

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Isolation, molecular characterization, and in vitro differentiation of bovine Wharton's jelly-derived multipotent mesenchymal cells**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1661007> since 2018-02-28T12:09:59Z

*Published version:*

DOI:10.1016/j.theriogenology.2016.09.042

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

# Accepted Manuscript

Isolation, molecular characterization and *in vitro* differentiation of bovine Wharton's jelly-derived multipotent mesenchymal cells

Anna Lange-Consiglio, Claudia Perrini, Alessia Bertero, Paola Esposti, Fausto Cremonesi, Leila Vincenti



PII: S0093-691X(16)30461-7

DOI: [10.1016/j.theriogenology.2016.09.042](https://doi.org/10.1016/j.theriogenology.2016.09.042)

Reference: THE 13841

To appear in: *Theriogenology*

Received Date: 12 July 2016

Revised Date: 21 September 2016

Accepted Date: 23 September 2016

Please cite this article as: Lange-Consiglio A, Perrini C, Bertero A, Esposti P, Cremonesi F, Vincenti L, Isolation, molecular characterization and *in vitro* differentiation of bovine Wharton's jelly-derived multipotent mesenchymal cells, *Theriogenology* (2016), doi: 10.1016/j.theriogenology.2016.09.042.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1  
2 **Isolation, molecular characterization and *in vitro* differentiation of bovine Wharton's jelly-**  
3 **derived multipotent mesenchymal cells**  
4

5 Anna Lange-Consiglio<sup>a</sup>, Claudia Perrini<sup>a</sup>, Alessia Bertero<sup>b</sup>, Paola Esposti<sup>a</sup>, Fausto Cremonesi<sup>a\*</sup>,  
6 Leila Vincenti<sup>b</sup>

7 <sup>a</sup>*Reproduction Unit, Large Animal Hospital, Università degli Studi di Milano, Italy*

8 <sup>b</sup>*Department of Animal Science, Torino, Italy*  
9

10 \*Corresponding author: Fausto Cremonesi, Università degli Studi di Milano, Large Animal  
11 Hospital, Reproduction Unit, Via dell'Università 6, 26900 Lodi. E-mail:  
12 [fausto.cremonesi@unimi.it](mailto:fausto.cremonesi@unimi.it). Telephone number: tel +39-0250331150; fax: +39-0250331115.  
13

14 **ABSTRACT**

15 Extra-fetal tissues are a non-controversial and inexhaustible source of mesenchymal stem cells  
16 (MSCs) that can be harvested non-invasively at low cost. In the veterinary field, as in man, stem  
17 cells derived from extra-fetal tissues express plasticity, reduced immunogenicity, and have high  
18 anti-inflammatory potential making them promising candidates for treatment of many diseases.  
19 Umbilical cord mesenchymal cells have been isolated and characterized in different species, and  
20 have recently been investigated as potential candidates in regenerative medicine. In this study, cells  
21 derived from bovine Wharton's jelly (WJ) were isolated for the first time by enzymatic methods,  
22 frozen/thawed, cultivated for at least ten passages and characterized. Wharton's jelly (WJ)-derived  
23 cells readily attached to plastic culture dishes displaying typical fibroblast-like morphology and,  
24 although their proliferative capacity decreased to the seventh passage, these cells showed a mean  
25 doubling time of  $34.55 \pm 6.33$  hours and a mean frequency of 1 colony forming unit fibroblast-like  
26 (CFU-F) for every 221.68 plated cells. The results of molecular biology studies and flow cytometry

27 analyses revealed that WJ-derived cells showed the typical antigen profile of MSCs and were  
28 positive for CD29, CD44, CD105, CD166, Oct-4 and c-Myc. They were negative for CD34 and  
29 CD14. Remarkably, WJ-derived cells showed differentiation ability. After culture in induced media,  
30 WJ-derived cells were able to differentiate into osteogenic, adipogenic, chondrogenic and  
31 neurogenic lines as shown by positive staining and expression of specific markers. On PCR  
32 analysis, these cells were negative for *MHC-II* and positive for *MHC-I*, thus reinforcing the role of  
33 extra-fetal tissue as an allogenic source for bovine cell-based therapies. These results provide  
34 evidence that bovine WJ-derived cells may have the potential to differentiate to repair damaged  
35 tissues and reinforce the importance of extra-fetal tissues as stem cell sources in veterinary  
36 regenerative medicine. A more detailed evaluation of their immunological properties is necessary to  
37 better understand their potential role in cellular therapy.

38

39 *Key words:* bovine, Wharton's jelly, stromal cells, characterization

40

## 41 **1. INTRODUCTION**

42 Increasing interest in veterinary stem cell therapy has led to research into new stem cell sources that  
43 can supply sufficient numbers of cells whilst minimizing risks to donors and recipients [1]. Extra-  
44 fetal stem cells are being investigated for this purpose in large animals [2]. Extra-fetal stem cells  
45 have been isolated from umbilical cord blood, amniotic fluid, amniotic membrane, umbilical cord  
46 matrix, yolk sac and placenta [3-9]. It has been shown that they possess properties of mesenchymal  
47 stem cells, and their application in regenerative medicine has increased over the past few years [10].  
48 These studies show that umbilical cord, previously considered as a biomedical waste, is a source of  
49 stem cells with promising therapeutic applications in man as well as in livestock species.  
50 The umbilical cord provides an inexhaustible source of stem cells and presents few, if any, ethical  
51 concerns. Indeed, umbilical cord can be collected after parturition since it is considered a waste  
52 tissue. In addition, the process of obtaining the cord tissue is relatively simple and non- invasive.

53 Mesenchymal stem cells (MSCs) have been isolated from different compartments of the umbilical  
54 cord in different species. In particular, they were obtained invasively, by a surgical intrauterine  
55 approach, from umbilical cord blood of sheep [11,12], non-invasively at the time of birth in the  
56 horse [13], in cattle, after caesarean section delivery [14], and from canine and feline fetuses at birth  
57 [15,16]. In 2006, for the first time in veterinary medicine, MSCs from cordon matrix, called  
58 Wharton's jelly (WJ), were obtained from porcine umbilical cord [17]. Wharton's jelly is the  
59 embryonic mucous connective tissue lying between the amniotic epithelium and the umbilical  
60 vessels [18]. It encloses the yolk sac, which is the source of primordial germ cells and the first  
61 hematopoietic stem cells [18]. Extra-embryonic tissues, which originate from the hypoblast and the  
62 trophoctoderm, do not participate in gastrulation, therefore they are less differentiated than adult  
63 somatic tissues such as bone marrow or adipose tissue [2]. Wharton's jelly is a rich source of MSCs  
64 [19-21]. Recently, primitive MSCs were isolated from the umbilical cord matrix in caprine [22,23],  
65 canine [24] and equine species [4,13,25]. These cells show specific markers of pluripotency and  
66 MSCs markers and are able to differentiate into adipogenic, chondrogenic, osteogenic and  
67 neurogenic tissues [2]. The similarities in growth kinetics and expression of markers of pluripotency  
68 indicate their close resemblance to embryonic stem cells [26]. These markers, found in mouse and  
69 human embryonic stem cells, include the POU (Pit/Oct/Unc) domain-containing protein Oct-4, Sox-  
70 2 and Nanog. Some authors have shown that human umbilical cord matrix expresses low levels of  
71 transcription factors that play a central role in the regulation of pluripotency and self-renewal [18].  
72 In contrast however, Carlin et al. [17] showed that cells derived from porcine umbilical cord matrix  
73 express markers of embryonic lineage Oct-3/4, Sox2 and Nanog. Contrary to observations in adult  
74 MSCs, WJ-MSCs share properties unique to fetal-derived MSCs, such as more rapid proliferation  
75 and greater *ex vivo* expansion capabilities [18,27]. Moreover, they have high potential to be  
76 differentiated *in vitro* [28], they express HLA-class I surface markers but do not express HLA-class  
77 II markers [29], and they are immunosuppressive in mixed lymphocyte assays by inhibition of T-  
78 cell proliferation [30,31]. For these reasons, these cells have raised interest for their potential uses in

79 cell and gene therapy, cloning, virological and biotechnological studies [32].  
80 Despite the importance of bovine species as models for *in vivo* studies, little it is known about  
81 bovine MSCs. So far, they have been derived from umbilical cord blood [14], bone marrow [33,34]  
82 and amniotic membrane or amniotic fluid [8]. To date, only one paper on isolation and  
83 characterization of MSCs from bovine umbilical cord matrix [32] had been published but these cells  
84 were isolated by migration techniques and not by classical enzymatic digestion. The mechanical and  
85 enzymatic disaggregation of the tissue avoids problems of selection by migration, but perhaps more  
86 importantly, yields a higher number of cells more representative of the whole tissue in a shorter  
87 time. However, as well as the primary explant technique selecting on the basis of cell migration, the  
88 dissociation technique selects cells resistant to disaggregation but still capable of attachment [35].  
89 The isolation of bovine MSCs from fetal adnexa is an interesting prospect because of the potential  
90 for these cells to be used for biotechnological applications. For the first time, we isolated, by  
91 enzymatic methods, presumptive MSCs from bovine WJ and were able to characterize them in  
92 terms of morphology, specific mesenchymal or pluripotent markers, proliferative and differentiation  
93 potential.

94

## 95 **2. MATERIALS AND METHODS**

### 96 *2.1 Materials*

97 Chemicals were obtained from Sigma Chemical (Milan, Italy) and tissue culture plastic dishes from  
98 Euroclone (Milan, Italy) unless otherwise specified.

99

### 100 *2.2 Tissue collection*

101 This study was approved by the Ethical Committee of the University of Milan and written owner  
102 consent was given. All procedures were conducted following standard veterinary practice and in  
103 accordance with 2010/63 EU directive on animal protection and Italian Law (D.L. No. 116/1992).

104 Fresh bovine umbilical cords were obtained after full-term births.

105 Bovine umbilical cords (n=3) were obtained from three cows following normal term pregnancies  
106 with spontaneous parturition in accordance with veterinary practice standard. Before the cows stood  
107 up breaking the cord, a surgical tape was placed at the calf junction and a second tie was tightened  
108 at approximately 30/40 cm from the first. The tie-limited cord portion was cut away with scissors.  
109 The harvested segment of the cord was washed twice in sterile saline solution and kept at 4°C in  
110 saline solution supplemented with 4 µg/mL amphotericin, 100 UI/mL penicillin and 100 µg/mL  
111 streptomycin and processed within 12 hours of collection.

112

### 113 *2.3 Isolation and culture of WJ-derived cells*

114 At the laboratory, the loose amnion was removed from the exterior of the cords and the cords were  
115 incised longitudinally to expose and remove umbilical vessels (arteries and veins). The remaining  
116 WJ-containing tissue was minced into small pieces using scissors. The tissue was digested in HG-  
117 DMEM supplemented with 1mg/mL collagenase type I at 38.5 °C for 8 hours. After incubation,  
118 collagenase was inactivated by diluting 1:1 with HG-DMEM supplemented with 10% fetal calf  
119 serum (FCS). The digested suspension was filtered on an 80 µm strainer, centrifuged at 300 g for 10  
120 minutes and washed twice in PBS. Before seeding, cells were counted using a Burker chamber with  
121 the Trypan Blue dye exclusion assay. WJ-cell cultures were established in HG-DMEM standard  
122 medium composed of 10% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL  
123 amphotericin B, 2 mM L-glutamine and 10 ng/mL EGF. Cultures were established at a density of  
124  $1 \times 10^5$  cells/cm<sup>2</sup> in T75 culture flasks. The flasks were incubated at 38.5 °C with 5% CO<sub>2</sub> and 90%  
125 humidity. The medium was replaced after 72 hours to remove non-adherent cells and then replaced  
126 twice weekly until cells reached approximately 80% confluence. Cells were then detached with  
127 0.05% trypsin-EDTA, counted, and redistributed into new culture flasks at a density of  $1 \times 10^4$   
128 cells/cm<sup>2</sup> to maintain and expand the culture for ten passages (P).

129

130

131 *2.4 Cryopreservation and thawing*

132 Cells at P3 were cryopreserved. Briefly, the confluent cultures were treated with 0.05% trypsin-  
133 EDTA and washed by centrifugation (200g at 4 °C, for 5 minutes) with cell culture medium  
134 supplemented with 10% FCS to neutralize trypsin-EDTA. The cell pellet obtained was resuspended  
135 in pre-cooled (4 °C) cryopreservation media in 1-mL cryovials. The cryopreservation medium was  
136 90% (v/v) FBS and 10% dimethyl sulfoxide. The cryovials were maintained at -80 °C overnight and  
137 then plunged into liquid nitrogen (-196 °C). After a minimum of one month of cryopreservation, the  
138 cells were thawed in a water bath at 37 °C. The cells were diluted in culture medium and  
139 centrifuged twice at 200g for 10 minutes. The cell pellet was resuspended in culture medium and  
140 plated in T25 culture flasks. Aliquots of these cells were kept to evaluate the cell viability using  
141 Burker hemocytometer chamber using the trypan blue dye exclusion method, under phase contrast  
142 microscopy (Nikon Eclipse Ti, Tokyo, Japan). After thawing, other aliquots were used for  
143 population doubling studies, or expanded until P3 to evaluate specific MSC marker expression and  
144 multipotent differentiation capacity in comparison to freshly isolated cells.

145

146 *2.5 Proliferation rate and CFU-assay*

147 Proliferation of MSCs was determined as previously reported [6]. Doubling time (DT) from P 1-10  
148 was assessed by plating  $9 \times 10^3$  cells into six-well tissue culture plates. Every 4 days, cells were  
149 trypsinized, counted and reseeded at the same density. Mean DT was calculated from day 0 to day  
150 4. The DT value was obtained for each P according to the formula  $DT = CT/CD$ , where CT  
151 represents the culture time and  $CD = \log(N_c/N_o)/\log 2$  represents the number of cell generations ( $N_c$   
152 represents the number of cells at confluence,  $N_o$  represents the number of seeded cells). Data  
153 representative of three independent experiments are reported.

154 To obtain the cell proliferation growth curve, cells at P0, P3 and P5 were seeded into six-well tissue  
155 culture dishes at a density of  $1 \times 10^3$  cells/cm<sup>2</sup>. Every 2 days, until 14 days of culture, one well out



156 of the six was trypsinized and cells were counted using the Trypan blue dye exclusion method with  
157 a Burker chamber.

158 Colony-forming unit (CFU) assays were performed at P0 on freshly isolated cells at different  
159 densities (100, 250, 500 and 1000 cells/cm<sup>2</sup>). Cells were plated in six-well plates for two weeks in  
160 HG-DMEM standard medium. Then, colonies were fixed for 1 hour with 4% formalin and stained  
161 with 1% methylene blue for 15 minutes in 10 mM borate buffer at room temperature. Colonies  
162 formed by 16 to 20 nucleated cells were counted under a BX71 microscope (Olympus Italia, Srl,  
163 Milano, Italy).

164

#### 165 *2.6 Osteogenic, adipogenic, chondrogenic and neurogenic cell differentiation*

166 All the differentiation tests were performed when cells reached P3.

167 For osteogenic differentiation, cells were placed in plastic six-well plates at a density of  $28 \times 10^3$   
168 cells per well. After the cells had adhered to the plastic, the inducer medium was added to the plate  
169 for 21 days and refreshed every three days. The medium was composed of HG-DMEM, 10% FCS,  
170 penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, 200 mM L-  
171 glutamine, 0.25 mM ascorbic acid, 10 mM β-Glycerophosphate, and 0.1 uM Dexamethasone [36].  
172 Osteogenic differentiation was confirmed by positive staining of the extracellular calcium matrix  
173 using Von Kossa staining.

174 For adipogenic differentiation, cells were placed in plastic six-well plates at a density of  $28 \times 10^3$   
175 cells per well. After the cells had adhered to the plastic, the inducer medium and the maintaining  
176 medium were added alternately, every 3 days for a total of 21 days. The inducer medium was  
177 composed of HG-DMEM, 10% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25  
178 µg/mL amphotericin B, 200 mM L-glutamine, 0.1% insulin, 0.1 uM dexamethasone, and 1%  
179 indomethacin. The maintaining medium was composed of HG-DMEM, 10% FCS, penicillin (100  
180 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, and 0.1% insulin [36]. Adipogenic  
181 differentiation was confirmed by positive staining of the lipid structures using Oil Red O staining.

182 For chondrogenic differentiation, cells were cultured in DMEM low-glucose, containing 100U/mL  
183 penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 2 mM/l L-glutamine, 100 nM  
184 dexamethasone, 50 µg/mL L-ascorbic acid 2-phosphate, 1 mM sodium pyruvate (BDH, Atlanta,  
185 USA), 40 µg/mL proline, ITS (insulin 5 µg/mL, transferrin 5 µg/mL, selenous acid 5 ng/mL) and 5  
186 ng/mL transforming growth factor-β3 (Peprovet, DBA Italia, 100-36E). Chondrogenic  
187 differentiation was assessed after incubating cells for up to 3 weeks [37]. Differentiation was  
188 evaluated by Alcian blue staining.

189 For neurogenic differentiation, cells were placed in plastic six-well plates at a density of  $28 \times 10^3$   
190 cells per well. After the cells had adhered to the plastic, the pre-inducer medium was administrated  
191 to the plates for 1 day, followed by administration of inducer medium for 7 days. The pre-inducer  
192 medium was composed of HG-DMEM, 20% FCS, penicillin (100 UI/mL)-streptomycin (100  
193 µg/mL), 0.25 µg/mL amphotericin B, and 0.0007% β-mercaptoethanol [15,38]. The inducer  
194 medium was composed of HG-DMEM, 2% FCS, penicillin (100 UI/mL)-streptomycin (100  
195 µg/mL), 0.25 µg/mL amphotericin B, 1% DMSO, and 0.36 mg/mL BHA [39]. Neurogenic  
196 differentiation was confirmed by positive staining of the Nissl substance and granules, using Nissl  
197 staining.

198 For each differentiation experiment, a control group was performed by seeding cells at lower  
199 density ( $9.5 \times 10^3$ ) and feeding with HG-DMEM standard medium. These cells were stained using  
200 the same protocol as the treated cells. At the end of the differentiations, aliquots of non-stained cells  
201 were harvested and stored at -80 °C for further molecular analysis.

## 202 203 *2.7 RNA extraction and Reverse Transcription-PCR analysis*

204 Expression of specific MSC (*CD29*, *CD44*, *CD105*, *CD166*), pluripotent- (*Oct-4* and *c-Myc*) and  
205 hematopoietic (*CD34*, *CD14*, *CD45*) markers was investigated by RT-PCR analysis on fresh and  
206 thawed undifferentiated cells at P3. To evaluate whether cells could be well tolerated by the host  
207 once transplanted, expression of the Major Histocompatibility Complex, class I (*MHC-I*) and II

208 (*MHC-II*) was assessed. Total RNA was extracted from bovine WJ-derived cells using Trizol  
209 reagent (Invitrogen, Monza, Italy), followed by DNase treatment according to the manufacturer's  
210 specifications. RNA concentration and purity were measured using a NanoDrop ND1000  
211 spectrophotometer (*NanoDrop* Technologies, Wilmington, DE, USA). cDNA was synthesized from  
212 500 ng total RNA, using the iScript retrotranscription kit (Bio-Rad Laboratories, Hercules, CA,  
213 USA). Conventional PCR was performed in a 25  $\mu$ L final volume with DreamTaq DNA  
214 Polymerase (Fermentas, St. Leon Rot, Germany) under the following conditions: initial  
215 denaturation at 95 °C for 2 minutes, 32 cycles at 95 °C for 30 seconds (denaturation), 55–63 °C for  
216 30 seconds (annealing), 72 °C for 30 seconds (elongation) and final elongation at 72 °C for 10  
217 minutes.

218 For differentiation experiments, total RNA was extracted from undifferentiated (control cells) and  
219 from induced WJ-derived cells and RT-PCR analysis was performed as described above. Bovine  
220 adult tissues (bone, fat, cartilage and spinal cord) were employed as positive controls for assessing  
221 the expression of *BGLAP*, *SPP1* and *SPARC* for osteogenic differentiation, peroxisome proliferator-  
222 activated receptor-gamma (*PPAR- $\gamma$* ), and leptin (*LEP*) for adipogenesis, collagen type 2 $\alpha$ 1  
223 (*COL2A1*) and aggrecan (*ACAN*) for chondrogenesis, and glial fibrillary acidic protein (*GFAP*) and  
224 nestin (*NES*) for neurogenesis. Bovine-specific oligonucleotide primers were designed using open  
225 source PerlPrimer software v. 1.1.17, based on available NCBI *Bos taurus* sequences or on Mammal  
226 multi-aligned sequences. Primers were designed across an exon–exon junction in order to avoid  
227 DNA amplification. Primers were used at 300 nM final concentrations. Their sequences and the  
228 conditions used to amplify each gene are shown in Table 2. *GAPDH* was employed as a reference  
229 gene.

230

### 231 2.8 Flow cytometry

232 WJ-derived cells were analyzed by flow cytometry to determine the percentage of mesenchymal-  
233 (CD105, CD166), hematopoietic (CD34) and immunogenic (MHC-II) markers after isolation (P0).

234 For CD105, CD166 and CD34, primary mouse monoclonal antibodies and secondary antibodies  
235 rabbit anti-mouse FITC (Sigma, Milan, Italy) were used. For MHC-II, primary rat monoclonal  
236 antibody and secondary rabbit anti-rat FITC (Sigma) were used. Staining was performed as  
237 previously reported [40]. Cells ( $1 \times 10^6$  cells/mL) were labeled with primary antibodies in PBS with  
238 3% of bovine serum albumin (BSA) (BDH; VWR International Ltd, Poole, UK) for 45 minutes at  
239 room temperature in the dark, followed by washing in cold PBS and a final incubation with  
240 secondary antibodies (1:50) for 30 minutes at room temperature in the dark. After incubation, cells  
241 were washed twice in ice-cold PBS and analyzed using a Millipore Guava easyCyte Single Sample  
242 Flow Cytometer. A minimum of 10,000 cells was acquired for each sample and analyzed in the FL1  
243 channel.

244 The negative pattern was examined by applying the same cell suspension with the first incubation,  
245 and the result was included on the global compensation, in order to exclude auto fluorescence. A  
246 488 nm filter was used in each analysis.

247 Off-line analyses of the flow cytometry standard (FCS) files were performed using Weasel software  
248 v.2.5 (<http://en.bio-soft.net/other/WEASEL.html>).

249

## 250 2.9 Statistical analysis

251 Statistical analysis was performed using GraphPad InStat 3.00 for Windows (GraphPad Software,  
252 La Jolla, CA, USA). Three replicates were performed for each experiment (DT and CFU) and the  
253 results reported as mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) for  
254 multiple comparisons by Student-Newman-Keuls multiple comparison tests was used.  $P < 0.05$  was  
255 considered as significant.

256

## 257 3. RESULTS

### 258 3.1 Cell morphology

259 The cellular yield was approximately  $3 \times 10^6$  cells per gram of minced WJ. The initial viability was

260 greater than 95%.

261 Cells were selected purely on their ability to adhere to plastic. Isolated cells readily attached to  
262 plastic culture dishes. Colonies started to appear within the first two days, reaching confluence after  
263 5 days. WJ-derived cells were a morphologically homogeneous population of fibroblast-like cells in  
264 all passages of cell culture (Figure 1 A).

265 After thawing (at P3), viability was 80%. WJ-derived cells conserved their fibroblast-like shape.

266

267 *3.2 Proliferation studies* Cells WJ-derived cells were able to proliferate, reaching confluence in up  
268 to 10 passages.

269 At P1, the growth curve showed an initial lag phase of 3-4 days longer than that seen in the other  
270 passages and a subsequent log phase until 14 days. The WJ-derived cells showed more extensive  
271 proliferation at P5 but the intensity of proliferation was similar in all passages in the final culture  
272 days (Fig 1B).

273 DT remained constant until the seventh passage, then decreased significantly ( $P < 0.05$ ) until passage  
274 10. The mean DT was  $1.44 \pm 0.24$  days or  $34.55 \pm 6.33$  hours (Fig 1C).

275 After thawing, the mean DT was  $1.59 \pm 0.06$  days or  $38.21 \pm 1.68$  hours (Fig 1C) with no statistical  
276 difference compared to fresh cells.

277 The number of cell colonies formed was counted at P0 on fresh cells. These cells were able to form  
278 an average of 1 CFU-F) (Fig 1D) for  $221.68 \pm 3.86$  seeded cells. The highest number of colonies  
279 was found at the greatest density of seeding (Table 1).

280

281 *3.3 Molecular characterization*

282 As shown by RT-PCR, WJ-derived cells expressed MSC-markers (*CD29*, *CD44*, *CD105*, *CD166*)  
283 and lacked hematopoietic ones (*CD34* and *CD14*) from P0 to P10. *MHC-I* expression was present  
284 while *MHC-II* was not. Moreover, undifferentiated MSCs were found to express *Oct-4* and *c-Myc*,  
285 essential transcription factors for maintaining the primitive pluripotent state of embryonic stem

286 cells. After thawing, cells studied at P3 expressed the same MSC-mRNA markers such as *CD29*,  
287 *CD44*, *CD105*, *CD166*, *Oct-4*, *c-Myc* and *MHC-I*, as freshly isolated cells, but not *CD34*, *CD14* and  
288 *MHC-II*. (Figure 2A).

289

### 290 3.4 Flow cytometry analysis

291 Flow cytometry was used to evaluate the homogeneity of the cell population and to identify the  
292 subset of mesenchymal, hematopoietic and immunogenic cells. The cell populations tested were all  
293 *CD105*<sup>+</sup> and *CD166*<sup>+</sup>, but negative for *CD34* and *MHC-II*, as shown in Fig 2B.

294

### 295 3.5 Differentiation assay

296 The results of all differentiation assays are shown in Fig 3.

297 *Osteogenic differentiation potential*. After 21 days of induction, osteogenic differentiation was  
298 confirmed by von Kossa staining. The control (non-induced cells) was negative for von Kossa  
299 staining. RT-PCR analysis of *SPP1* and *SPARC* mRNA expression confirmed osteogenic induction  
300 but *BGLAP* was not expressed in induced cells.

301 *Adipogenic differentiation potential*. Cells were able to undergo adipogenic differentiation, as  
302 demonstrated by the development of positive staining for Oil Red O after 3 weeks of culture in  
303 adipogenic induction medium. Control cells, maintained in regular control medium, showed no lipid  
304 deposits. RT-PCR analysis of *PPAR-γ* and *LEP* mRNA expression confirmed adipogenic induction.

305 *Chondrogenic differentiation potential*. Differentiation was identified by marked deposition of  
306 glycosaminoglycans in the matrix, stained with Alcian blue. The presence of *COL2A1* and *ACAN*  
307 mRNA confirmed chondrogenic induction for this cell population.

308 *Neurogenic differentiation potential*. After 3 days of induction, neurogenic differentiation was  
309 confirmed by the morphology of the cells. The WJ-derived cells adopted the typical morphology of  
310 neural cells with dendrite-like processes. The presence of *GFAP* mRNA suggested that under these  
311 culture conditions, WJ-derived cells were induced to differentiate into glial cells. RT-PCR analysis

312 of bovine adult tissues (bone, fat, cartilage and spinal cord) showed expression of the specific  
313 studied genes.

314 The frozen-thawed cells were able to differentiate toward the same lineages as freshly isolated cells  
315 (data not shown).

316

#### 317 **4. DISCUSSION**

318 This work allowed the isolation, characterization and differentiation of bovine stem cells derived  
319 from WJ. Our findings suggest that this tissue is a reliable source of presumptive stem cells,  
320 displaying intermediate features between adult and embryonic stem cells. These cells have wide  
321 potential clinical applications, because of their low immunogenicity and high differentiation  
322 potential. After digestion, large numbers of WJ-derived cells with > 95% viability (optimal value in  
323 terms of plating efficiency and cellular growth) were obtained. When cultured, these cells  
324 demonstrated strong adherence to plastic dishes and developed fibroblast-like morphology over  
325 time. Adherence is a fundamental property for the culture of stem cells [41]. The proliferation  
326 studies showed that WJ-derived cells reached high plating efficiency and had a high proliferation  
327 rate *in vitro* until P10, demonstrating a growth curve with a *lag* phase of few hours and an intensive  
328 *log* phase of 12 days. Moreover, the mean value of DT for 10 passages was 34.55 hours. During this  
329 intensive proliferation, the cells maintained their morphological characteristics. These data are in  
330 agreement with those obtained by other researchers who reported a high proliferation rate of human  
331 [37,42], equine [6], bovine [8] and feline [43] extra-fetal derived cells. It is very difficult to  
332 compare these data with those obtained from Cardoso et al. [32] because this study only reported  
333 the number of cells per mL found at different passages and the DT value was not calculated.

334 Bovine WJ-derived cells also showed the ability to produce clones. When seeded at different  
335 densities, they were able to form clones with a frequency that increased with the cell-seeding  
336 density, suggesting paracrine signaling between cells at P0 [44]. Moreover, WJ-derived cells  
337 showed a typical expression pattern expected for cultured stem cells [41] when analyzed by RT-



338 PCR. Indeed, these cells expressed a pattern of mesenchymal (*CD29*, *CD44*, *CD105*, *CD73*,  
339 *CD166*) and pluripotency (*Oct4*, *c-Myc*) genes with no expression of the hematopoietic *CD34*. The  
340 pluripotency genes are essential transcription factors for maintaining the primitive pluripotent state  
341 of embryonic stem cells. These data confirm the results obtained in equine, canine and bovine WJ  
342 [6,8,24,32,45-47] where pluripotent- and mesenchymal-associated markers were expressed.

343 For the first time, the expression of *MHC-I* and *MHC-II*, related to cell immunogenicity, was also  
344 evaluated to assess the usefulness of bovine WJ-derived cells for cell therapy. At each passage,  
345 these cells were negative for *MHC-II* and positive for *MHC-I*, consistent with findings of previous  
346 publications [6,8]. These findings reinforce the role of the extra-fetal tissue as an allogenic source  
347 for cell-based therapies in cattle. It is important to underline that RT-PCR alone is not useful for  
348 characterizing WJ-derived cells and that quantitative analyses are needed to make meaningful  
349 statements about their gene expression. Flow cytometry provides useful quantitative data on the  
350 percentage of cell reactivity. Indeed, we showed that >90% cells were positive for *CD105* and  
351 *CD73* while < 10% were positive for *CD34* and *MHC-II*. These data confirm the mesenchymal  
352 nature of isolated cells, the lack of immunogenicity and underline the homogeneity of this cell line.

353 The capacity of MSCs to differentiate into a variety of cell types (adipocytes, osteocytes and  
354 chondrocytes) [48,49], has aroused interest in cell and gene therapy. Bovine WJ-derived cells,  
355 obtained by enzymatic digestion, were able to differentiate into osteocytes, adipocytes,  
356 chondrocytes and neuron-like cells in the same way as cells obtained by non-enzymatic digestion  
357 [32]. This suggests that these cells are capable of differentiation into multiple germ layers, an  
358 essential characteristic also observed in the pig [17], dog [24], horse [4] and chicken [50]. After 21  
359 days of induction, mineral deposits were confirmed by Von Kossa staining and by the expression of  
360 *SPP1* and *SPARC* but not of *BGLAP*. This might be because *BGLAP* is expressed in terminally  
361 differentiated osteoblasts [51]. When stimulated to differentiate towards the adipogenic lineage,  
362 bovine WJ-derived cells were positive for Oil Red O staining and expressed genes involved in lipid  
363 biosynthesis and storage. mRNAs for *PPAR- $\gamma$* , crucial for the preadipocyte commitment [52], and



364 *LEP*, regarded as a late marker of adipocyte differentiation, were detected. The potential of bovine  
365 WJ-derived cells to undergo chondrogenesis was confirmed by positive Alcian blue staining and  
366 identification of markers commonly associated with the chondrocyte phenotype such as collagen  
367 type II and aggrecan, the most essential cartilage proteoglycan and key markers of chondrocyte  
368 differentiation [53]. *ACAN* expression was demonstrated, whereas a basal level of *COL2A1* was  
369 detected. The low expression of *COL2A1* might be related to the culture conditions in this study  
370 since chondrogenic differentiation of MSCs in monolayer culture appears to be dose-dependent and  
371 time-dependent in relation to the bioactive factors used [32,33]. *GFAP* and nestin, markers,  
372 expressed in neuronal precursor stem cells, have been detected in WJ-derived cells. The expression  
373 of both markers is probably related to the ability of these cells to differentiate either towards the  
374 glial cell lineage, as previously shown by Miki et al. [54] for 95% of cells isolated from human  
375 amnion, or toward neurogenic line as previously observed in pigs [55].

376 Whatever the reason, this cell line converted into a typical neuron-like morphology when  
377 appropriately induced.

378 Our findings suggest that, in agreement with reports of other researchers in several species [6,45-  
379 47,56], bovine WJ represents an alternative source of progenitor cells, that can be obtained by  
380 enzymatic methods for use in cell-based therapies. In our study, the digestion of tissue did not result  
381 in a reduced cellular viability or degradation of cellular surface receptors or alteration of cellular  
382 function as reported by Jeschke et al. [57].

383 Moreover, after thawing, the cryopreserved cells had a high level of viability (80%) and could be  
384 successfully expanded and differentiated. This demonstrates that bovine umbilical cord matrix cells  
385 can tolerate freezing without loss of functional integrity in terms of morphology, presence of  
386 specific stemness markers and differentiation potential, although renewal capacity was slightly  
387 lower than that observed in freshly isolated cells.

388 In conclusion, these data confirm that bovine WJ contain a niche of MSCs. However, further  
389 investigation, including pre-clinical studies and further study of immunological properties, are

390 needed to better understand their role in cellular therapy before *in vivo* applications of WJ-derived  
391 cells are considered. To date, there is only a single literature report of transplantation of caprine WJ-  
392 derived cells for wound healing, which showed promising effects [23]. The findings of this study  
393 reinforce the emerging importance of extra-embryonic tissues for derivation of cells that may be  
394 ideal tools in veterinary regenerative medicine.

395

#### 396 **ACKNOWLEDGEMENTS**

397 This work was supported by grants from Università degli Studi di Milano and by “Bando ricerca  
398 scientifica 2011 - Sezione innovazione tecnologica - Approcci metodologici per la valorizzazione  
399 della razza Piemontese, Cassa di Risparmio di Cuneo”.

400

#### 401 **REFERENCES**

- 402 [1] Marcus-Sekura C, Richardson JC, Harston RK, Sane N, Sheets RL. Evaluation of the human  
403 host range of bovine and porcine viruses that may contaminate bovine serum and porcine  
404 trypsin used in the manufacture of biological products. *Biologicals* 2011;39(6):359–369.
- 405
- 406 [2] Cremonesi F, Corradetti B, Lange Consiglio A. Fetal adnexa derived stem cells from domestic  
407 animal: progress and perspectives. *Theriogenology* 2011;75:1400–1415.
- 408
- 409 [3] In't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FHJ, Willemze R,  
410 Fibbe WE, Kanhai HHH. Amniotic fluid as a novel source of mesenchymal stem cells for  
411 therapeutic transplantation. *Blood* 2003;102:1548–1549.
- 412
- 413 [4] Lange-Consiglio A, Corradetti B, Rutigliano L, Cremonesi F, Bizzaro D. *In vitro* studies of  
414 horse umbilical cord matrix-derived cells and labeling efficiency with magnetic resonance  
415 contrast agents. *The Open Tissue Eng Regen Med J* 2011;4:120-133.

416

417 [5] Yadav PS, Mann A, Singh V, Yashveer S, Sharma RK, Singh I. Expression of pluripotency genes  
418 in buffalo (*Bubalus bubalis*) amniotic fluid cells. *Reprod Dom Anim* 2011;46(4):705–711.

419

420 [6] Lange-Consiglio A, Corradetti B, Bizzaro D, Magatti M, Ressel L, Tassan S, Parolini O &  
421 Cremonesi F. Characterization and potential applications of progenitor-like cells isolated from  
422 horse amniotic membrane. *J Tissue Eng Regen Med* 2012;6:622–635.

423

424 [7] Mann A, Yadav RP, Singh J, Kumar D, Singh B, Yadav PS. Culture, characterization and  
425 differentiation of cells from buffalo (*Bubalus bubalis*) amnion. *Cytotechnology* 2012;65(1):23-  
426 30.

427

428 [8] Corradetti B, Meucci A, Bizzaro D, Cremonesi F, Lange-Consiglio A. Mesenchymal stem cells  
429 from amnion and amniotic fluid in the bovine. *Reproduction* 2013;145:391–400.

430

431 [9] Mançanares CAF, Oliveira VC, Oliveira LJ, Carvalho AF, Sampaio RV, Mançanares ACF,  
432 Souza AF, Meirelles FV, Miglino MA, Ambrósio CE. Isolation and characterization of  
433 mesenchymal stem cells from the yolk sac of bovine embryos. *Theriogenology* 2015;84:887-  
434 898.

435

436 [10] Abdulrazzak H, Moschidou D, Jones G, Guillot PV. Biological characteristics of stem cells  
437 from foetal, cord blood and extraembryonic tissues. *J R Soc Interface* 2010;6: 689–706.

438

439 [11] Hall JG, Martin PL, Wood S, Kurtzberg J. Unrelated umbilical cord blood transplantation for an  
440 infant with beta-thalassemia major. *Am J Pediatr Hematol Oncol* 2004;26:382–385.

441

- 442 [12] Fuchs JR, Hannouche D, Terada S, Zand S, Vacanti JP, Fauza DO. Cartilage engineering from  
443 ovine umbilical cord blood mesenchymal progenitor cells. *Stem Cells* 2005;23:958–964.  
444
- 445 [13] Koch TG, Heerkens T, Thomsen PD, Betts DH. Isolation of mesenchymal stem cells from  
446 equine umbilical cord blood. *BMC Biotechnol* 2007;7(30):26.  
447
- 448 [14] Raoufi MF, Tajik P, Dehghan MM, Eini F, Barin A. Isolation and differentiation of  
449 mesenchymal stem cells from bovine umbilical cord blood. *Reprod Dom Anim* 2011;46(1):95–  
450 99.  
451
- 452 [15] Seo MS, Jeong YH, Park JR, Park SB, Rho KH, Kim HS, Yu KR, Lee SH, Jung JW, Lee YS,  
453 Kang KS. Isolation and characterization of canine umbilical cord blood-derived mesenchymal  
454 stem cells. *J Vet Sci.* 2009;10(3):181-187.  
455
- 456 [16] Brólio MP, Vidane AS, Zomer HD, Wenceslau CV, Ozório JJ, Martins DS, Miglino MA,  
457 Ambrósio CE. Morphological characterization of the progenitor blood cells in canine and feline  
458 umbilical cord. *Microsc Res Tech.* 2012;75(6):766-770.  
459
- 460 [17] Carlin R, Davis D, Weiss M, Schultz B, Troyer D: Expression of early transcription factors  
461 Oct-4, Sox-2 and nanog by porcine umbilical cord (PUC) matrix cells. *Reprod Biol Endocrinol*  
462 2006;4(6):8.  
463
- 464 [18] Troyer DL, Weiss ML. Concise review: Wharton’s Jelly-derived cells are primitive stromal cell  
465 population. *Stem Cells* 2008;26(3):591–599.  
466
- 467 [19] Lee MW, Choi J, Yang MS, Moon YJ, Park JS, Kim HC, Kim YJ. Mesenchymal stem cells

- 468 from cryopreserved human umbilical cord blood. *Biochem Biophys Res Commun*  
469 2004;320:273–278.
- 470
- 471 [20] Nekanti U, Mohanty L, Venugopal P, Balasubramanian S, Totey S, Ta M. Optimization and  
472 scale-up of Wharton's jelly-derived mesenchymal stem cells for clinical applications. *Stem Cell*  
473 *Res* 2010;5:244–254.
- 474
- 475 [21] Seshareddy K, Troyer D, Weiss ML. Method to isolate mesenchymal-like cells from Wharton's  
476 jelly of umbilical cord. *Methods Cell Biol* 2008;86:101–119.
- 477
- 478 [22] Babaei H, Moshrefi M, Golchin M, Nematollahi-Mahani SN. Assess The Pluripotency of  
479 Caprine Umbilical Cord Wharton's Jelly Mesenchymal Cells By RT-PCR Analysis of Early  
480 Transcription Factor Nanog. *Iran J Vet Surg* 2008;3:57-66.
- 481
- 482 [23] Azari O, Babaei H, Derakhshanfar A, Nematollahi-Mahani SN, Poursahebi R, Moshrefi M.  
483 Effects of transplanted mesenchymal stem cells isolated from Wharton's jelly of caprine  
484 umbilical cord on cutaneous wound healing; histopathological evaluation. *Vet Res Commun*  
485 2011;35(4):211-222.
- 486
- 487 [24] Filioli Uranio M, Valentini L., Lange-Consiglio A, Caira M, Guaricci A, L'abbate A, Catacchio  
488 CR, Ventura M, Cremonesi F, Dell'aquila ME. Isolation, proliferation, cytogenetic, and  
489 molecular characterization and in vitro differentiation potency of canine stem cells from foetal  
490 adnexa: a comparative study of amniotic fluid, amnion, and umbilical cord matrix. *Mol Reprod*  
491 *Dev* 2011;78:361–373.
- 492
- 493 [25] Reed SA, Johnson SE. Equine umbilical cord blood contains a population of stem cells that

- 494 express Oct4 and differentiate into mesodermal and endodermal cell types. *J Cell Physiol*  
495 2007;215(2):329–336.
- 496
- 497 [26] Pappa K, Anagnou N. Novel sources of fetal stem cells: where do they fit on the  
498 developmental continuum? *Regen Med* 2009;4:423–433.
- 499
- 500 [27] Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, Troyer D,  
501 McIntosh KR. Immune Properties of human umbilical cord Wharton’s Jelly-derived cells. *Stem*  
502 *Cells* 2008;26(11):2865–2874.
- 503
- 504 [28] Hartmann I, Hollweck T, Haffner S, Krebs M, Meiser B, Reichart B, Eissner G. Umbilical cord  
505 tissue-derived mesenchymal stem cells grow best under GMP-compliant culture conditions and  
506 maintain their phenotypic and functional properties. *J Immunol Methods* 2010;363(1):80–89.
- 507
- 508 [29] La Rocca G, Anzalone R, Corrao S, Magno F, Loria T, Lo Iacono M, Di Stefano A, Giannuzzi  
509 P, Marasà L, Cappello F, Zummo G, Farina F. Isolation and characterization of Oct-4+/HLA-G+  
510 mesenchymal stem cells from human umbilical cord matrix: differentiation potential and  
511 detection of new markers. *Histochem Cell Biol* 2009;131(2):267–282.
- 512
- 513 [30] Francese R, Fiorina P. Immunological and regenerative properties of cord blood stem cells.  
514 *Clinical Immunol* 2010;136(3):309–322.
- 515
- 516 [31] Anzalone R, Lo Iacono M, Corrao S, Magno F, Loria T, Cappello F, Zummo G, Farina F, La  
517 Rocca G. New emerging potentials for human Wharton’s jelly mesenchymal stem cells:  
518 Immunological features and hepatocyte-like differentiative capacity. *Stem Cells Dev*  
519 2010;19(4):423–438.

520

521 [32] Cardoso TC, Ferrari HF, Garcia AF, Novais JB, Silva-Frade C, Ferrarezi MC, Andrade AL,  
522 Gameiro R. Isolation and characterization of Wharton's jelly-derived multipotent mesenchymal  
523 stromal cells obtained from bovine umbilical cord and maintained in a defined serum-free  
524 three-dimensional system. *BMC Biotechnology* 2012;12:18

525

526 [33] Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Isolation and  
527 multilineage differentiation of bovine bone marrow mesenchymal stem cells. *Cell Tissue Res*  
528 2005;319:243–253.

529

530 [34] Bosnakovski D, Mizuno M, Kim G, Ishiguro T, Okumura M, Iwanaga T, Kadosawa T, Fujinaga  
531 T. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet  
532 cultural system. *Exp Hematol* 2004;32:502–509.

533

534

535 [35] Freshney RI. *Culture of animal cells. A manual of basic technique.* Third Edition. Wiley-Liss.  
536 A John Wiley & Sons, Inc., Publication, New York, 1994.

537

538 [36] Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal  
539 human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells*  
540 2003;21:105–110.

541

542 [37] Soncini M, Vertua E, Gibelli L. Isolation and characterization of mesenchymal cells from  
543 human fetal membranes. *J Tissue Eng Regen Med* 2007;1:296–305.

544

545 [38] Mitchell KE, Weiss ML, Mitchell BM. Matrix cells from Wharton's jelly form neurons and

- 546 glia. *Stem Cells* 2003;21:50–60.
- 547
- 548 [39] Woodbury D, Schwarz EJ, Prockop DJ. Adult rat and human bone marrow stromal cells  
549 differentiate into neurons. *J Neurosci Res* 2000;61: 364–370.
- 550
- 551 [40] Corradetti B, Lange-Consiglio A, Barucca M, Cremonesi F, Bizzaro D. Size-sieved  
552 subpopulations of mesenchymal stem cells from intervacular and perivascular equine  
553 umbilical cord matrix. *Cell Prolif* 2011;44:330-342
- 554
- 555 [41] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R,  
556 Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal  
557 stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*  
558 2006;8:315-317.
- 559
- 560 [42] Miki T, Marongiu F, Ellis E, Strom C. Isolation of amniotic epithelial stem cells. *Curr. Protoc.*  
561 *Stem Cell Biol.* 2010;1:1E 3.
- 562
- 563 [43] Rutigliano L, Coradetti B, Valentini L, Bizzaro D, Meucci A, Cremonesi F, Lange-Consiglio A.  
564 Molecular characterization and in vitro differentiation of feline progenitor-like amniotic  
565 epithelial cells. *Stem Cell Res Ther* 2013;4:133.
- 566
- 567 [44] Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord  
568 perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells* 2005;23:220–  
569 229.
- 570
- 571 [45] Lovati AB, Corradetti B, Lange Consiglio A, Recordati C, Bonacina E, Bizzaro D, Cremonesi



- 572 F. Comparison of equine bone marrow-, umbilical cord matrix and amniotic fluid-derived  
573 progenitor cells. *Vet Res Commun* 2011;35:103-121.
- 574
- 575 [46] Iacono E, Brunori L, Pirrone A, Pagliaro PP, Ricci F, Tazzari PL, Merlo B. Isolation,  
576 characterization and differentiation of mesenchymal stem cells from amniotic fluid, umbilical  
577 cord blood and Wharton's jelly in the horse. *Reproduction* 2012a;143:455-468.
- 578
- 579 [47] Chen J, Lu Z, Cheng D, Peng S, Wang H. Isolation and characterization of porcine amniotic  
580 fluid-derived multipotent stem cells. *PLoS One* 2011;6:e19964.
- 581
- 582 [48] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA,  
583 Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal  
584 stem cells. *Science* 1999;284:143–147.
- 585
- 586 [49] Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult  
587 stem cells from bone marrow stroma defines the sequence of cellular and molecular events  
588 during chondrogenesis. *Proc Natl Acad Sci USA* 2002;99:4397–402.
- 589
- 590 [50] Khatri M, O'Brien TD, Sharma JM. Isolation and differentiation of chicken mesenchymal stem  
591 cells from bone marrow. *Stem Cells Dev* 2009;18(10):1485–1492.
- 592
- 593 [51] Wagner E, Luther G, Zhu G, Luo Q, Shi Q, Kim SH, Gao JL, Huang E, Gao Y, Yang K, Wang  
594 L. Review article: Defective osteogenic differentiation in the development of osteosarcoma.  
595 *Sarcoma* 2011;[http:// dx.doi.org/10.1155/2011/325238](http://dx.doi.org/10.1155/2011/325238).
- 596
- 597 [52] Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPARg2: tissue-specific

598 regulator of an adipocyte enhancer. *Genes Dev* 1994;8:1224–1234.

599

600 [53] Han Y, Lefebvre V. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by  
601 securing binding of Sox9 to a far-upstream enhancer. *Mol Cell Biol* 2008;28:4999–5013.

602

603 [54] Miki T, Lehmann T, Cai H, Stolz DB, Strom SC. Stem cell characteristics of amniotic epithelial  
604 cells. *Stem Cell* 2005;23:1549-1559.

605

606 [55] Zheng YM, Zhao XE, An ZX. Neurogenic differentiation of EGFP gene transfected amniotic  
607 fluid-derived stem cells from pigs at intermediate and late gestational ages. *Reprod Domest*  
608 *Anim* 2010;45:e78–e82.

609

610 [56] Iacono E, Cunto M, Zambelli D, Ricci F, Tazzari PL, Merlo B. Could fetal fluid and  
611 membranes be an alternative source for Mesenchymal Stem Cells (MSCs) in the feline species?  
612 A preliminary study. *Vet Res Commun* 2012b; 36(2):107-18.

613

614 [57] Jeschke MG, Gauglitz GG, Phan TT, Herdon DN, Kita K. Umbilical cord lining membrane and  
615 Wharton's jelly-derived mesenchymal stem cells: the similarities and differences. *The Open*  
616 *Tissue Eng Regen Med J* 2011;4:21–27.

617

## 618 **DECLARATION OF INTEREST**

619 The authors declare that there are no conflicts of interest that could be perceived as prejudicing the  
620 impartiality of the reported research.

621

## 622 **CONTRIBUTORS**

623 ALC designed the study, performed isolation cells, acquired, analyzed and interpreted data, and  
624 wrote the article

625 CP performed proliferation study, molecular characterization and approved the final version of  
626 manuscript

627 AB performed cytofluorimetric analyses and approved the final version of manuscript

628 PE performed differentiation study and approved the final version of manuscript

629 FC designed the study, analyzed and interpreted data and revised the manuscript

630 LV conceived and designed the study, collected the umbilical cords, analyzed and interpreted data,  
631 and wrote the article

632

633 **Table 1.** CFU assay

634

Density cells/cm <sup>2</sup>	Total cells	CFU	1 CFU each
100	950	1.5±0.71 <sup>a</sup>	633.33
250	2375	22.33±5.17 <sup>b</sup>	106.36
500	4750	57.92±4.31 <sup>c</sup>	82.01
1000	9500	146.15±2.78 <sup>c</sup>	65.00

635 Different small letters superscripts (a,b,c) indicate statistically different comparisons ( $P<0.05$ ) between cell densities in  
636 the WJ group.  
637

638  
639**Table 2.** Oligonucleotide sequences used for RT-PCR analysis.

Markers	Forward (5'→3')	Reverse (5'→3')	Annealing T	Product length
Integrin $\beta$ -1 ( <i>CD29</i> )	GTTGGTTCTGCAGTTACGATCAG	AACCAAACCCAATTCGGAAGTC	52°C	203
CD44 antigen ( <i>CD44</i> )	AACAGTAGGAGAAGGTGTGG	TCATGAACTGGTCTTGGGTC	61°C	166
Endoglin ( <i>CD105</i> )	ACAAGTCTTGAGAAACAGTC	GATGTCTGGAGAGTCAGCTC	61°C	182
ALCAM ( <i>CD166</i> )	GTATTTATGCTTCAGGTCCT	TCTACCAGGGAGCATTATAGTC	59°C	755
octamer-binding transcription factor 4 ( <i>Oct-4</i> )	CACACTAGGATATACCCAGGC	GGAGATATGCAAGGCAGAGA	60°C	177
v-myc avian myelocytomatosis viral oncogene homolog ( <i>c-Myc</i> )	GCGCCGATTTCGCGAAACTT	TGAGGGGCATCGCTGCAAGC	58°C	214
CD34 molecule ( <i>CD34</i> )	CCTGAAGCTAAATGAGACCT	AACTTCTGTCTGTTGGTC	58°C	173
CD14 molecule ( <i>CD14</i> )	TCCGAAGCCTGACTGGTCTA	TGTCGGCTCCCTTGAGAAAC	56°C	104
Major histocompatibility complex I ( <i>MHC-I</i> )	GATCTCACTGACCTGGCA	CTGAGGAGGTTCCCATCTC	60°C	199
Major histocompatibility complex II ( <i>MHC-II</i> )	CCTCGCTTGCTGAATTTGC	ACAGGTGCCGACTGATGC	53°C	299
Bone Gamma-Carboxyglutamate (Gla) Protein ( <i>BGLAP</i> )	TCGGGCAAAGGCGCAGCCTTC	GCAGGGCTGCAAGCTTAGACG	55°C	231
Secreted Phosphoprotein 1 ( <i>SPP1</i> )	CGCCGATCTAACGTTTCAGAGTC	GACTCTCAATCAGATTGGAATGC	55°C	199
secreted protein acidic and rich in cysteine ( <i>SPARC</i> )	CTGGTCACGCTGTACGAGAG	CGGTGTGAGACAGGTACCCGT	55°C	232
Leptin ( <i>LEP</i> )	CAATGACATCTCACACACGAG	CGGCCAGCAGGTGGAGAAG	55°C	212
Peroxisome Proliferator-activated Receptor ( <i>PPAR-y</i> )	CGCACTGGAATTAGATGACAGC	CACAATCTGTCTGAGGTCTGTC	55°C	199
Collagen type 1, alpha I ( <i>COL1A1</i> )	CGCGGATTTGTTGCTGCTTGC	AGGTCCCATCAGCCCCATTGGT	55°C	269
Aggrecan ( <i>ACAN</i> )	CGCTGTCTCGCCAAGTGTATGG	CGGTTTCAGGGATGCTGACACTC	60°C	175
Glial Fibrillary Acidic Protein ( <i>GFAP</i> )	GGCACCTTGAGGCAGAAGCTC	CTCCTGGAGCTCCCGCACCT	60°C	195
Nestin ( <i>NES</i> )	ACCACTGAGCAGTTCAGCTGG	TTGCAGGTGTCTGCAGCCGT	55°C	187
Glyceraldehyde-3-phosphate dehydrogenase ( <i>GAPDH</i> )	ATGAGATCAAGAAGGTGGTG	CCAAATTCATTGTCGTACCAG	60°C	190

640

641

642

643

644

645

646

647

648

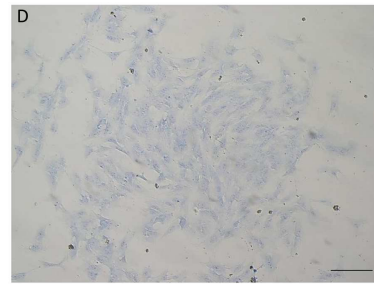
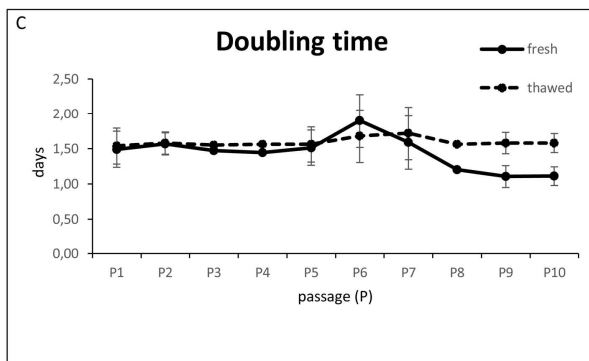
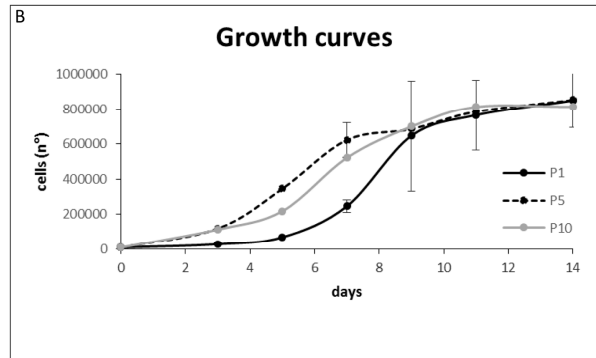
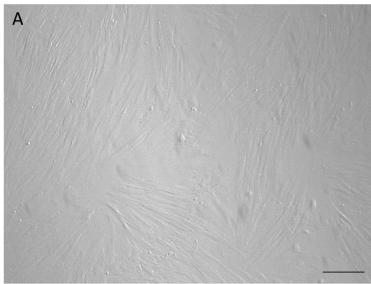
649 **Figure Captions**

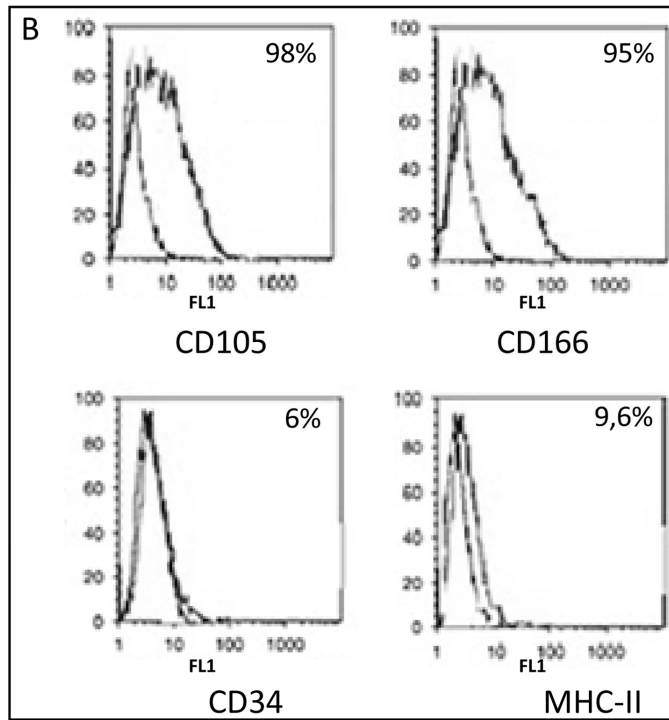
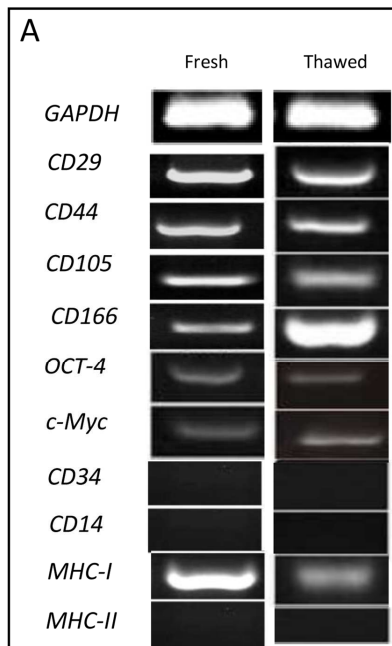
650 **Figure 1.** Cell characteristics. (A) Monolayer of WJ-derived cells (A); magnification 20X; scale  
651 bar= 20  $\mu\text{m}$ . (B) growth curve at passages 1 (P1), 5 (P5) and 10 (P10); (C) doubling times for both  
652 fresh and thawed cells. (D) colony of WJ-derived cells for CFU study. Magnification: X 20; scale  
653 bar= 20  $\mu\text{m}$ .

654  
655 **Figure 2.** (A) RT-PCR analysis of mesenchymal (*CD29*, *CD44*, *CD105* and *CD166*), pluripotent  
656 (*Oct-4* and *c-Myc*), hematopoietic (*CD34* and *CD14*) specific gene expression on WJ-derived fresh  
657 and thawed cells. Major histocompatibility complex (*MHC*) I and II gene expression is also  
658 reported. *GAPDH* was used as the reference gene. (B) Flow cytometry analysis of the expression of  
659 mesenchymal (*CD105* and *CD166*), hematopoietic (*CD34*) and immunogenic (*MHCII*) markers.  
660 Histograms represent relative number of cells vs. fluorescence intensity (FL1). Black histograms  
661 indicate background fluorescence intensity of cells labeled with isotype control antibodies only gray  
662 histograms show positivity to the studied antibodies.

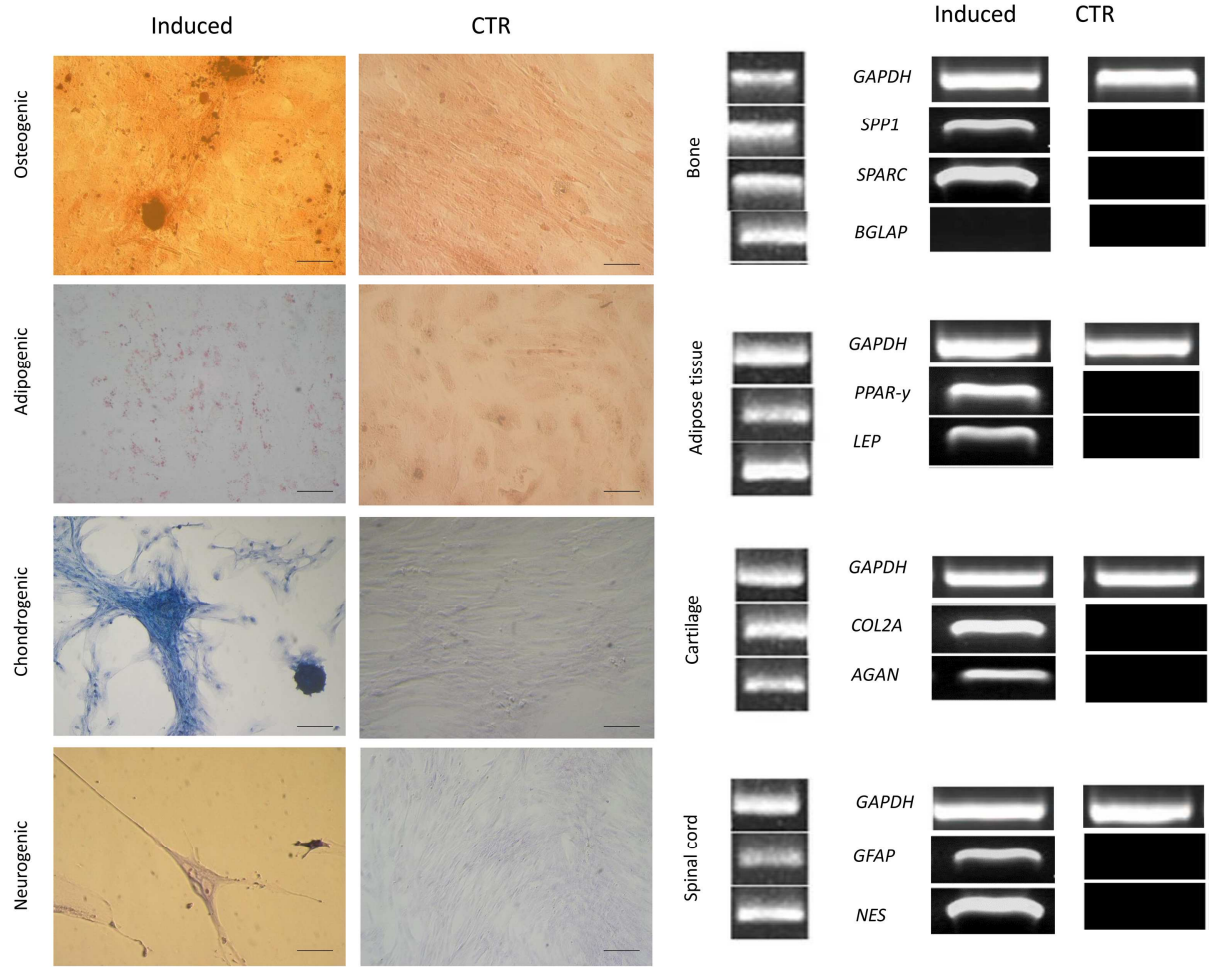
663  
664 **Figure 3.** Staining of differentiated and control (undifferentiated) WJ-derived cells and respective  
665 molecular expression. Osteogenic induced cells were evaluated for von Kossa staining and RT-PCR  
666 analysis of *SPPI*, *SPARC* and *BGLAP*. Adipogenic induced cells were evaluated for Oil Red O-  
667 stained cytoplasmic neutral lipids and RT-PCR of *PPAR- $\gamma$*  and *LEP*. Chondrogenic induced cells  
668 were evaluated for Alcian blue staining and RT-PCR of *COL2A1* and *ACAN*. Neurogenic induced  
669 cells were evaluated for Nissl staining and RT-PCR of *NES* and *GFAP*; magnification: X 20; scale  
670 bar=20  $\mu\text{m}$ . *GAPDH* was employed as the reference gene. Bone, adipose tissue, cartilage and spinal  
671 cord were used as positive controls.

672









ACCEPTED

Bovine Wharton's jelly (WJ)-derived cells were isolated for the first time by enzymatic method  
Molecular biology analyses revealed that these cells showed the CD antigen profile of MSCs  
These cells possessed ability to differentiate in mesodermic and ectodermic lines  
Their negativity to *MHC-II* reinforce the role of these cells as an allogenic source

ACCEPTED MANUSCRIPT