 (Spaas *et al.*)