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REGULAR ARTICLE

Isolation, characterization and immunomodulatory-associated gene transcription of Wharton's jelly-derived multipotent mesenchymal stromal cells at different trimesters of cow pregnancy

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Abstract The possibility of isolating bovine mesenchymal multipotent stromal cells (MSCs) from fetal adnexa is an interesting prospect due to the potential use of these cells in biotechnological applications. However, little is known about the properties of these progenitor cells in bovine species. Wharton's jelly (WJ) MSC cells were obtained from the umbilical cord of bovine fetuses at three different stages of pregnancy and divided into groups 1, 2 and 3 according to gestational trimester. Cell morphology, from the three stages of pregnancy, typically appeared fibroblast-like spindle-shaped, presenting the same viability and number. Moreover, the proliferative ability of T-cells in response to a mitogenic stimulus was suppressed when WJMSC cells were added to the culture. Multilineage properties were confirmed by their ability to undergo adipogenic, osteogenic/chondrogenic and neurogenic differentiation. Mesenchymal phenotyping, CD105+, CD29+, CD73+ and CD90+ cell markers were detected in all three cell groups, yet these markers were considered more expressed in MSCs of group 2 ($p < 0.005$). Expression of cytokines *IL2*, *IL6RR*, *INFAC*, *INFB1*, *IFNG*, *TNF* and *LTBR* were downregulated, whereas *IL1F10* expression was upregulated in all tested WJMSCs. The present study demonstrated that WJMSCs harvested from the bovine umbilical cord at different gestational stages showed proliferative capacity, immune privilege and stemness potential.

Keywords Stem cells · Umbilical cord · Immunomodulation · Bovine

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Introduction

Wharton's jelly (WJ) is the primitive mucous, connective tissue of the umbilical cord, lying between the amniotic epithelium and umbilical vessels (Troyer and Weiss 2008; Taghizadeh et al. 2011). First described by Thomas Wharton in 1656, this structure is composed mainly of proteoglycans and collagen (Cremonesi et al. 2011; Corrao et al. 2013). In 2006, in order to pursue standardization, the Mesenchymal and Tissue Stem Cell Committee (ISCT) proposed that: mesenchymal cells must be designed as multipotent mesenchymal stromal cells (MSC); be adherent to plastic culture ware; show specific surface antigen expression; and multipotent differentiation potential (Dominici et al. 2008; Calloni et al. 2014; Iacono and Merlo 2015).

The MSC population in WJ of the umbilical cord (UC) present properties that make it of interest (Pham et al. 2016).

For example, these cells are easy to harvest by non-invasive procedures, provide large number of cells without risk to the donor and can be expanded, genetically manipulated and differentiated in vitro (Troyer and Weiss 2008; Cremonesi et al. 2011; Corrao et al. 2013; Calloni et al. 2014). The immunogenicity of WJMSC has been proposed but is still not clear at present, although immunossupressive effects of bone marrow MSC have been extensively studied and tested in several animal species (Weiss et al. 2008; De Miguel et al. 2012; Mukonoweshuro et al. 2014). Several studies have described WJMSC properties harvested from pregnant women at birth (Prasanna et al. 2010). Nonetheless, WJMSC from bovines (Cardoso et al. 2012) and from buffaloes have been reported only recently (Singh et al. 2013). However, bovine MSCs derived from umbilical cord blood (UCBMSCs), amniotic fluid (AFMSCs) and bone marrow (BMMSCs) have been described (Lu et al. 2011; Raoufi et al. 2011; Corradetti et al. 2013; Cortes et al. 2013). Despite the importance of bovine species as a model for in vitro studies because bovine pregnancy lasts 280 days, as in human beings, there is a lack of information about WJMSCs isolated during cow pregnancy. In contrast to MSCs from different sources, the characterization of bovine MSCs is far from being completely understood, and contradictory information emerges from the literature.

The aim of this study was to isolate and propagate bovine WJMCS cells collected from umbilical cords at three trimesters of pregnancy. Additionally, comparisons were made regarding cell viability, T-cell inhibition, telomerase activity, cell proliferation, phenotype, multipotency and immunomodulatory gene expression. This study highlights a possible potential source of multipotent MSCs and may support their therapeutic and biotechnological use in large animals.

Materials and methods

Isolation of WJMSC from bovine umbilical cord at different stages of pregnancy

Bovine UC were harvested at a slaughterhouse from pregnant Nelore cows ($n = 18$). The bovine pregnancy was divided into three trimesters, as done in humans and gestational periods were estimated by measuring the crown rump length of the fetuses. The first trimester corresponded from 0 to 93 days ($n = 6$ UC; group1 ; Fig. 1a), the second trimester from 94 to 187 days of pregnancy ($n = 6$ UC; group 2; Fig. 1b) and a further 6 umbilical cords were from the third trimester, with the 188-term of pregnancy corresponding to group 3 (Fig. 1c). UCs were collected according to the Animal Care Committee at the University of São Paulo State, Brazil and were conserved at room temperature in sterile phosphate-buffered saline (PBS) supplemented with a penicillin/streptomycin solution containing penicillin 100 μg/ml, streptomycin 10 μg/ml and amphotericin B 250 μg/ml (Sigma-Aldrich, St Louis, MO, USA) until use (within 3 h). Bovine umbilical segments were sectioned longitudinally to expose the WJ after 3 h. Some incisions were made on the matrix and UC fragments were transferred to 25-cm^2 tissue culture flasks (TPP®, Zollstr, SW, Brazil). The initial culture and cell expansion were performed as described previously (Cardoso et al. 2012). Images were taken to observe cell morphology for each group at passage 6 (P6) (Fig. 1d–f), harvested and expanded until they reached subconfluence and then analyzed for their capacity for colony-forming, viability, T-cell proliferation, telomerase activity, phenotype and differentiation as well as for immunomodulatory transcripts.

Cell viability, T-cell proliferation, telomerase activity and cell expansion

Assays for cell viability, T-cell proliferation and telomerase activity were performed according to a previous study (Cardoso et al. 2012). Briefly, cell viability analysis was performed using the In Vitro Toxicology Assay® Kit, MTT-based assay (TOXI-1 Kit; Sigma-Aldrich) following the manufacturer's instructions. In order to evaluate T-cell proliferation, culture and stimulation were performed according to a previous study (Cardoso et al. 2012). Lymphoproliferation was evaluated as counts per minute by a Matrix9600 beta counter (Packard Instrument, Meridien, CT, USA). The ConA was used at 5 μg/ml and PMA and ionomycin at concentrations of 50 ng/ml and 1 μg/ml, respectively (Sigma-Aldrich). A TRAPeze® Telomerase Detection Kit (Millipore, CA, USA) was used to assess the telomerase activity in all groups (Cardoso et al. 2012). The samples were considered positive when the optical density (OD) was ≥0.2 and negative when OD was ≤0.2. All reported values are means of triplicate samples.

Doubling time for passages 1–10 was performed following the procedure described previously (Corradetti et al. 2013). Data representative of three independent experiments were recorded.

In vitro multilineage differentiation assay

The differentiation potential of bovine-derived WJMSC cells was examined using cells at passage number 6 (P6) in all cell groups according to a previous study (Cardoso et al. 2012; Silva et al. 2016). For all procedures 2×10^5 cells/ml were submitted to osteogenic, chondrogenic, adipogenic and neurogenic differentiation according to the manufacturer's instructions (STEMPRO® differentiation medium; Invitrogen). The neurogenic differentiation was adapted from previous studies (Oda et al. 2013). For osteogenic/chondrogenic differentiation, 2 ml of STEMPRO® osteogenic/chondrogenic

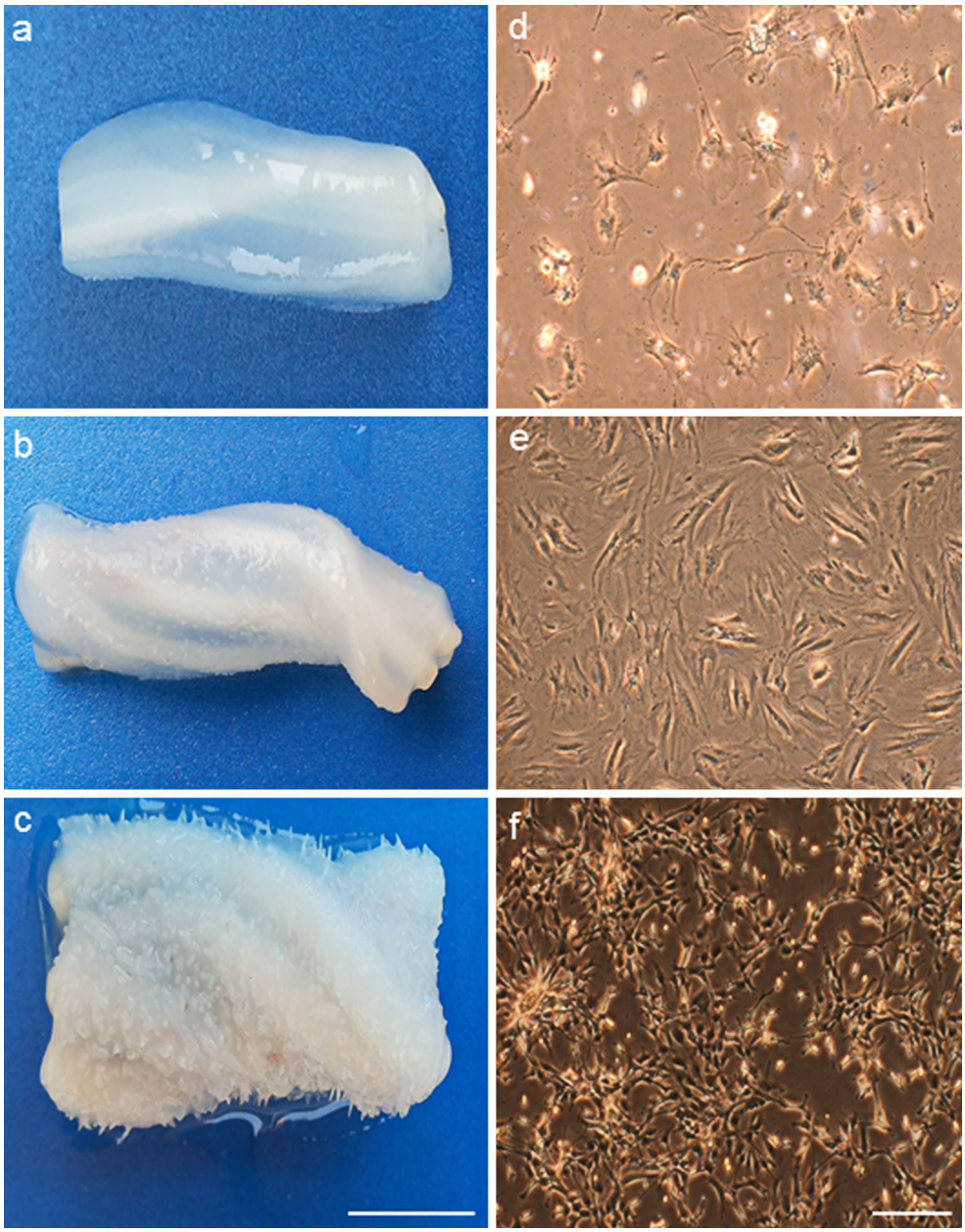


Fig. 1 Bovine umbilical cords analyzed in this study. **a** 0–93 days corresponding to group 1; **b** 94–187 days corresponding to group 2; **c** 188-term of pregnancy corresponding to group 3, *bar* 500 μm. **d** Ex vivo cultured cell obtained from Wharton's jelly zone corresponded to group 1, **e** group 2 and **f** group 3. After P6, spindle-shaped fibroblast-like appearance can be observed under phase contrast microscopy. *Bar* 40 μm

differentiation medium was added to undifferentiated cultures comprising osteogenic and chondrogenic commercial inducers (STEMPRO®). After 15 days of differentiation, cells were fixed with 4 % paraformaldehyde (Sigma-Aldrich). For osteogenic differentiation, Alizarin Red staining (Sigma-Aldrich) was performed (Yang et al. 2015); and for chondrogenic differentiation 0.5 % toluidine blue solution was added (Cardoso et al. 2012).

The adipogenic differentiation followed the described protocol (Cardoso et al. 2012; Silva et al. 2016). In order to verify adipocytes, Oil Red staining was performed (Cardoso et al. 2012). The differentiation of bovine-derived WJMSC cells into neural-like cells followed the procedure described previously, with some modifications (Cardoso et al. 2012; Oda et al. 2013; Silva et al. 2016). The neuronal differentiation was confirmed by immunofluorescence for GFAP and nestin cell markers, as described previously (Cardoso et al. 2012). From each experiment, samples from cell differentiation and undifferentiated cells were harvested for multilineage gene transcription among the three studied groups as described below.

Flow cytometry

Briefly, 2×10^5 cells at P6 were harvested, washed in PBS and incubated for 18 h at 4 °C with monoclonal antibodies: CD34 (hematopoietic precursor cells and MSCs); CD45 (anti-bone marrow lymphoid cells); CD90 (anti-THy1 antigen); CD105

Table 1 Specifications of (*Bos taurus*) cattle gene name, description and location searched by microarray

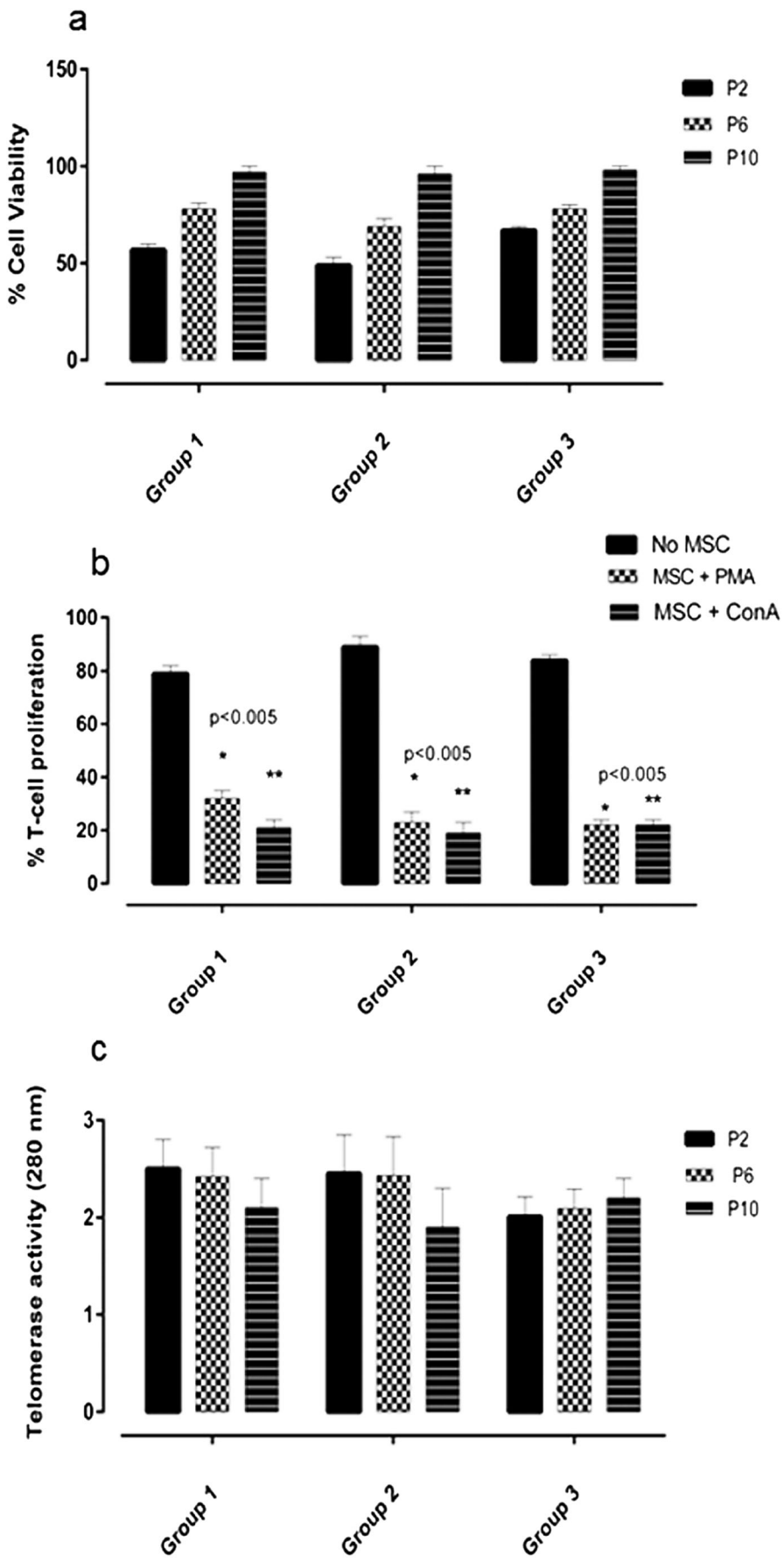
Gene symbol/ID		Description	Location
Positive markers of MSCs			
ENG	615.844	Endoglin (CD105)	Chromosome 11, AC_000.168.1
ITGB1	281.876	Integrin, beta 1 (CD29)	Chromosome 13 AC_000.170.1
NT5E	281.363	5′nucleosidase ecto (CD73)	Chromosome 9, AC_000.166.1
THY1	614.712	Thy-1 cells surface antigen (CD90)	Chromosome 15, AC_ 000.171.1
CD34	281.051	Hematopoietic progenitor cell antigen (CD34)	Chromosome 16, AC_000.954.1
PTPRC	407.152	Protein tyrosine phosphatase, receptor type C (CD45)	Chromosome 16, AC_000.173.1
JSP.1	407.173	Major histocompatibility complex class I (MHCI)	Chromosome 23, AC_ 000.180.1
DSB	618.722	Major histocompatibility complex class II, antigen DS beta (MHC II)	Chromosome 23, AC_000.180.1
Immune-related genes			
IFNAC	281.236	Interferon alpha C (INF-alpha C)	Chromosome 8, AC_000.165.1
INFB1	281.845	Interferon, beta 1, fibroblast	Chromosome 8, AC_000.165.1
IFNG	281.237	Interferon, gamma	Chromosome 5, AC_000.162.1
IL2	280.822	Interleukin 2	Chromosome 17, AC_000.174.1
IL6R	507.359	Interleukin 6 receptor	Chromosome 5, AC_000.162.1
IL1F10	615.702	Interleukin 1, family member 10	Chromosome 11, AC_000.168.1
TNF	280.943	Tumor necrosis factor (TNF alpha)	Chromosome 23, AC_000.180.1
LTBR	280.845	Lymphotoxin beta receptor (TNF superfamily member 3)	Chromosome 5, AC_000.162.1
Positive markers of MSCs multipotency			
LEP	280.836	Leptin	Chromosome 4, AC_000.161.1
FABP4	281.759	Fatty acid binding protein 4, adipocyte	Chromosome 14, AC_000.171.1
PPARD	353.106	Perixome proliferator-activated receptor delta	Chromosme 23, AC_ 000.180.1
COL1A1	282.187	Collagen type 1, alpha 1	Chromosome 19, AC_000.176.1
SOX9	353.115	SRY (sex determining region Y)-box 10	Chromosome 5, AC_000.162.1
GFAP	281.189	Glial fibrillary acidic protein	Chromosome 19,AC_000.176.1
NES	522.383	Nestin	Chromosome 3, AC_000.160.1
OMD	280.885	Osteomodulin	Chromosome 8, AC_000.0149.1
POST	281.960	Osteoblast specific factor	Chromosome 12, AC_000.034.1
OSTF1	281.961	Osteoclast stimulating factor 1	Chromosome 8, AC_000.0610.1

(anti-endoglin); CD29 (anti-integrin β1) all diluted at 1:50; CD73 (anti-nucleotidase) diluted at 1:25 (Sigma-Aldrich). Next, cells were washed three times with PBS plus 0.1 % Triton X-100 and 1:50 dilution of the secondary antibody, represented by goat anti-mouse labeled to FITC (Sigma-Aldrich), was added to 100 μl of cell suspension and incubated at 37 °C for 30 min. The cell suspension was washed as previously described and after the final wash, cells were fixed with 4 % paraformaldehyde. Data were captured with the Attune™ acoustic focusing cytometer system (Applied Biosystems, Foster City, CA, USA). The equipmental settings were defined as an initial threshold: 2.500 events/s, BL1A filter (488 nm emission) 300 voltage, SSC (scatter complexity) 250 V and FSC (forward scatter) 220 V. After the first acquisition, a dot plot graph was obtained and a global compensation was performed to exclude unspecific signals and cell debris (>10^3 cells were excluded). Only one fluorophore was used in this analysis, so these parameters could be applied in all analyses. The data were expressed in histograms.

Microarray analysis

Total RNA was isolated from all groups, corresponding to WJMSCs (5 × 10^5 cells) in triplicate after trypsinization, according to the Qiagen RNeasy System™ (Qiagen, Hilden, Germany) manufacturer's guide. The total RNA was treated

Fig. 2 Cell viability, inhibition of T-cell proliferation and telomerase activity. **a** Viability of WJMSC cells measured by MTT based assay at 2, 6 and 10P. Data are expressed as mean ± standard deviation (SD) of values obtained from four different experiments. **b** Ability of bovine WJMSCs cells to inhibit T-cell proliferation in response to mitogens at 6P, $P < 0.005$ obtained from four different experiments. **c** Telomerase repeat amplification results obtained from four different experiments. *Bars* represent all groups at 2P, 6P and 10P


a
% Cell Viability
150
100
50
0
P2
P6
P10
Group 1
Group 2
Group 3
b
% T-cell proliferation
100
80
60
40
20
0
No MSC
MSC + PMA
MSC + ConA
p<0.005
p<0.005
p<0.005
*
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Group 1
Group 2
Group 3
c
Telomerase activity (280 nm)
3
2
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P2
P6
P10
Group 1
Group 2
Group 3

with DNAse and reverse transcribed into cDNA using a reverse transcriptase (Superscript III; Life Technologies, Carlsbad, CA, USA). The Axiom® Genome-Wide BOS 1 Array was used for transcriptome analysis (Affymetrix, Santa Clara, CA, USA). This commercial array was designed to maximize genetic coverage of commercially important cattle breeds, including *Bos taurus*, *Bos indicus* and dairy and beef cattle breeds. The array covers more than 640,000 validated transcript markers representing the genetic diversity of approximately 3 million from the Affymetrix Bovine Genomic Database. After hybridization, the gene chips were washed and stained with SA-PE and read using an Affymetrix Gene Chip fluidic station and scanner. Analyzed genes, corresponding to positive markers of MSCs, immune-related genes and MSCs multipotency, are detailed in Table 1. The average expression was calculated and $\log_2$-transformed for each gene by Affymetrix Microarray Suite 5.0.

Statistical analysis

All statistical analyses were performed using the SAS 9.1.2 software package (SAS Institute). Data are presented as mean ± SD. Three replicates for each experiment were performed and the results represent these replicates. We executed one-way analysis of variance (ANOVA) for multiple comparisons or two-tailed Student's *t* test, whenever applicable by GraphPad Prism 6.05. A level of $P < 0.005$ was accepted as significant.

Results

Isolation and characterization of WJMSCs

WJMSCs were cultured individually only during the first passage. After P1, all WJMSCs from each group were transferred to a unique culture flask and proceeded as lineages. The formation of fibroblast-like cells was observed around the second day and in vitro cell expansion was performed until 10 consecutive passages for all groups (Fig. 1d–f).

The culture conditions were able to promote good cell viability, >80 % after 10 P for all groups; induced inhibition of T-cell proliferation; telomerase activity at satisfactory levels (Fig. 2a–c, respectively). The percentage of living cells was maintained approximately constant when the passage number remained at a constant level of 10 (Fig. 2a). The addition of WJMSC cells to blood monocytes stimulated with ConA or PMA/ionomycin inhibited their proliferation less than 20 % in comparison to no addition of WJMSC cells, whereas 80 % of proliferation for all groups was observed ($P < 0.005$). In addition, the activity of telomerase was verified and in all groups after 6P the same activity could be observed (Fig. 2c). The doubling time was measured, calculated and drawn as a graph, where a consistent increasing rate of growth at P6 was observed for each group, respectively (Figs. 3a–c and 4).

Multilineage differentiation and phenotypic characterization

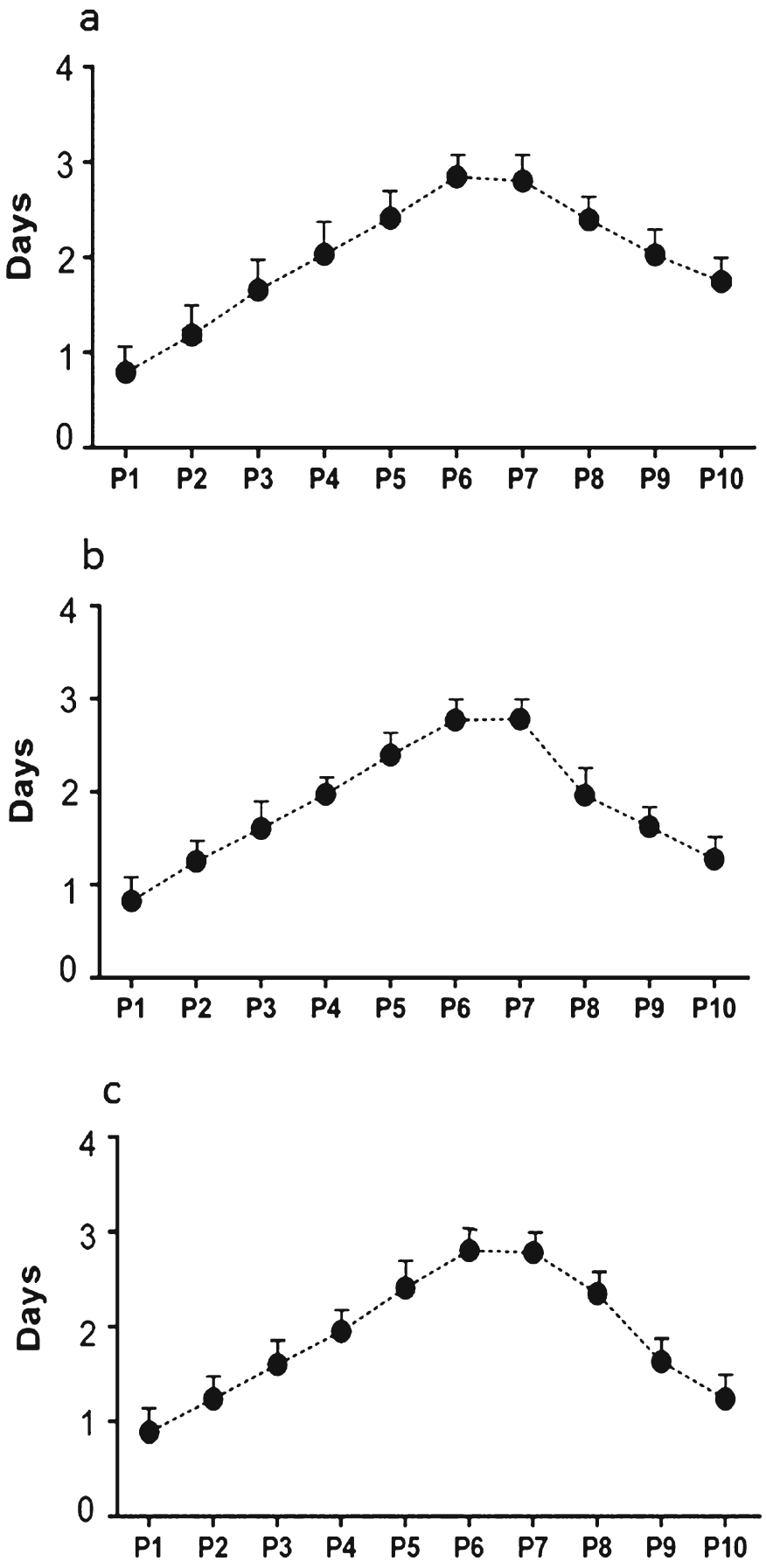
Pluripotency was confirmed by the ability of WJMSCs cells to differentiate into osteocytes, adipocytes, chondrocytes and neuron-like cells. Undifferentiated cells were included in all analyses (Fig. 3a, e, i, corresponding to groups 1, 2 and 3, respectively). Osteogenic differentiation was detected by the matrix calcification shown by Alizarin Red staining (Fig. 3b, f, j, corresponding to groups 1, 2 and 3, respectively). After induction, adipogenic differentiation with a high number of very small lipid vacuoles that stained positively using Oil Red solution was visualized in groups 1, 2 and 3 (Fig. 3c, g, l, respectively). Chondrogenic differentiation was confirmed by blue deposits representing glycosaminoglycans (Fig. 3d, h, m, corresponding to groups 1, 2 and 3, respectively). The neurogenic induction was confirmed by positive staining for GFAP and nestin neuro markers in group 1 (Fig. 5b, c), group 2 (Fig. 5e, f) and group 3 (Fig. 5h, i). Undifferentiated cells were also included in groups 1, 2 and 3 as control (Fig. 5a, d, g, respectively).

The phenotype of WJMSCs obtained from three groups was characterized using flow cytometry (FC) analysis (Figs. 6, 7 and 8 corresponding to groups 1, 2 and 3, respectively). All WJMSCs revealed negative results for CD45 and CD34 surface markers at FC analysis in groups 1, 2 and 3 (Figs. 6a, b, 7a, b and 8a, b, respectively). However, positive results for CD105, CD29, CD73 and CD90 surface markers were recorded at the same rate of 10^4 cells for all groups (Figs. 6, 7, 8). In group 1 (Fig. 6c), CD105-positive cells were considered at a lower rate (45 %) when compared to group 2 (81 %; Fig. 7c). However, in groups 2 and 3 (Figs. 7e, f, and 8e, f, respectively), CD73 (92 % and 78 %; $P < 0.005$) and CD90 (96 % and 79 %) were more highly expressed, respectively ($P < 0.005$). In comparison, group 2 revealed superior expression of positive markers of MSCs in this study (Fig. 7a–f).

Gene expression profile

Genes described as being involved in MSCs characterization revealed consistent results with flow cytometric analysis. High transcription levels for MSCs markers *THY1* (CD90), *NT5E* (CD73), *ITGB1* (CD29), *ENG* (CD105) and low levels for *CD34* and *PTPRC* (CD45) were found among WJMSCs

Fig. 3 Doubling time over 10 passages during cell culture from group 1 (**a**), 2 (**b**) and 3 (**c**). *X-axis* is represented by number of cell passage and *Y-axis* by days of culture. Data are expressed as mean ± standard deviation (SD of values obtained from four different experiments). *$P < 0.05$


a
Days
4
3
2
1
0
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
b
Days
4
3
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P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
c
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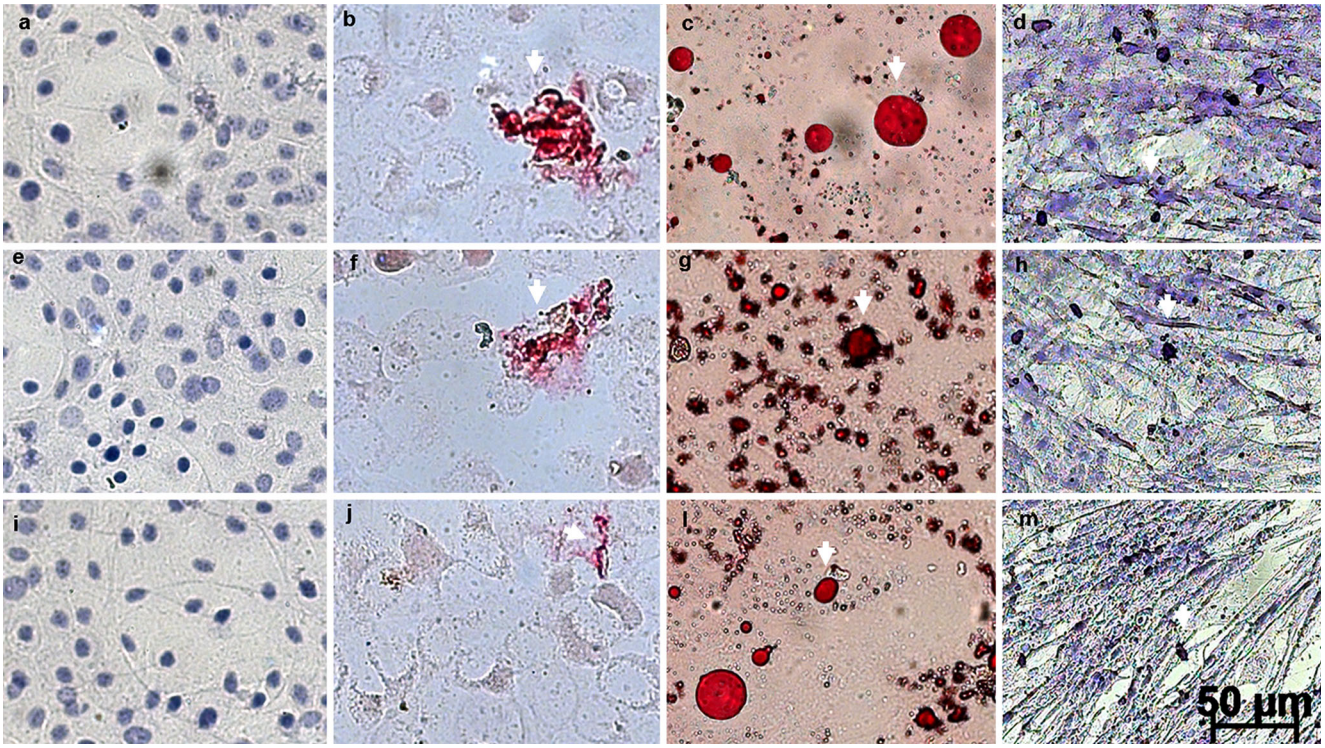

Fig. 4 Photomicrographs representative of the morphological appearance. For each differentiation protocol, undifferentiated cells were kept as controls for group 1 (**a**), 2 (**e**) and 3 (**i**). Osteogenic differentiation was confirmed after Alizarin Red staining (group 4 **b**, group 4 **f** and group 4 **j**; *arrows*), adipogenic differentiation after Oil Red staining (group 4 **c**, group 4 **g** and group 4 **l**; *arrows*) and chondrogenic differentiation after Toluidine blue staining (group 4 **d**, group 4 **h** and group 4 **m**; *arrows*) and differentiation of bovine-derived WJMSCs cells at P6

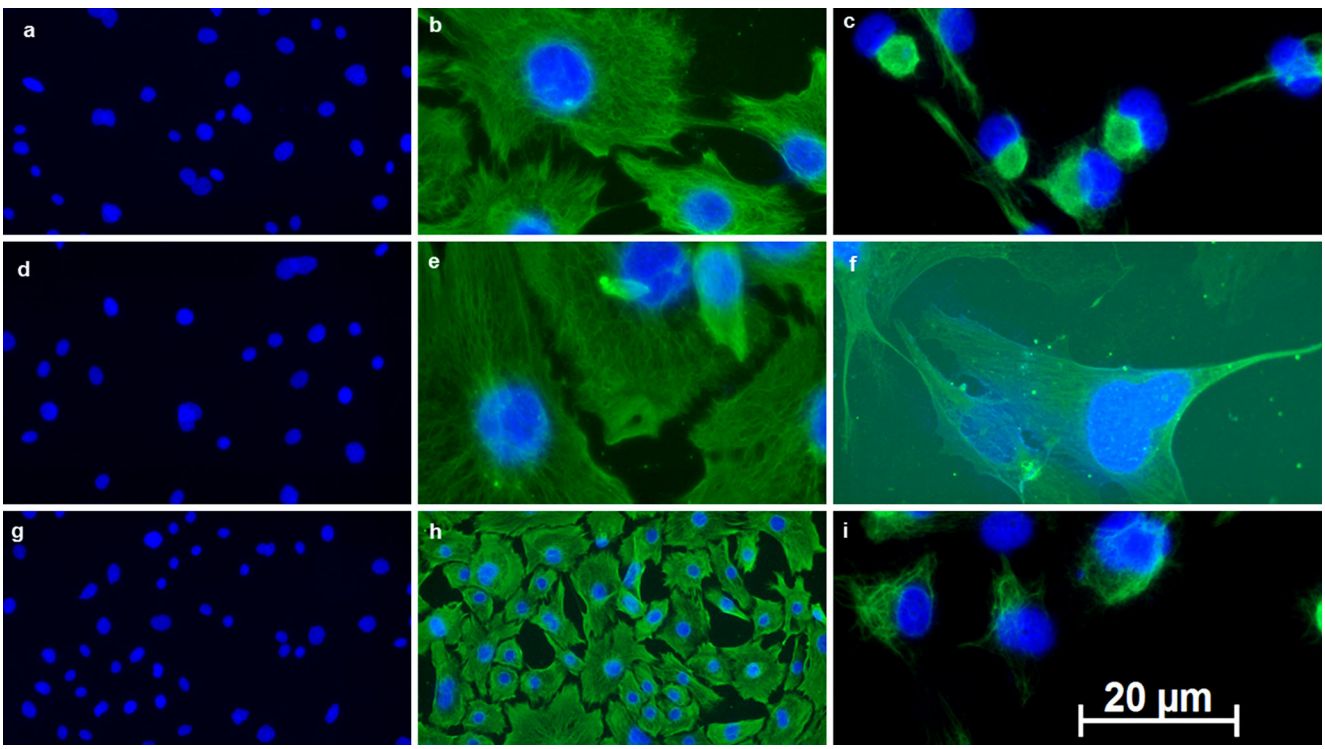

Fig. 5 Photomicrographs representative of the morphological appearance of neuro-like cells after neurogenic induction visible under immunofluorescence microscopy positive GFAP (group 1 **b**; group 2 **e**; group 3 **h**) and Nestin (group 1 **c**; group 2 **f** and group 3 **i**) cell markers (*scale bar* 20 μm). Undifferentiated cells were kept as controls for groups 1 (**a**), 2 (**d**) and 3 (**g**)

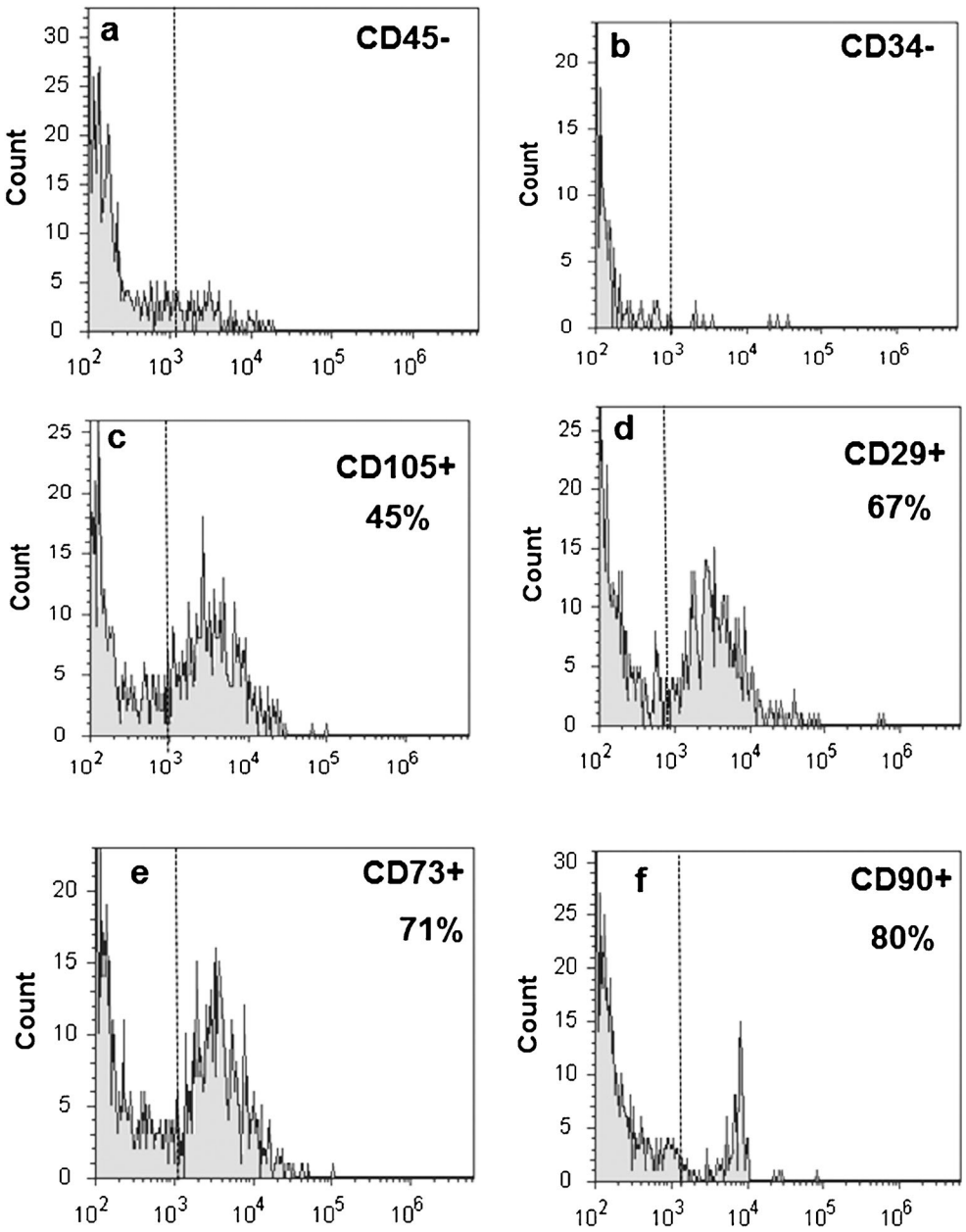

Fig. 6 Flow cytometry analysis of WJMSCs surface markers corresponded to group 1. *X-axis* corresponds to the number of positive labeled cells detected by the BL1A filter (488 nm). *Y-axis* is cell count (log scale). The data obtained from four different experiments were processed using an Attune™ acoustic focusing cytometer. For CD45 and CD34, data are illustrated in (**a**, **b**) (group 1). For CD105, CD29, CD73 and CD90 the results are illustrated in (**c**–**f**)

(Fig. 9a–c). When stimulated to differentiate towards adipogenic, chondrogenic and neurogenic lineages, WJMSCs showed substantial transcriptional expression of *LEP*, *FABP4*, *PPARD*, *COL1A1*, *SOX9*, *GFAP* and *NES* for all WJMSCs (Fig. 9a–c). The WJMSCs potential to undergo chondrogenesis showed a higher pattern of *COL1A1* gene expression at group 2 in comparison to groups 1 and 3 (Fig. 9b). From the factors measured in this study, *IL2*, *IL6R*, *INFAC*, *INFB1*, *INFG*, *TNF* and *LTB*, considered pro-inflammatory cytokines, were genetically downregulated in all WJMSCs tested (Fig. 9ac; $P < 0.005$). However, *IL1F10* was noticeably upregulated, in microarray analysis (Fig. 9a). The lack of *JSP.1* and *DSB* (MHCI and II) expression could be observed in this study amongst WJMSCs from all groups (Fig. 9a–c).

Discussion

Extra gestational tissues have been widely suggested as ideal sources of mesenchymal cells due to their non-invasive harvest and most of the time being discarded biological material (Troyer and Weiss 2008; Iacono and Merlo 2015). Currently, limited reports are available regarding the isolation and characterization of Wharton's jelly-derived MSCs in farm animal species (Carlin et al. 2006; Corradetti et al. 2008; Cardoso et al. 2012; Singh et al. 2013). WJMSCs demonstrated, at all gestational periods, satisfactory telomerase activity, viability and MSCs surface markers according to previous studies (Cardoso et al. 2012). In spite of the fact that non-

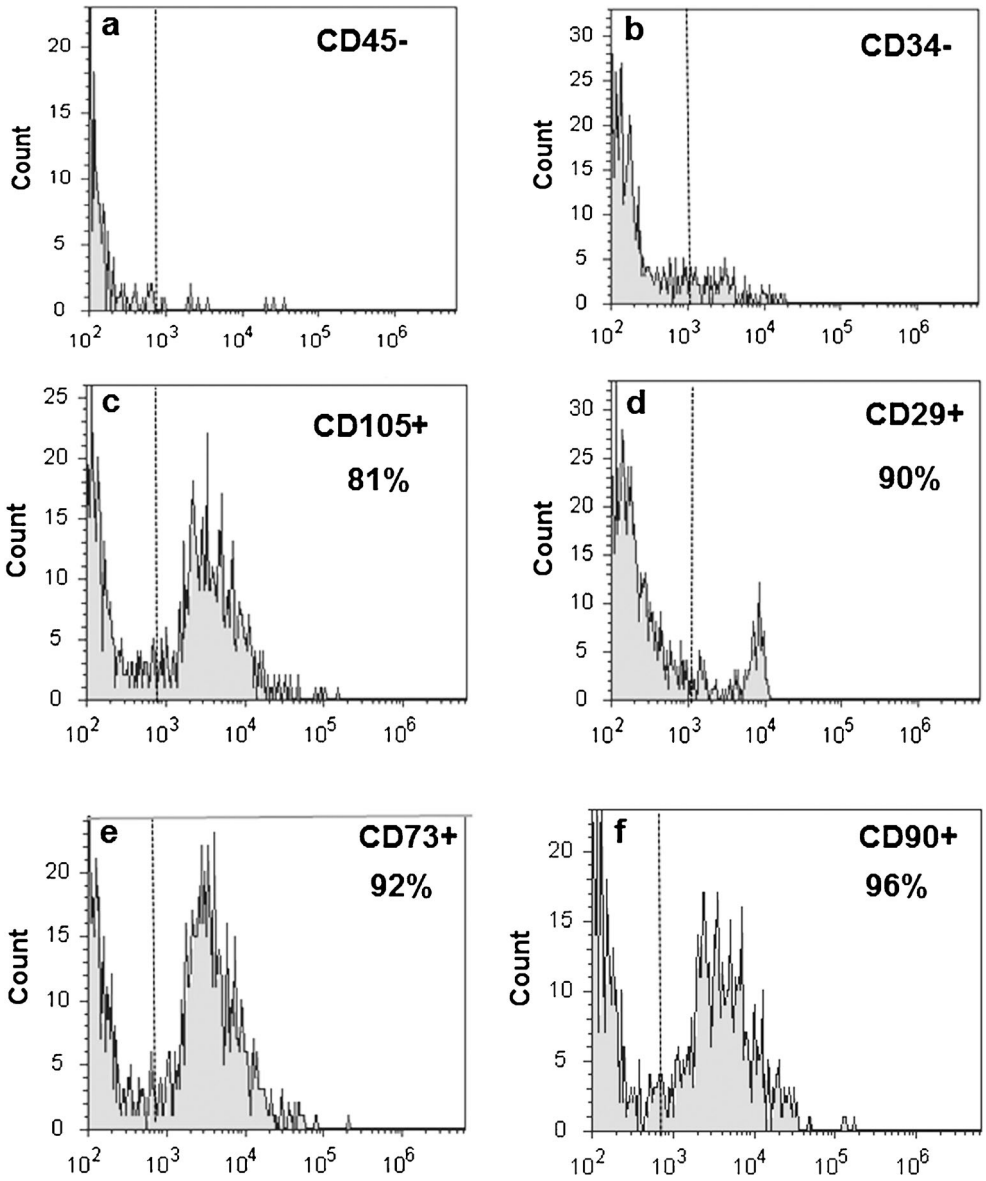

Fig. 7 Flow cytometry analysis of WJMSCs surface markers corresponded to group 2. *X-axis* corresponds to the number of positive labeled cells detected by the BL1A filter (488 nm). *Y-axis* is cell count (log scale). The data obtained from four different experiments were processed using an Attune™ acoustic focusing cytometer. For CD45 and CD34, data are illustrated in (**a**, **b**) (group 2). For CD105, CD29, CD73 and CD90, the results are illustrated in (**c**–**f**)

bovine monoclonal antibodies were used here, several reports have described cross-reaction between animals and human MSCs epitopes (Godoy et al. 2014). Moreover, *THY1* (CD90), *NT5E* (CD73), *ITGB1* (CD29) and *ENG* (CD105) transcripts were confirmed by flow cytometric analysis, confirming the stem cell potential of WJMSCs in this study.

Studies on the isolation and characterization of MSCs from fetal adnexa in humans are advancing rapidly (Troyer and Weiss 2008; Weiss et al. 2008; Corrao et al. 2013; Gottipamula et al. 2013). Some authors have reported that MSCs isolated from human umbilical cord matrix, precisely from Wharton's jelly, could be used for therapy of some diseases such as amyotrophic lateral sclerosis and Parkinson's disease, even in cancer treatment (Troyer and Weiss 2008; Corrao et al. 2013). However, studies in animal models are still in their infancy. MSCs have been isolated from umbilical cord matrix of cattle, pigs, goats, horses and dogs (Uranio et al. 2011; Cremonesi et al. 2008; Cardoso et al. 2012). The bovine model could have a critical role in studying fetal adnexa MSC sources, mainly through the similarity to human gestational time. Our study aimed specifically to isolate, expand in vitro, and characterize WJMSCs harvested at different gestational stages. Previous studies have reported the isolation of WJMSCs from bovine umbilical cord at birth, successfully growing them in culture without fetal calf serum showing pluripotency capacity (Cardoso et al. 2012; Silva et al. 2016). Moreover, a recent study demonstrated that bovine MSCs isolated from amniotic fluid and adipose tissue could be an alternative for nuclear

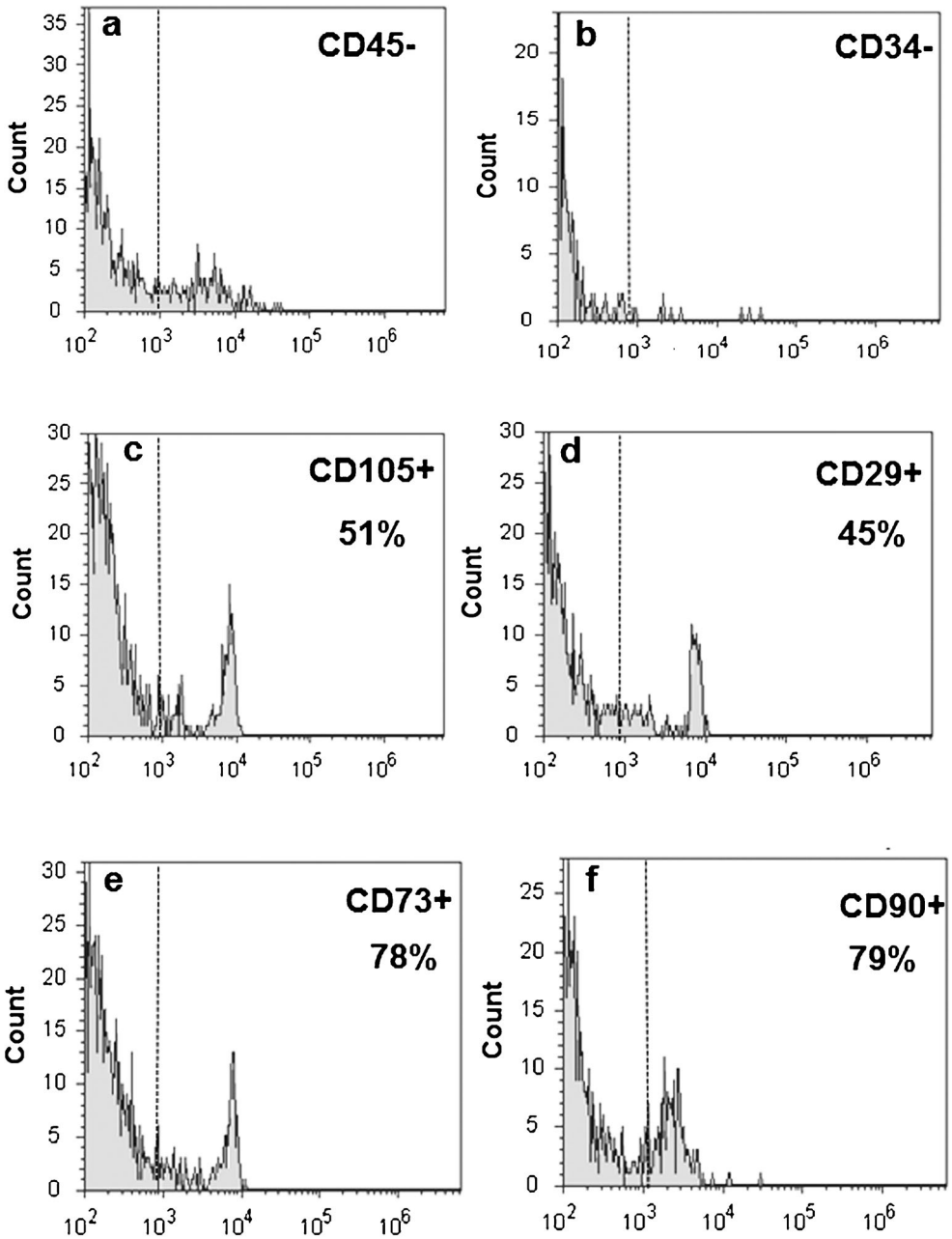

Fig. 8 Flow cytometry analysis of WJMSCs surface markers corresponded to group 3. *X-axis* corresponds to the number of positive labeled cells detected by the BL1A filter (488 nm). *Y-axis* is cell count (log scale). The data obtained from four different experiments were processed using an Attune™ acoustic focusing cytometer. For CD45 and CD34, data are illustrated in (**a**, **b**) (group 3). For CD105, CD29, CD73 and CD90, the results are illustrated in (**c**–**f**)

transfer (Silva et al. 2016). In both studies, the size of bovine MSC-derived WJ cells was found to be smaller when compared to human studies (Taghizadeh et al. 2011; Gottipamula et al. 2013; Pham et al. 2016) and ruminant MSCs were derived from different fetal adnexa sources (Cardoso et al. 2012; Corradetti et al. 2013; Cortes et al. 2013; Raoufi et al. 2011; Somal et al. 2016). This finding must be investigated in future studies.

The in vitro differentiation results support the findings already reported for bovine MSC (Lu et al. 2011; Raoufi et al. 2011; Corradetti et al. 2013; Cortes et al. 2013). All WJMSCs show high plasticity, being able to differentiate into multiple germ layers, mesoderm and ectoderm. This is in agreement with all studies regarding bovine MSC and other species (Cremonesi et al. 2008; Uranio et al. 2011; Oda et al. 2013). WJMSCs, when stimulated to differentiate toward adipogenic lineage, expressed high levels of the *LEP* gene that is regarded as an intermediate and late marker of adipocyte differentiation, which may lead to a distinct differentiation characteristic of stem cells in all gestational periods. The WJMSCs potential to undergo osteogenesis/chondrogenesis showed a higher pattern of expression of *OMD*, *POST*, *OSTF1* and *COL1A1* in all groups. As demonstrated previously, WJMSCs have a proven ability to undergo astrocyte differentiation, confirmed by *GFAP* expression already demonstrated for other species (Oda et al. 2013). Taking these results together, it seems that

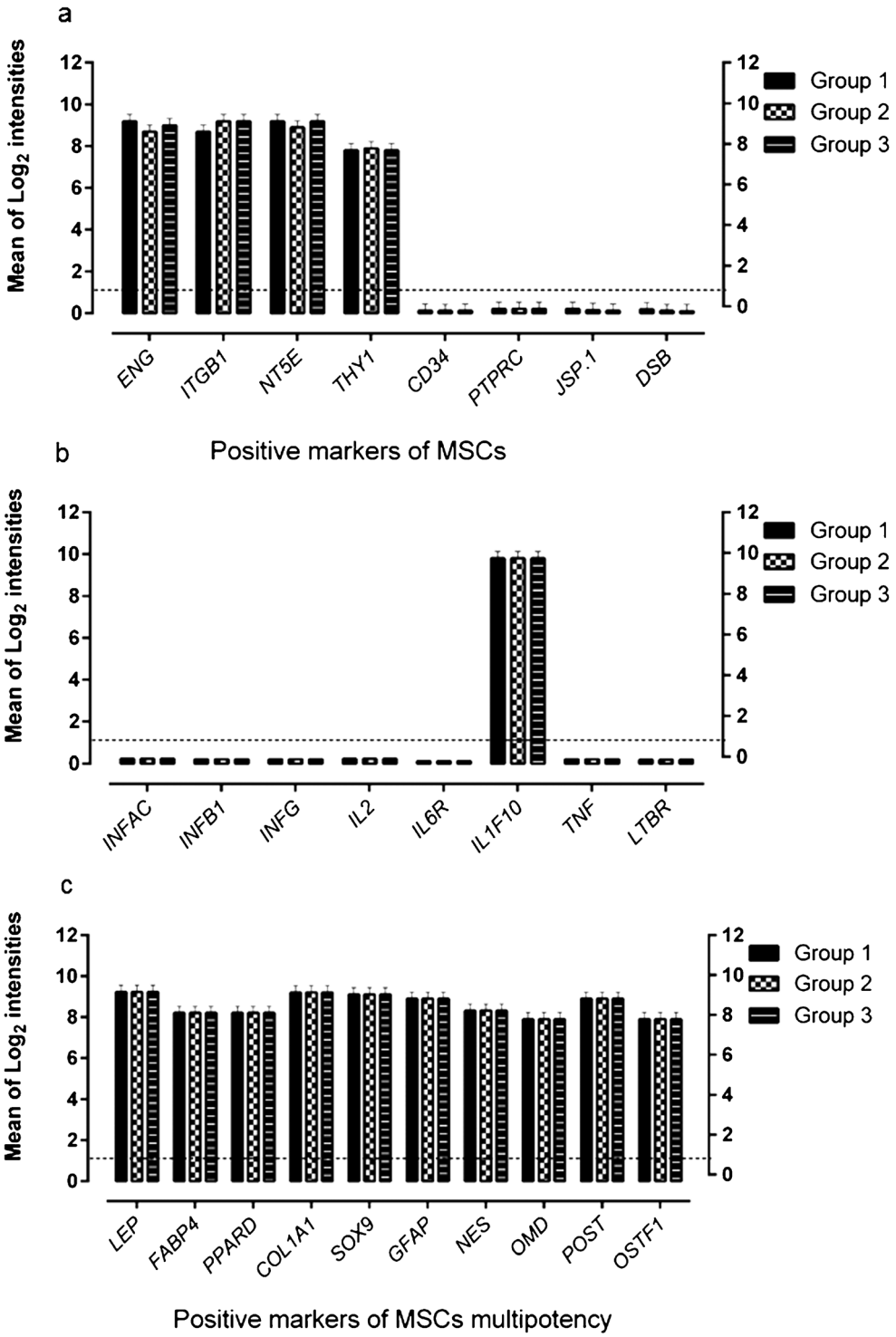

Fig. 9 Transcriptome analysis of WJMSCs surface markers, multipotency differentiation and immune related genes from groups 1, 2 and 3. Relative gene expression are represented as mean + SD obtained from four different experiments. *Unbroken line* is positioned on the average negative known markers of MSCs. Genes with relative expression values above this line were considered upregulated and were analyzed in comparison to control comprising the bovine T-lymphocyte population. **a** Group 1, **b** group 2 and **c** group 3

all gestational periods have provided WJMSCs with stemness attributed to MSCs according to the International Society for Cellular Therapy (Dominici et al. 2008).

Since MSCs are trapped within the Wharton's jelly between days 4 and 12 of embryonic development and reside there for the whole gestation, they can be harvested after the birth of the newborn and during pregnancy (Taghizadeh et al. 2011). Therefore, WJMSCs that formed during the earliest ontogenic period result in a significant expansion potential compared to bone marrow mesenchymal cells (Troyer and Weiss 2008).

There is considerable controversy in the literature regarding the immunogenicity of human MSCs (Weiss et al. 2008; De Miguel et al. 2012; Mukonoweshuro

et al. 2014) and a lack of information about bovine MSCs. However, porcine umbilical cord-derived stem cells did not induce a considerable immune response in vivo but stimulation with interferon gamma or injection in an inflamed region resulted in immunogenicity (Poncelet et al. 2007). Inflammatory situations prevail during any injury and MSCs could be exposed to such stimuli in many clinical conditions. Not only neighboring cells but also environmental factors like systemic or local inflammation can influence the immune behavior of MSCs (Poncelet et al. 2007). In fact, recent reports indicate the role of inflammatory cytokines in affecting functions of mouse MSCs (Mukonoweshuro et al. 2014).

From the factors measured in this study, *IL2*, *IL6R*, *INFAC*, *INFB1*, *INFG*, *TNF* and *LTBR*, considered pro-inflammatory cytokines, were genetically downregulated in all the bMSCs tested. IL10 cytokyne is produced by both myeloid and lymphoid cells. However, it is a good immune suppressor, although some stimulatory effects have been described. Therefore, IL10 cytokine is recognized by its effect on T-cells, macrophages and monocytes in suppressing inflammation processes (Prasanna et al. 2010; Mukonoweshuro et al. 2014). However, when *IL1F10* is expressed it will downregulate *JSP.1* (MHC I) as revealed in WJMSCs cultures. These findings are in accordance with what has been described in human MSCs (Weiss et al. 2008). The results described previously revealed that MSC possess immunosuppressive properties; however, they might not be immunoprivileged (De Miguel et al. 2012). The lack of *DSB* and low *JSP.1* expression observed in this study is thought to be, in part, responsible for their WJMSC immunoprivileged status, which would mean allogeneic bovine MSC could be used without the risk of immune rejection, a scenario that is attractive for tissue comparative studies.

These findings demonstrated the complexity of studying the immunological properties of WJMSCs in vitro, as well as the difficulty of distinguishing between optimal gestational stages in order to collect Wharton's jelly cells with optimal stemness properties. Finally, WJMSCs collected from bovine umbilical cord at all gestational periods showed similar stemness properties.

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Author's contributions TCC participated in the design of the study, performed the cell culture in all steps and flow cytometric analysis. LHO and JCB participated in the preparation of respective umbilical cords. RG and HLF participated also in the design of the study, performed the statistical analysis and drafted the manuscript. MM and EFF drafted the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors indicate no conflicts of interest.

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