Size-sieved subpopulations of mesenchymal stem cells from intervacular and perivascular equine umbilical cord matrix

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Abstract

Objectives: Umbilical cord matrix (UCM) has been recently proposed as an alternative source of mesenchymal stem cells (MSCs). The aim of this study was to isolate and characterize presumptive stem cells from intervacular and perivascular equine UCM and to obtain homogeneous subpopulations from both sites.

Materials and methods: Umbilical cords were processed for retrieval of MSCs. Unsieved cells from intervacular and perivascular portions were evaluated for cell cycle analysis and for immunophenotyping by flow cytometry. Cells from each site were separated into larger and smaller sieved populations using multi-dishes with 8-µm pore transwell inserts. Each cell population was characterized in terms of renewal capability, specific marker expression and differentiation potential. Cryopreservation was performed on sieved cells only.

Results: Cells from both areas expressed MSC and pluripotential specific markers and were able to differentiate into mesodermic and ectodermic lineages. The sieving procedure yielded two relatively homogeneous subpopulations with comparable characteristics. Surprisingly, after sieving, large intervacular and small perivascular cells were the most rapidly replicating cells [20.53 and 19.49 cell population doublings (PD) after 31 days respectively] and also showed higher fibroblast colony forming unit frequency. Unsieved cell populations were used as

controls, and showed PD of 9.42(intervacular cells) and 8.54 (perivascular cells) after 31 days.

Conclusions: Here, cells from UCM represented an intermediate stage between pluripotent embryonic and adult stem cells. Size-sieving can be used to isolate more rapidly proliferating cell populations.

Introduction

Mesenchymal stem cells (MSCs) are multipotent precursor cells with great self-renewal and differentiation potential, that play an important role in both human and veterinary regenerative medicine (1,2). Minimum criteria for defining MSCs are adherence to dish plastic, formation of fibroblast colony forming units (CFU-F), differentiation competence towards one or more tissue lineages (ectoderm, mesoderm or endoderm), and specific pattern of surface antigen expression including CD105, CD90 and CD73 (3). The best characterized source of adult MSCs is bone marrow; however, cell number, proliferation and differentiation capabilities decrease with donor age and culture passages *in vitro* (4). Stem cells derived from adult tissues are considered to have more limited potential compared to embryonic stem cells, although they are currently the more versatile cells used in the clinical field. Umbilical cord has been recently proposed as an alternative source of MSCs in view of these cells' pluripotent characteristics, non-invasive nature of the isolation procedure and their relatively low ethical implications.

Studies conducted on human cells from foetal and extra-foetal tissues, including amniotic membrane (5), amniotic fluid (6), umbilical cord blood (7) and different compartments of the umbilical cord matrix (UCM) (8,9) have revealed highly efficient MSC recovery at birth, and greater expansion capacity *in vitro* as well as faster doubling times than adult MSCs, probably due to their high telomerase activity (10).

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UCM is a mucoid connective tissue surrounding umbilical vessels and in human umbilical cord is comprised of specialized fibroblast-like cells and occasional mast cells embedded in an amorphous ground substance rich in proteoglycans, mainly hyaluronic acid. Human umbilical cord also has tissue compartmentalization in which cell characteristics and extracellular matrix elements differ from one another. Based on structural and functional studies, at least six distinctive zones are now recognized in the umbilical cord: (i) surface epithelium (amniotic epithelium), (ii) subamniotic stroma, (iii) clefts, (iv) intervascular stroma (named classically as Wharton's jelly), (v) perivascular stroma and (vi) vessels. In previous reports, equine MSCs have been isolated from umbilical cord blood (11,12) and UCM (13,14). Despite the possible clinical importance of these cells for treating musculoskeletal tissue injuries, there is no well-defined protocol for isolation and expansion of MSCs from foetal adnexa in the horse. Generally, MSCs are isolated from UCM primarily by their tight adherence to culture dishes (15), without distinction between intervascular and perivascular stroma. Cells obtained in this way appear heterogeneous.

In this context, the aims of the present study were to isolate cells from intervascular and perivascular cord matrix and to obtain homogeneous cell lineages. To obtain homogeneous subpopulations of stem cells from human UCM, Majore *et al.* applied the counterflow centrifugal elutriation method and separated cells with distinct characteristics in terms of size, morphology and proliferative activity (16). Other approaches, including size-sieving methods (17), fluorescence activated cell sorting (18) and long-term culture under specific conditions (19) have also been described for identification of differentially sized subpopulations from bone marrow aspirates. Particularly in the horse, as there are no surface epitopes that can usefully distinguish stem cell populations in cultures (for example, distinguishing early progenitors from mature cells), the size-sieving protocol might provide a simple and inexpensive technique for cell separation. With regard to size-sieving methods, previous studies (18,20) have proposed certain differentially sized subpopulations of small, rapidly proliferating cells with high capacity for differentiation, which are of interest for regenerative medicine. The size of these rapidly self-renewing cells (RS) has been described to be in the range of 7 μm (21) or smaller (22). But, use of smaller pores, for example, 3 μm according to Hung *et al.*, would result in isolating very small cells with polygonal shapes and little renewal capacity in the lower dish, while leaving a rather heterogeneous population in the upper dish (17). Taking these into account, to sieve out homogeneous equine MSC-like subpopulations from both portions of UCM, a specific device (a plastic culture dish comprising a plate with 8- μm pores)

has been used to separate cells of this size range which are meant to be the very small embryonic-like stem cells.

In this study, we describe characterization of homogeneous MSC-like cells from each umbilical cord area, their specific expression patterns, capacity for self-renewal and their potential to differentiate into several lineages.

Materials and methods

Collection of samples

Umbilical cords ($n = 3$) were collected at the end of delivery from three mares hospitalized at the Large Animal Hospital of Milan University for parturition assistance, in accordance with standard veterinary practice. Before each mare stood up breaking the cord, surgical tape was placed at the junction of the cord with the foal and a second tie was tightened approximately 30/40 cm from the first; tie-limited cord portion was excised using scissors. The harvested cord segment was washed twice in 10% betadine solution and then in 70% alcohol and maintained at 4 °C in saline solution supplemented with 4 $\mu\text{g}/\text{ml}$ amphotericin (Sigma Aldrich, St. Louis, MO, USA; <http://www.sigmaaldrich.com>), 100 IU/ml penicillin (Sigma) and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma) and processed within 12 h of collection.

Histology

Small samples of each umbilical cord were sectioned and fixed in 10% buffered formalin for 24 h. After fixation, tissues were dehydrated in a graded series of ethanol solutions and embedded in paraffin wax. Serial sections were cut at 4 μm , dewaxed and routinely stained in haematoxylin and eosin (H&E), for histological examination.

Immunohistochemistry

Further sections of umbilical cord, after removal of paraffin wax with xylene, were rehydrated through graded alcohol solutions (100%, 90% and 70% ethanol in distilled water) and immunostained with anti-vimentin (Biogenex, San Ramon, CA, USA) or anti-desmin antibodies (Dako-Cytomation, Carpinteria, CA, USA). Antibody binding was detected using the peroxidase anti-peroxidase method and immunoreaction sites were developed with DAB (Sigma). Sections were examined using an Olympus BX 51 microscope (Tokyo, Japan).

Isolation of unsieved umbilical cord cells

Umbilical arteries and vein were removed and remaining tissue was divided into intervascular and perivascular

portions. Intervascular regions (Fig. 1a) represent stroma connecting vein, arteries and urachus while perivascular regions are the matrix surrounding these vessels (Fig. 1b). Details of perivascular stromal detachment are shown in Fig. 1c,d. Tissues from both portions were minced and digested with 0.75 mg/ml collagenase (Sigma) for 16 h at 37 °C. After incubation, suspensions were filtered with 80-µm filters (Millipore, Milan, Italy; <http://www.millipore.com>). Enzyme was removed by washing and centrifuging at 250 *g* for 10 min and digested solutions were washed twice in PBS.

Isolation of sieved umbilical cord cells

To identify subpopulations, cells from each intervascular and perivascular matrix were plated at a density of 1×10^6 cells/cm² in the upper compartment of a culture device made up of two chambers divided by a polycarbonate membrane with 8-µm pores (Transwell 8-µm Pore Size; Corning Inc., Corning, NY, USA; <http://www.corning.com>).

Culture and expansion of umbilical cord-derived cells

Cells were cultured in high glucose-Dulbecco's Modified Eagle's medium (HG-DMEM; EuroClone, Milan, Italy; <http://www.euroclone.com>) supplemented with 10% foetal bovine serum (FBS; Sigma), 10 ng/ml epidermal growth

factor, 1% penicillin (100 IU/ml) – 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine (Gibco Invitrogen, San Giuliano Milanese, Mi, Italy). Cell cultures were maintained in 5% CO₂, at 90% humidity and 38.5 °C for experiments described below. Culture medium was replaced initially after 72 h to remove non-adherent cells and then twice weekly or according to experimental design. Adherent cells were detached with 0.05% trypsin-EDTA (EuroClone) just prior to reaching confluence (80–90%) and then reseeded for culture maintenance.

All studies, except cell cycle and flow cytometry analyses, were performed on cells of all experimental conditions represented by unsieved inter- and perivascular portions, and larger and smaller sieved inter- and perivascular portions. Post-cryopreservation studies were performed on sieved cells only.

Cell cycle analysis

Isolated unsieved cells from both tissue portions at passage 1 (P1) were trypsinized and resuspended at $1\text{--}2 \times 10^6$ cells/ml. Cells were fixed by treatment with 70% ethanol for 45 min at 4 °C and then washed twice in cold PBS. Flow cytometry was used to evaluate DNA content using cells labelled with 1 ml PBS containing 40 µg/ml propidium iodide (Fluka BioChemika, Buchs, Switzerland) and RNase A (Sigma) for 45 min at 37 °C.

Figure 1. Equine umbilical cord. Intervascular (a: red arrow) and perivascular (b: black arrow) Wharton's Jelly portions. Details of perivascular stroma detachment (c and d). (e) Urachus and vessels immersed in the cord matrix. Area included into the square is enlarged in (f), showing inter- and perivascular areas (H&E staining; 2x magnification). Anti-desmin (g and i) and anti-vimentin (h and l) immunoreactivity in intervascular (g and h) and perivascular portion (i and l) (20x magnification). Scale bars: 100 µm

Flow cytometry (FCM) analysis

No commercially available species-specific antibodies exist to characterize equine stem cells. Antibodies were chosen according to results obtained by Hoyonowsky *et al.* and were used according to the manufacturer's instructions (13). Tested markers included matrix receptors (endoglin, CD105), haematopoietic lineage marker (CD34), early stage specific antigens (SSEA-3 and SSEA-4), pluripotency markers (OCT-4, c-Myc, TRA-1-60) and immunogenic antigen (HLA-ABC). OCT-4, CD34 and CD105, SSEA-3, SSEA-4, TRA-1-60, c-Myc were purchased from Abcam (Cambridge, MA, USA; <http://www.abcam.com>); HLA-ABC was purchased from Chemicon Inc. (Temecula, CA, USA; <http://www.chemicon.com>). AlexaFluor-488 conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA; <http://www.invitrogen.com>). Isotype-specific IgM and IgG were purchased from Abcam and Immunotools GmbH (Germany, <http://www.immunotools.de>) respectively. Antibodies are listed in Table 1. Unsieved cells from intervascular and perivascular portions (2×10^6 cells/ml) were labelled at passages 3 (P3) and 5 (P5) with primary antibodies in PBS 3% BSA (BDH; VWR International Ltd, Poole, UK) for 45 min at room temperature in the dark, followed by washing in cold PBS and final incubation with secondary AlexaFluor-488 conjugated antibodies (1:250) for 30 min at room temperature in the dark. For evaluation of nuclear markers including OCT-4 and c-Myc, cells were fixed in 0.01% paraformaldehyde (in PBS) at 4 °C for 15 min, washed in 3% BSA in PBS and then treated to promote permeability for 10 min at room temperature in 1% Triton-X 100 diluted in

PBS. Labelled cells were washed twice in ice cold PBS and analysed using an Epics Coulter flow cytometer (Beckman Coulter-IL, Fullerton, CA, USA). Minimum of 10 000 cells were acquired for each sample and analysed in the FL1 channel. All analyses were based on control cells incubated with isotype-specific IgGs or IgM to establish background signal. Off-line analysis of the FCS files was performed using Weasel software v.2.5 (<http://www.biotechcentre.net.au/cytometry/index.html>).

Cell proliferation studies

CFU-F assay. To assess CFU-F, cells from each population were seeded at P1 at concentration of 1800 cells/cm² in six-well plates (Euroclone) and kept in 5% CO₂, at 90% humidity and 38.5 °C. Colonies were fixed in 4% formalin, stained with 1% methylene blue in borate buffer 10 mM (pH 8.8; Fluka) at room temperature and washed twice. Colonies formed by at least 16–20 nucleate cells were counted using an Olympus BX71 microscope.

Cell population doublings

Cell proliferation was evaluated for each population from P1 to P10. Numbers of viable cells were counted using the trypan blue dye exclusion method using a Burkner chamber. Mean population doublings (PD) was obtained for each passage according to the following formula:

$$CD = \log (N_i/N_f)/\log_2, \quad PD = CD/CT,$$

where N_i represents seeded cells, N_f , number of cells at confluence and CT, culture time. Data of three independent experiments were reported.

Multilineage differentiation studies

Multilineage potential of cells, towards osteogenic, adipogenic, chondrogenic and neurogenic differentiation at P3, was tested *in vitro*. To induce differentiation, cells were seeded at density of 3000 cells/cm² in six-well plates and cultured until they reached approximately 80–90% confluence.

Osteogenic induction. Medium consisted of HG-DMEM, supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine, 10 mM β-glycerophosphate (Sigma), 0.1 µM dexamethasone (Sigma) and 250 µM ascorbic acid (Sigma). Non-induced control cells were cultured for the same length of time in standard medium (HG-DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine). Osteogenesis was assessed by von

Table 1. Primary and secondary antibodies for FCM analysis

Markers	Primary antibody	Ig
Oct-4	Rabbit polyclonal	IgG
c-Myc	Mouse monoclonal	IgG1
SSEA-4	Mouse monoclonal	IgG3
SSEA-3	Rat monoclonal	IgM
CD105	Mouse monoclonal	IgG1
CD34	Rat monoclonal	IgG2a
TRA 1-60	Mouse monoclonal	IgM
Secondary antibody		
Goat Anti-Rabbit		IgG
Goat Anti-Mouse		IgG
Goat Anti-Mouse		IgM
Goat Anti-Rat		IgG
Goat Anti-Rat		IgM

Ig, immunoglobulin.

Kossa staining (1% silver nitrate and 5% sodium thio-sulphate) for detection of calcium deposits.

Adipogenic induction. Differentiation was induced by three repeated changes of adipogenic induction and maintenance medium. Cells were cultured for 3 days in induction medium containing HG-DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine, 10 µg/ml insulin (Sigma), 150 µM indomethacin (Sigma), 1 µM dexamethasone and 500 µM 3-isobutyl-1-methyl-xanthine (Sigma). Induction medium was replaced by maintenance medium containing only HG-DMEM, supplemented with 10% FBS and 10 µg/ml insulin. Non-induced control cells were kept in adipogenic maintenance medium. Adipogenesis was assessed using Oil red O staining (0.1% in 60% isopropanol) to visualize lipid droplets.

Chondrogenic induction. Cells were incubated for 3 weeks in DMEM low glucose containing 100 nM dexamethasone; 50 µg/ml L-ascorbic acid 2-phosphate, 1 mM sodium pyruvate (BDH), 40 µg/ml proline, ITS (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite; Sigma) and 5 ng/ml TGF-β3 (Peprovect, DBA, Milan, Italy). Non-induced control cells were cultured for the same time in a standard medium. Presence of metachromatic matrix was detected after alcian blue staining, pH 2.5.

Neurogenic induction. Twenty-four hour prior to induction, preinduction was performed using medium consisting of HG-DMEM, 20% FBS and 1 mM β-mercaptoethanol (Sigma). Neuronal induction medium was composed of HG-DMEM supplemented with 2% FBS, 2% dimethylsulphoxide (DMSO; Sigma), 200 µM butylated hydroxyanisole (Sigma). Neurogenic induction required 3 days. Non-induced control cells were cultured for the same time, in standard medium. Neurogenic differentiation was demonstrated after Nissl staining (0.1% cresyl violet solution).

Molecular characterization

Specific MSC-markers were investigated by reverse transcription (RT) followed by polymerase chain reaction (PCR) analysis, on undifferentiated cells from P1 to P10. Total RNA was isolated using TRIZOL[®] Reagent (Invitrogen) and then samples were treated with DNase (Sigma). Both steps were performed according to the manufacturers' specifications. RNA concentration and purity were measured using NanoDrop Spectrophotometer (NanoDrop[®] ND1000, Wilmington, DE, USA). Complementary DNA was synthesized from 200 ng of

total RNA using iScript (Bio-Rad Laboratories, Hercules, CA, USA; <http://www.bio-rad.com>) retrotranscription kit. Conditions used were 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. Qualitative PCR was performed using 1 µl of the obtained cDNA in 25 µl final volume with DreamTaq DNA polymerase (Fermentas GmbH, St. Leon Rot, Germany; <http://www.fermentas.com>) under the following conditions: initial denaturation at 95 °C for 2 min, 32 cycles at 95 °C for 30 s (denaturation), 55–63 °C for 30 s (annealing), 72 °C for 30 s (elongation) and final elongation at 72 °C for 10 min. Equine specific oligonucleotide primers were initially designed using open source PerlPrimer software v.1.1.17 based on NCBI *Equus caballus* available sequences or on mammal multi-aligned sequences and manually improved. Oligonucleotides were designed across an exon-exon junction to avoid DNA amplification. Primers were used at 200 nM final concentration and their sequences are shown in Table 2. *GAPDH* was employed as reference gene. Total RNA was extracted from induced cells and controls as specified for MSC extraction and was then treated with DNase. RNA from equine mature bone, adipose tissue, cartilage and spinal bone marrow was used as positive control for expression of osteocalcin (*BGLAP*) and osteopontin (*OPN*) for osteogenesis, peroxisome proliferator-activated receptor gamma (*PPAR-γ*) and adiponectin (*ADIPQ*) for adipogenesis, collagen type 2 alpha I (*COL2A1*) and aggrecan (*ACAN*) for chondrogenesis, glialfibrillary acidic protein (*GFAP*) and nestin (*NES*) for neurogenesis respectively. RT-PCR reactions were performed as described above.

Cryopreservation

Cells from both sieved portions were frozen at P1 in HG-DMEM supplemented with 50% FCS and 10% DMSO for 6 months and expanded after thawing until P3 to study whether cryopreservation would alter characteristics of the cell subpopulations in terms of morphology, renewal capacity and presence of specific MSC markers.

Statistical analysis

All statistical analyses were performed using the Statistics Package for the Social Sciences, version 11.0 (SPSS Inc., Chicago, IL, USA). Three replicates for each experiment were performed and results are presented as mean ± SD. Kolmogorov–Smirnov test was used to determine whether data were random samples from a normal distribution. For normally distributed variables, the independent-samples *t*-test was applied. A level of *P* < 0.05 was accepted as statistically significant.

Table 2. Oligonucleotide sequences used for RT-PCR analysis

Markers	Sequences (5' → 3')	Product size (bp)	Annealing temperature (°C)
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	S: AGATCAAGAAGGTGGTGAAG A: TTGTCATACCAGGAAATGAGC	178	60
Endoglin (<i>CD105</i>)	S: AAGAGCTCATCTCGAGTCTG A: TGACGACCACCTCATTACTG	162	56
CD34 molecule (<i>CD34</i>)	S: CAGAAATTCCCAGCAAGCTC A: ATAGCAAATGAGGCCCAAGA	207	56
Integrin β -1 (<i>CD29</i>)	S: CTTATTGGCCTTGCAATTGCT A: TTCCTCTGTA CTTCGGATTG	184	63
CD44 antigen (<i>CD44</i>)	S: ATCCTCACGTCCAACACCTC A: CTCGCCTTTCTTGGTGTAGC	165	63
ALCAM (<i>CD166</i>)	S: CCGTTCACTATTTGGATTGT A: CGTTTCACAGACATAGTTTCC	199	55
CD14 molecule (<i>CD14</i>)	S: TTGATCTCAGCTGCAACAGG A: GTGGGGATATCAGGAACCT	184	56
Major histocompatibility complex I (<i>MHC-I</i>)	S: GGAGAGGAGCAGAGATACA A: CTGTCACTGTTTGCACTCT	218	55
Major histocompatibility complex II (<i>MHC-II</i>)	S: TCTACACCTGCCAAGTG A: CCACCATGCCCTTTCTG	178	55
Osteocalcin (<i>BGLAP</i>)	S: TGAAGACCAGTATCCTGATGC A: GCTGACTTGTTTCTGACTG	174	60
Osteopontin (<i>OPN</i>)	S: GTCTGGCAGAGGTGCAGCCT A: ATGTGGTCAGCCAGCTCGTC	173	56
Peroxisome Proliferator-activated Receptor (<i>PPAR-γ</i>)	S: TGCCCTTCAACGAAATTACC A: TGGAATGTCTTCATAGTGTGG	176	55
Adiponectin (<i>ADIPQ</i>)	S: GGAGACAGCTACTCCCAAGAT A: GTCCAGTCTTACCTCTCAAACCT	187	64
Aggrecan (<i>ACAN</i>)	S: TCTGCTACACAGGTGAAGAC A: AAGATGGGTTTCACTGTGAG	174	60
Collagen type 2, alpha I (<i>COL2A1</i>)	S: GGAGACTACTGGATTGACCC A: TCCATAGCTGAAGTGAAGC	201	60
Glial Fibrillary Acidic Protein (<i>GFAP</i>)	S: CAGAAGCTCCAGGATGAAACC A: TGGATCTTCCCTCAAGAACCGGA	155	58
Nestin (<i>NES</i>)	S: TGCCCTCAGCTTGCAGGAC A: GTGTCTCGAGAGTATCAGGCAAG	134	60

S, sense; A, antisense; bp, base pairs.

Results

Histology and immunostaining

H&E staining demonstrated typical formation of umbilical cord with two arteries and one vein surrounded by matrix (Fig. 1e). Details of intervascular and perivascular portions are also shown in Fig. 1f. Immunohistochemical reaction with anti-desmin antibody showed sparse distribution of positively staining muscle fibres in the intervascular matrix (Fig. 1g). Stromal cells positively expressing vimentin were also seen (Fig. 1h) and residual trabecular spaces of umbilical cord matrix identified. Figure 1i shows the perivascular matrix with more intense distribution of muscle fibres positively stained for desmin, while in Fig. 1l, more sparse distribution of anti-vimentin immunoreacting stromal cells was observed.

Cell cycle analysis

Cell cycle analysis was evaluated on cells derived from both anatomical areas (Fig. 2a) indicating existence of a high percentage (60%) of quiescent cells (G_0/G_1) along with a lower percentage (~40%) of proliferating cells ($S+G_2/M$) in the intervascular fraction. The perivascular portion had relatively uniform cell distribution between quiescent (49% in G_0/G_1) and proliferating cells (51% in $S+G_2/M$).

FCM analysis: UCM-derived cells are primitive mesenchymal stem cells

FCM analysis of unsieved cells revealed the presence of two distinct cell populations within both the intervascular and perivascular stroma (Fig. 2b). No differences

Figure 2. Characterization of intervascular- and perivascular-derived cells. (a) Histograms indicating representative cell cycle study for intervascular- and perivascular- derived cells during log-phase growth. (b) Morphological dotplot showing two subpopulations within each portion. (c) Immunophenotype of cultured cells. Flow cytometry analysis of antigen expression with Alexafluor-488 labelled antibodies: CD34, SSEA-4, Oct-4, TRA-1-60, SSEA-3, HLA-ABC, c-Myc and CD105. Histograms represent relative number of cells vs. fluorescence intensity (FL1). Black histograms indicate background fluorescence intensity of cells labelled with isotype control antibodies only; grey histograms show positivity to the studied antibodies.

were observed in markers of expression between the portions. The representative immunophenotype pattern for the intervascular and perivascular portions is reported in Fig. 2c. All populations were negative for CD105 and CD34 and positive for HLA-ABC. Expression of c-Myc, proto-oncogene implicated in regulation of proliferation, differentiation and in many cell types in self-renewal maintenance in stem cells (23,24), was detected on intervascular- and perivascular- derived cells. OCT-4 expression was reported in both the intervascular and perivascular portions, while 10% and 20% of cells respectively showed SSEA-4 reactivity. No positivity for TRA-1-60 and SSEA-3 was detected in cells from intervascular portions, while cells from perivascular portions had weak expression of both markers

(~10%). Table 3 reports values of positive expressions at P3 and P5.

Cell morphology: all cell populations displayed the same spindle-shaped fibroblast like morphology

UCM-derived cells from both perivascular and intervascular fractions were a population of morphologically fibroblast-like cells, in early stages of primary monolayer culture. However, on reaching confluence, these cells formed spheroid clusters that appeared as three-dimensional structures. This behaviour was consistently observed when cultures reached confluence at every subsequent passage. After sieving, cells with diameters <8- μ m, that adhered to lower plate surfaces formed a

Table 3. Comparison between markers expression at P3 and P5 assessed by FCM analysis on unsieved intervacular- and perivascular-derived cells

Samples	Passages	OCT-4 (%)	C-Myc (%)	TRA-1-60 (%)	SSEA-3 (%)	SSEA-4 (%)	CD105 (%)	HLA-ABC (%)	CD34 (%)
Intervascular	P3	65	50	<1	<1	10	<1	85	<1
	P5	68	55	<1	<1	10	<1	85	<1
Perivascular	P3	70	40	10	10	20	<1	80	<1
	P5	68	38	7	12	20	<1	82	<1

Figure 3. Sieved-MSCs characteristics. (a) Phase contrast images of cells that adhered to the lower and upper plate surface of either intervacular (a and c, respectively) or perivascular (b and d, respectively) Wharton's Jelly. Representative spheroid cluster is also showed (e). (20x magnification). Scale bars: 20 μ m. (b) Cell doubling time: comparison between MSCs (lower and upper than 8- μ m) from intervacular and perivascular fraction with controls (unsieved intervacular and perivascular cells); ** $P < 0.01$ and * $P < 0.05$. (c) CFU colonies obtained after plating 17,500 cells from each population at P1. Mean $\pm$ SD. (d) Cell doubling time: comparison between fresh and cryopreserved MSCs. Mean $\pm$ DS.

morphological homogeneous population (Fig. 3A-a,b) compared to the larger sized population of either intervacular or perivascular matrix (Fig. 3A-c,d, respectively). Formation of clusters was also observed during culture of sieved cells (Fig. 3A-e).

Proliferation studies: sieved cells had more proliferative potential than unsieved cells

Every subpopulation proliferated over passages studied, reaching confluence even after 10 passages. Large cells of the perivascular portion propagated slowly and passed 15.89 ± 0.34 PD after 31 days, whereas over the same time range, small ones reached 19.13 ± 0.45 PD. After the seventh passage, proliferating properties of the two-cell population became similar (data not shown). As control, non-fractionated perivascular portions surpassed 8.54 ± 1 cell PD. In contrast, in the intervacular portions, large cells propagated more rapidly compared to small

ones (20 ± 0.13 PD versus 13.19 ± 0.57 PD), while non-fractionated intervacular portions, as control, reached 9.42 ± 0.42 PD (Fig. 3B).

Unsieved cells isolated from intervacular and perivascular matrix were able to form fibroblast-colony forming units with the rates of $1:289.93 \pm 7.14$ and $1:366.11 \pm 9.87$ respectively. Of sieved cells, CFU-F assay supported differences reported above: higher CFU for small perivascular cells and large intervacular cells (rates $1:140.54 \pm 11.88$ and $1:166.59 \pm 1.12$, respectively) (Fig. 3C).

Molecular characterization: isolated cells were mesenchymal cells with low immunogenicity

As shown by RT-PCR, every population expressed MSC- (*CD29*, *CD44*, *CD166*) markers and lacked *CD34* and *CD14* over the passages studied (from P1 to P10). *CD105* mRNA was detected in all cells and at every passage, but

Table 4. Summary of MSC-markers expression on sieved intervascular- and perivascular-derived cells

Samples	Passages	CD29	CD105	CD44	CD166	CD34	CD14	MHC-I	MHC-II
Intervascular <8 µm	P1	+	+	+	+	–	–	+	–
	P2	+	+	+	+	–	–	+	–
	P5	+	+	+	+	–	–	+	–
	P7	+	+	+	+	–	–	+	–
	P10	+	–	+	+	–	–	+	–
Intervascular >8 µm	P1	+	+	+	+	–	–	+	–
	P2	+	+	+	+	–	–	+	–
	P5	+	+	+	+	–	–	+	–
	P7	+	+	+	+	–	–	+	–
	P10	+	+	+	+	–	–	+	–
Perivascular <8 µm	P1	+	+	+	+	–	–	+	–
	P2	+	+	+	+	–	–	+	–
	P5	+	+	+	+	–	–	+	–
	P7	+	+	+	+	–	–	+	–
	P10	+	+	+	+	–	–	+	–
Perivascular >8 µm	P1	+	+	+	+	–	–	+	–
	P2	+	+	+	+	–	–	+	–
	P5	+	+	+	+	–	–	+	–
	P7	+	+	+	+	–	–	+	–
	P10	+	–	+	+	–	–	+	–

disappeared in small intervascular and large perivascular cells at P10. *MHC-I* expression was demonstrated in each cell population, while *MHC-II* was not (Table 4).

Cryopreservation: six months-cryopreservation did not affect MSC properties

Recovery rate after thawing was 80% and 70% for large and small intervascular cells and 75% and 80% for large and small perivascular cells respectively. After cryopreservation, every subpopulation maintained its morphological characteristics. Proliferation capability showed the same trends observed with fresh cells, although each subpopulation's doubling time was slightly longer than previously observed. Large cells of the perivascular portion propagated slowly and passed 12.19 PD after 31 days, whereas over the same time, small ones reached 14.91 PD (Fig. 3D). Conversely, in the intervascular portion, large cells propagated more rapidly compared to small ones (15.26 PD versus 10.96 PD, respectively). Frozen cells showed the same surface molecule expression as fresh cells. They were positive for *CD44*, *CD29*, *CD166*, *CD105* and *MHC-I* (data not shown). Every subpopu-

lation was negative for expression of haematopoietic markers (*CD34* and *CD14*) and *MHC-II*.

Multi-lineage differentiation studies: committed equine UCM-derived populations gave rise to several lineages

Differentiating potential was evaluated in cells from all experimental conditions by histochemical staining and RT-PCR analysis. Figure 4 shows differentiation staining and molecular studies from unsieved intervascular and perivascular cells.

Osteogenic differentiation. After 10 days induction, every cell population was positive under von Kossa staining. Minerals associated with matrix of induced cells were observed. RT-PCR analysis of *BGLAP* and *OPN* mRNA expression confirmed osteogenic induction. Each population expressed *BGLAP*, but only cells from intervascular portions expressed *OPN*. Controls did not express any osteogenic markers studied.

Adipogenic differentiation. Each cell population was positive under oil red O staining, revealing formation of

Figure 4. Differentiation studies. Intervascular- and perivascular- derived cells induced to osteogenic (von Kossa stain), adipogenic (Oil red O stain), chondrogenic (Alcian blue stain) and neurogenic (Nissl stain) differentiation. Undifferentiated cells are reported as control. 20x magnification. Scale bars: 40 µm. Panel on the right shows specific gene expression on intervascular and perivascular cells, induced (1 and 2, respectively) and controls (3 and 4, respectively). *BGLAP* and *OPN* mRNA were investigated for osteogenesis, *PPARY* and *ADIPOQ* for adipogenesis, *ACAN* and *COL2A1* for chondrogenesis and *NES* and *GFAP* for neurogenesis. *GAPDH* was employed as reference gene. Bone, adipose tissue, cartilage and spinal cord were used as positive control.

cytoplasmic inclusion of neutral lipids. *PPAR- γ* and *ADIPQ* mRNA expression was detected on induced cells. Undifferentiated controls did not show any positivity.

Chondrogenic differentiation. Cells from each cell population after 15 days formed spherical masses, which stained with alcian blue when induced to chondrogenic differentiation and were negative when not. Treated cells expressed *COL2A1* and *ACAN* mRNA, while controls did not.

Neurogenic differentiation. After induction, changes in cells' morphology were apparent. Cells developed rounded cell bodies with bipolar or multipolar neurite like extensions, similar to morphology of neural stem cells, as assessed by Nissl staining. Each induced cell population expressed classical glial astrocyte marker *GFAP*, whilst weak expression was reported for *NES* mRNA. Controls did not express any neural marker.

Discussion

Close to the foetus, the umbilical cord is composed of two arteries and one vein, surrounded by embryonic connective tissue or matrix. H&E staining revealed that within the UCM, it is possible to distinguish between intervascular (or Wharton's jelly) and perivascular portions. Immunohistochemical reactions with anti-vimentin and anti-desmin antibodies showed different intensity of distribution of vimentin and desmin between intervascular and perivascular portions. Vimentin is an intermediate filament protein specifically expressed in mesenchyme-derived cells, such as fibroblasts, and not expressed in smooth muscle cells (25), while desmin is a muscle-specific cytoskeletal filament strongly expressed in perivascular portions of the umbilical cord, near blood vessel walls. Coexistence of vimentin and desmin in these cells confirms the thesis of other authors (26,27) that UCM stromal cells rather than fibroblasts or smooth muscle cells are true 'myofibroblasts'. This term was first used by Majno *et al.* (28) to define cells that exhibit some ultrastructural features of both smooth muscle cells and of fibroblasts. The myofibroblastic cells of the intervascular stroma might be derived from adjacent vascular smooth muscle cells or, alternatively, from pre-existing fibroblasts (29) while in the perivascular portion, meaning in closer proximity to the umbilical vessels, the cells are more highly differentiated and more similar to vascular myofibrils.

Cells from UCM have been demonstrated to have faster proliferation and greater *ex vivo* expansion capabilities than adult MSCs, including bone marrow MSCs (30). Moreover, recently in human medicine, differentiation potential of these cells has been extended over the classi-

cal mesenchymal lineages and UCM-derived cells have been induced to differentiate towards hepatocytes and pancreatic cells (31,32).

Cells from the intervascular portions have faster doubling time and higher CFU-F frequency than cells from the perivascular portions (33) due to the more differentiated nature of perivascular cells. As reported by Nanaev *et al.*, the immature cells, retaining ability to proliferate, are located close to the amniotic surface, whereas highly differentiated, non-proliferating fibroblasts are located in closer proximity to vessels (34). In our study, for the first time, we isolated and characterized MSCs from both areas of the cord matrix and provided additional support for existence of primitive cells in equine UCM. Of particular importance is the finding that a large percentage of the cultured cells, mainly in Wharton's jelly, remained in the quiescent state (G_0/G_1). Quiescence has been reported as a peculiar feature of stem cells (35), although they are characterized by a high proliferative capability and show a huge self renewal potential *in vitro* (36,37).

To confirm this hypothesis, we characterized presumptive MSCs in terms of specific marker expression and, as reported in previous work (13,38), detected presence of some pluripotency-specific antigens (OCT-4 and c-Myc), with some other weakly expressed antigens (TRA-1-60 and SSEA-4). We also investigated presence of MSC-specific markers (*CD44*, *CD29*, *CD105*, *CD166*) by RT-PCR and expression of haematopoietic markers that remained undetectable in terms of presence of mRNA (*CD34* and *CD14*) and antigen (*CD34*). This pattern was conserved through the passages, from P1 to P10 in both portions.

RT-PCR revealed presence of *CD105* mRNA although flow cytometry analysis failed to detect any *CD105* antigen. This finding could be attributed to non species-specific antibodies used or to lack of translation of mRNA.

These results suggest that cells from UCM represent an intermediate stage between pluripotent embryonic stem cells and adult stem cells and that MSCs from each portion are primitive cells. Furthermore, we identified two distinct subpopulations within the intervascular and perivascular stroma (Fig. 2a) and hence the focus of the work became improving isolation and purification protocols of MSCs from both areas of equine UCM, employing a size-sieving method based on a polycarbonate membrane with 8- μ m pores. This procedure yielded two relatively homogeneous subpopulations from the intervascular and perivascular portions with comparable marker expression and differentiation potential, although the smaller intervascular and larger perivascular cells lost *CD105* expression at P10. It is possible that modifications in expression of markers, which frequently occur due to genetic changes in karyotype and tumorigenicity, are induced by extended culture. The four subpopulations (smaller and larger

intervascular and perivascular cells) proliferated reaching confluence over the passages studied, without spontaneous differentiation or senescence. Surprisingly, every sieved-cell population propagated faster when compared to the corresponding control (unsieved cells). Moreover, our data show differences between smaller and larger cell subpopulations isolated from each area. In particular, large intervascular and small perivascular cells had fastest doubling times and exhibited very similar and higher CFU-F frequency. This could indicate that unsieved portions of UCM are composed of heterogeneous cell populations with lower proliferation potential. When the portions are sieved, we hypothesize, in agreement with Colter *et al.* (21), that large perivascular cells, which propagate slowly, are mature MSCs, whereas the smaller ones that propagate more rapidly, are recycling stem cells. Large intervascular MSCs do not divide so rapidly until after they are separated from non-MSCs in the primary culture.

To assess usefulness of our cells for cell therapy, we also evaluated expression of markers related to cell immunogenicity including *MHC-I* and *MHC-II*. *MHC-I* was positive in terms of mRNA and antigen, this being in contrast with the negative result obtained by Hoynowski *et al.* (13). Every cell population was negative for *MHC-II* expression from P1 to P10 consistent with most publications (3,39), suggesting a potential role of UCM as an allogenic cell source for cell-based therapies.

During primary culture, sieved and unsieved cells attached to plastic dish surfaces and had spindle-shaped fibroblast-like morphology. After reaching confluence, some of these cells formed spheroid colonies that grew upward from the substratum surface overlying a layer of confluent cells. We suggest that these colonies represent more primitive progenitor cells; they were similar to embryoid bodies found after culture of mouse embryonic stem cells (12). Differentiation data confirmed the nature of these cells and they can be used as progenitors for mesodermal (including osteogenic, adipogenic and chondrogenic) and ectodermal (neurogenic) lineages. After 10 days of osteogenic induction, we detected presence of *BGLAP* mRNA in every cell population, although only intervascular cells expressed *OPN* – suggesting faster differentiation of these cells, as this gene is considered highly expressed during matrix maturation and mineralization phases (40). Committed equine UCM-derived populations gave rise to both chondrogenic and adipogenic lineages, although much literature claims inefficient differentiation capability into adipogenic lineage in equine MSCs compared to human and rodent MSCs, maybe due to heterogeneity of the starting population (41) and/or culture conditions (12). Furthermore, here, we show morphologically neuron-like cells and specific neuronal marker expression after differentiation *in vitro*. Expression of

classical *GFAP* was detected, suggesting that astrocyte differentiation had occurred. Presence of nestin, a marker expressed in neuronal precursor stem cells, was also investigated and weak expression was observed, probably due to long-term differentiation (more than 1 day) (42); expression of the nestin gene decreases with neuronal maturation (43). These data confirm that equine, as well as human UCM, represents an attractive source of MSCs for regenerative medicine, due to their high proliferation and multipotential differentiation properties, even after 6 months cryopreservation. Cells from intervascular portions apparently have better proliferation capabilities than perivascular cells, which are probably more highly differentiated. Moreover, size-sieved procedure here is shown to be a rapid and efficient tool to isolate more proliferative cells, with different ontological and anatomical origins, which probably influence their proliferation capabilities and, consequently, affect their performance as cell sources for tissue engineering.

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Disclosure

No competing financial interests exist.

References

- 1 Bruder SP, Fink DJ, Caplan AI (1994) Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J. Cell. Biochem.* **56**, 283–294.
- 2 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD *et al.* (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.
- 3 Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D *et al.* (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315–317.
- 4 D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA (1999) Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *J. Bone Miner. Res.* **14**, 1115–1122.
- 5 Alviano F, Fossati V, Marchionni C, Arpinati M, Bonsi L, Franchina M *et al.* (2007) Term amniotic membrane is a high throughput source

- for multipotent mesenchymal stem cells with the ability to differentiate into endothelial cells in vitro. *BMC Dev. Biol.* **7**, 11.
- 6 Tsai MS, Lee JL, Chang YJ, Hwang SM (2004) Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum. Reprod.* **19**, 1450–1456.
 - 7 Lee MW, Choi J, Yang MS, Moon YJ, Park JS, Kim HC *et al.* (2004) Mesenchymal stem cells from cryopreserved human umbilical cord blood. *Biochem. Biophys. Res. Commun.* **320**, 273–278.
 - 8 Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE (2005) Human umbilical cord perivascular (Hucpv) cells: a source of mesenchymal progenitors. *Stem Cells* **23**, 220–229.
 - 9 Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ *et al.* (2004) Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* **22**, 1330–1337.
 - 10 Guillot PV, Gotherstrom C, Chan J, Kurata H, Fisk NM (2007) Human first-trimester fetal Msc express pluripotency markers and grow faster and have longer telomeres than adult Msc. *Stem Cells* **25**, 646–654.
 - 11 Koch TG, Heerkens T, Thomsen PD, Betts DH (2007) Isolation of mesenchymal stem cells from equine umbilical cord blood. *BMC Biotechnol.* **7**, 26.
 - 12 Reed SA, Johnson SE (2008) Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types. *J. Cell. Physiol.* **215**, 329–336.
 - 13 Hoynowski SM, Fry MM, Gardner BM, Leming MT, Tucker JR, Black L *et al.* (2007) Characterization and differentiation of equine umbilical cord-derived matrix cells. *Biochem. Biophys. Res. Commun.* **362**, 347–353.
 - 14 Passeri S, Nocchi F, Lamanna R, Lapi S, Miragliotta V, Giannessi E *et al.* (2009) Isolation and expansion of equine umbilical cord-derived matrix cells (Eucmcs). *Cell Biol. Int.* **33**, 100–105.
 - 15 Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* **4**, 267–274.
 - 16 Majore I, Moretti P, Hass R, Kasper C (2009) Identification of subpopulations in mesenchymal stem cell-like cultures from human umbilical cord. *Cell Commun. Signal.* **7**, 6.
 - 17 Hung SC, Chen NJ, Hsieh SL, Li H, Ma HL, Lo WH (2002) Isolation and characterization of size-sieved stem cells from human bone marrow. *Stem Cells* **20**, 249–258.
 - 18 Smith JR, Pochampally R, Perry A, Hsu SC, Prockop DJ (2004) Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. *Stem Cells* **22**, 823–831.
 - 19 D'Ippolito G, Howard GA, Roos BA, Schiller PC (2006) Sustained stromal stem cell self-renewal and osteoblastic differentiation during aging. *Rejuvenation Res.* **9**, 10–19.
 - 20 Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Wan W, Rachakatla J, Kucia M (2008) Hunt for pluripotent stem cell-regenerative medicine search for almighty cell. *J. Autoimmun.* **30**, 151–162.
 - 21 Colter DC, Class R, DiGirolamo CM, Prockop DJ (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA* **97**, 3213–3218.
 - 22 Zuba-Surma EK, Kucia M, Abdel-Latif A, Dawn B, Hall B, Singh R *et al.* (2007) Morphological characterization of very small embryonic-like stem cells (Vsels) by imagestream system analysis. *J. Cell Mol. Med.* **12**, 292–303.
 - 23 Lin CH, Jackson AL, Guo J, Linsley PS, Eisenman RN (2009) Myc-regulated micromas attenuate embryonic stem cell differentiation. *EMBO J.* **28**, 3157–3170.
 - 24 Satoh Y, Matsumura I, Tanaka H, Ezoe S, Sugahara H, Mizuki M *et al.* (2004) Roles for C-Myc in self-renewal of hematopoietic stem cells. *J. Biol. Chem.* **279**, 24986–24993.
 - 25 Kreis T, Vale RS (1999) *Guidebook to the Cytoskeletal and Motor Proteins*, 1–551, 2nd edn. Oxford University Press, Oxford, New York.
 - 26 Kadner A, Zund G, Maurus C, Breymann C, Yakarisik S, Kadner G *et al.* (2004) Human umbilical cord cells for cardiovascular tissue engineering: a comparative study. *Eur. J. Cardiothorac. Surg.* **25**, 635–641.
 - 27 Takechi K, Kuwabara Y, Mizuno M (1993) Ultrastructural and immunohistochemical studies of Wharton's jelly umbilical cord cells. *Placenta* **14**, 235–245.
 - 28 Majno G, Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR (1971) Contraction of granulation tissue in vitro: similarity to smooth muscle. *Science* **173**, 548–550.
 - 29 Can A, Karahuseyinoglu S (2007) Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells* **25**, 2886–2895.
 - 30 Troyer DL, Weiss ML (2008) Wharton's Jelly-derived cells are a primitive stromal cell population. *Stem Cells* **26**, 591–599.
 - 31 Anzalone R, Lo Iacono M, Corrao S, Magno F, Loria T, Cappello F *et al.* (2010) New emerging potentials for human Wharton's jelly mesenchymal stem cells: immunological features and hepatocyte-like differentiative capacity. *Stem Cells Dev.* **19**, 423–438.
 - 32 Anzalone R, Lo Iacono M, Loria T, Di Stefano A, Giannuzzi P, Farina F *et al.* (2010) Wharton's Jelly mesenchymal stem cells as candidates for beta cells regeneration: extending the differentiative and immunomodulatory benefits of adult mesenchymal stem cells for the treatment of type 1 diabetes. *Stem Cell Rev.* **7**, 342–363.
 - 33 Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO *et al.* (2007) Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. *Stem Cells* **25**, 319–331.
 - 34 Nanaev AK, Kohnen G, Milovanov AP, Domogatsky SP, Kaufmann P (1997) Stromal differentiation and architecture of the human umbilical cord. *Placenta* **18**, 53–64.
 - 35 Owen M, Friedenstein AJ (1988) Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found. Symp.* **136**, 42–60.
 - 36 Baksh D, Song L, Tuan RS (2004) Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J. Cell Mol. Med.* **8**, 301–316.
 - 37 Prockop DJ (1997) Marrow stromal cells as stem cells for not hematopoietic tissues. *Science* **276**, 71–74.
 - 38 Carlin R, Davis D, Weiss M, Schultz B, Troyer D (2006) Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (Puc) matrix cells. *Reprod. Biol. Endocrinol.* **4**, 8.
 - 39 Deans RJ, Moseley AB (2000) Mesenchymal stem cells: biology and potential clinical uses. *Exp. Hematol.* **28**, 875–884.
 - 40 Raouf A, Seth A (2000) Ets transcription factors and targets in osteogenesis. *Oncogene* **19**, 6455–6463.
 - 41 Koerner J, Nesic D, Romero JD, Brehm W, Mainil-Varlet P, Grogan SP (2006) Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. *Stem Cells* **24**, 1613–1619.
 - 42 Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* **61**, 364–370.
 - 43 Lendahl U, Zimmerman LB, McKay RD (1990) Cns stem cells express a new class of intermediate filament protein. *Cell* **60**, 585–595.