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Research Article

Isolation and characterization of equine amnion mesenchymal stem cells

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Abstract

The amnion is a particular tissue whose cells show features of multipotent stem cells proposed for use in cellular therapy and regenerative medicine. From equine amnion collected after the foal birth we have isolated MSCs (mesenchymal stem cells), namely EAMSCs (equine amnion mesenchymal stem cells), from the mesoblastic layer. The cells were grown in α-MEM (α-modified minimum essential medium) and the effect of EGF (epidermal growth factor) supplementation was evaluated. To assess the growth kinetic of EAMSCs we have taken into account some parameters [PD (population doubling), fold increase and DT (doubling time)]. The differentiation in chondrogenic, adipogenic and osteogenic types of cells and their epitope expression by a cytofluorimetric study have been reported. EGF supplementation of the culture medium resulted in a significant increase in PD growth parameter and in the formation of bone nodules for the osteogenic differentiation. By immunohistochemistry the amnion tissue shows a positivity for the c-Kit (cluster tyrosine-protein kinase), CD105 and Oct-4 (octamer-binding transcription factor 4) antigens that confirmed the presence of MSCs with embryonic phenotype.

Keywords: amnion; equine; growth kinetics; histochemistry; immunohistochemistry; mesenchymal stem cells (MSCs)

1. Introduction

The equine placenta is classified as diffuse. It involves the entire surface of the chorioallantois and is vascularized from allantois, with tufts of villi distributed over the entire surface of the chorion. The allantoamnion results from fusion of the allantois with the amnion. The amnion is avascular and the blood vessels are on the surface of the allantois. The amnion assumes the appearance of a kidney-shaped bag completely separated from the chorion by the allantois. In mammals, the amnion is constituted by an ectoblastic and a mesoblastic layer separated by a basal membrane. In the horse the ectoblastic layer consists of epithelial cells, is directed toward the lumen and it is bathed by the amniotic fluid, while the mesoblastic layer adheres weakly to the allantois. Both epithelial and mesenchymal cells isolated from the amnion showed embryonic features and possess differentiative pluripotency. Several studies on amniotic epithelial cells showed that these cell lines can differentiate into ectodermal and endodermal lineages once isolated and grown in special culture media (Miki and Strom, 2006). MSCs (mesenchymal stem cells) isolated from human amnion can differentiate *in vitro* into neuroglial cells, cardiomyocytes, osteoblasts, adipocytes and chondroblasts, and express the Oct-4 (octamer-binding transcription factor 4) marker specific for embryonic stem cells (Kim et al., 2007). Therefore amniotic mesenchymal cells can be considered primitive cells capable of differentiating into mesoderm and ectoderm tissue. Due to low immunogenicity and anti-inflammatory effect (Toda et al., 2007) amnion stem cells were considered an advantage in transplant therapy than other adult stem cells. To make a successful stem cell allograft, it is important to consider the histocompatibility of stem cells for transplant candidates. Cells derived from amnion are immunologically immature and do not cause acute rejection after stem cells allograft: Kobayashi et al. (2008) identified a subpopulation doubly negative for MHC I and MHC II from the mesenchymal layer of the amnion, indicating these cells as good candidates for use in allografts and also describing these cells as useful in regenerative medicine. Recently, MSCs were isolated in the horse from bone marrow (Koerner et al., 2006; Vidal et al., 2006; Arnhold et al., 2007; Kisiday et al., 2008), adipose tissue (Vidal et al., 2007; Kisiday et al., 2008), peripheral blood (Koerner et al., 2006) and cord blood (Koch et al., 2007, Reed and Johnson, 2008) The extraembryonic tissues of the horse represent another source of stem cells: Hoynowski et al. (2007) isolated and characterized a stem cell population from equine umbilical cord matrix (Wharton's jelly) that expresses markers associated with an embryonic phenotype [Oct -4, SSEA-4 (stage-specific embryonic antigen-4) and c-Kit (cluster tyrosine-protein kinase)] and an adult phenotype [CD54 (cluster of differentiation 54), CD90, CD105 and CD146]. They also

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Abbreviations used: AM, amniotic membrane; α-MEM, α-modified minimum essential medium; CFU, colony-forming unit; CFU-F, CFU-fibroblast; c-Kit, cluster tyrosine-protein kinase; CPD, cumulative population doubling; DT, doubling time; EAMSC, equine amnion mesenchymal stem cell; EGF, epidermal growth factor; FCS, foetal calf serum; HS, horse serum; H/E, haematoxylin and eosin; MSC, mesenchymal stem cell; Oct-4, octamer-binding transcription factor 4; P0, passage 0; PD, population doubling; P/S, penicillin/streptomycin.

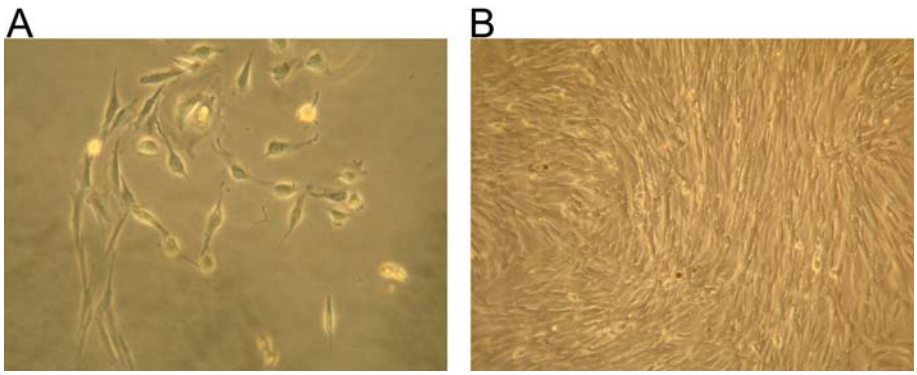

Figure 1 EAMSCs at the beginning of culture (A) and at confluence (B)

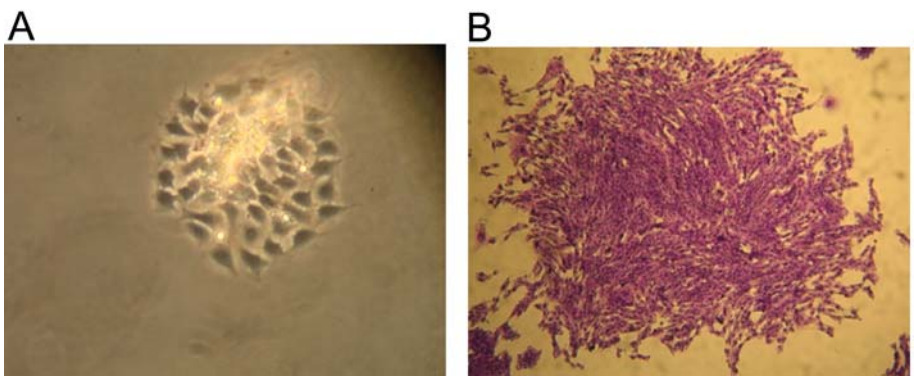

Figure 2 A CFU fixed (A) and stained with Crystal Violet (B)

highlighted their ability for osteogenic, chondrogenic and adipogenic differentiation. In order to improve non-invasive techniques aimed at the isolation of MSCs and minimize the risk of contamination during the expansion, we conducted a study evaluating different methods to expand equine MSCs; culture media were supplemented with EGF (epidermal growth factor)-like growth factor that formerly showed good results (Passeri et al., 2009). The results reported on the matrix of the umbilical cord in horses (Hoynowski et al., 2007) and humans (Wang et al., 2004; Fong et al., 2007; Secco et al., 2008; Qiao et al., 2008) pointed out that this tissue is a rich and easily accessible source of MSCs. Also equine amnion is easy to sample immediately after birth because it envelopes the foal from the surrounding chorion and did not show relations with the rest of the placenta that the mare delivers

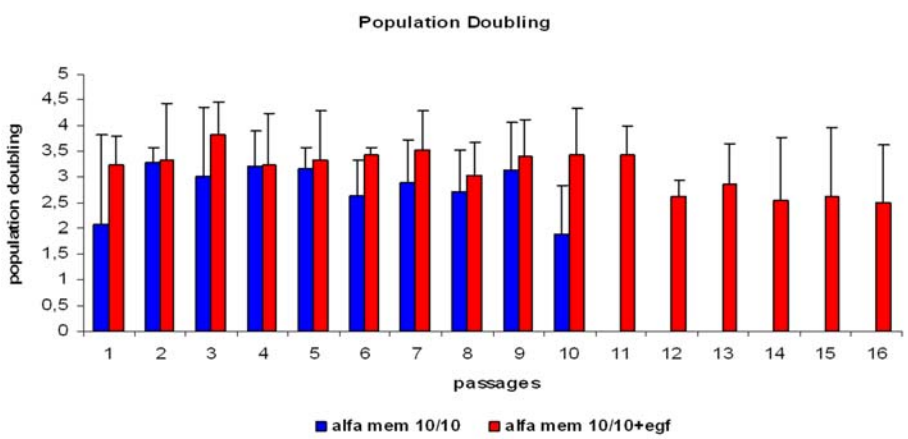

Figure 3 PD of EAMSCs

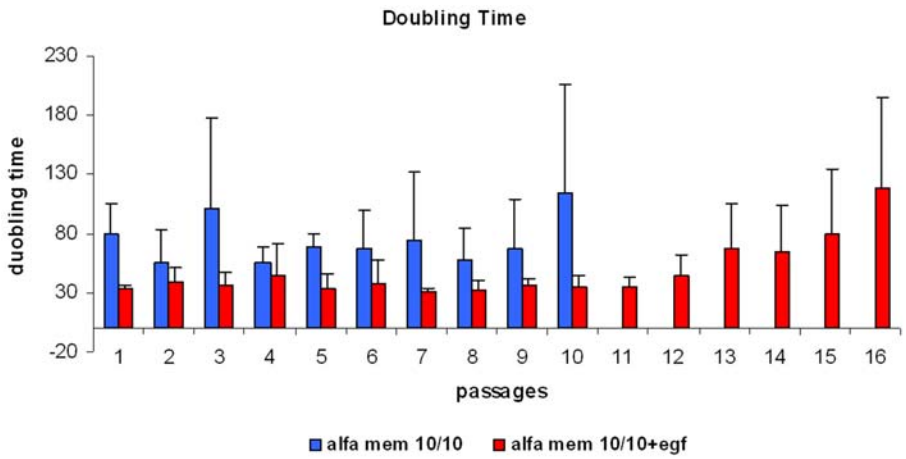

Figure 4 DT of EAMSCs

subsequently. For this purpose we focused on isolation and characterization of EAMSCs (equine amnion mesenchymal stem cells), studying their growth kinetics and properties of differentiation.

2. Materials and methods

2.1. Amnion sampling

AMs (amniotic membranes) were obtained from four 10–13-year-old standard bred mares. All the experimental procedures were performed as per guidelines determined by the local ethical committee. An AM portion of approx. 20×20 cm^2 was collected soon after delivery, put into RPMI 1640 medium (Invitrogen) supplemented with 2% P/S (penicillin/streptomycin; Cambrex) and 1% amphotericin B (Invitrogen) and stored at 4°C. Subsequently, each AM was cut into pieces (2–3 cm in length) and soaked in RPMI 1640+5% P/S+2% amphotericin B, stored at 4°C for 24 h and then processed.

2.2. Isolation and expansion of EAMSCs

AM samples were washed with PBS solution (Euroclone, Milan, Italy), thus removing blood traces, minced in 1 mm^3 pieces and soaked in 10 ml of a collagenase solution (1 mg/ml) for 30 min at 37°C. The suspension was then filtered through a 100 μm filter (Millipore, Billerica, MA, U.S.A.).

Nucleated cells were counted in a haemocytometer by staining with 0.4% Trypan Blue (Sigma, St. Louis, MO, U.S.A.) and centrifuged at 500 ***g*** for 10 min, and the pellets were resuspended in α-MEM (α-modified minimum essential medium; Cambrex) supplemented with 10% (v/v) FCS (foetal calf serum; Eurobio) and 10% HS (horse serum), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Euroclone).

Cells were plated at 10^5 cells/cm^2 on 25 cm^2 flasks (Sarstedt, Nümbrecht, Germany). After 24 h non-adherent cells were removed by washing with PBS and fresh medium was added twice a week for approx. 14 days or until adherent cells reached 90% confluence [P0 (passage 0)].

Cells were then harvested [P1 (passage 1)] for further expansion using 0.25% trypsin solution and 1 mM EDTA (Euroclone) for 5 min at 37°C, replated at 5000 cells/cm^2, grown to near confluence and harvested with the same protocol. At the end of each passage, the cells were counted by a haemocytometer and living cells were identified by Trypan Blue.

2.3. Selection of culture medium

Cells were grown in α-MEM supplemented with 10% FCS and 10% HS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Media were replaced every 3 days.

To evaluate the effect of growth factor supplementation, EGF (Sigma) was added to α-MEM to a concentration of 10 ng/ml.

EAMSCs were plated at 5000 cells/cm^2 in a 25 cm^2 tissue culture flask with either α-MEM or α-MEM+EGF to determine growth kinetics. Cells at 90% confluence were trypsinized, counted with a haemocytometer and replated as mentioned above.

2.4. Growth kinetics of EAMSCs

CFU-F [CFU (colony forming units)-fibroblast] test: To assess the number of EAMSCs in primary culture, cells isolated from amnion were seeded at 10^5 cells/cm^2 on 6-well plates and incubated for 8 days.

Then the cells were washed with PBS and stained with Crystal Violet (0.5%) in methanol at room temperature for 10 min, rinsed twice with PBS and counted using a phase-contrast microscope.

The frequency of EAMSCs extracted from amnion was estimated by dividing the total number of nucleated cells seeded in step 0 with the number of CFU-F colonies counted in primary culture.

PD (population doubling): PD was calculated by comparing the number of cells at the end of P0 with the estimated number of

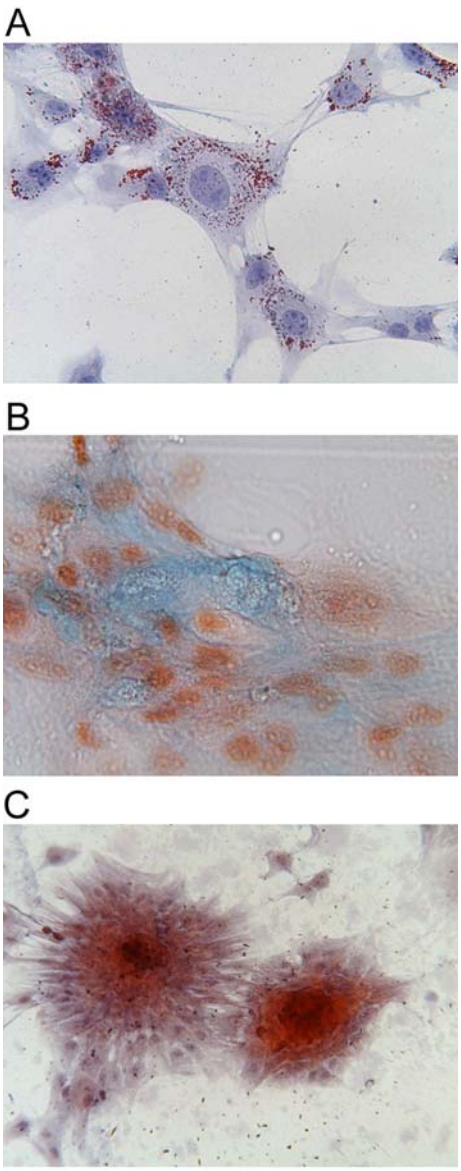

Figure 5 **EAMSCs differentiation: adipogenic (Oil Red O) (A) (magnification: ×25), chondrogenic (Alcian Blue) (B) (magnification: ×40) and osteogenic (Alizarin Red) (C) (magnification: ×25)**

EAMSCs at the beginning of the primary culture, according to the following formula: $(\log N_h - \log N_0)/\log 2$, where N_h is the cell number at the end of the passage and N_0 the initial cell number.

Fold increase: This was calculated by dividing the number of harvested cells at 90% confluence by the number of plated cells for each passage.

DT (doubling time): This represents the time between the initial seeding and the confluence of 90% for each step.

2.5. Statistical analysis

Values are reported as means ± S.D. All statistical analyses were performed using Graph-Pad Prism software (GraphPad, San Diego, CA, U.S.A.). The PD for each passage and each medium was compared using ANOVA and Student's *t* test. The correlation between CFU-F at P0 and CPD (cumulative population doubling) was determined by regression analysis. Differences were considered statistically significant at $P<0.05$.

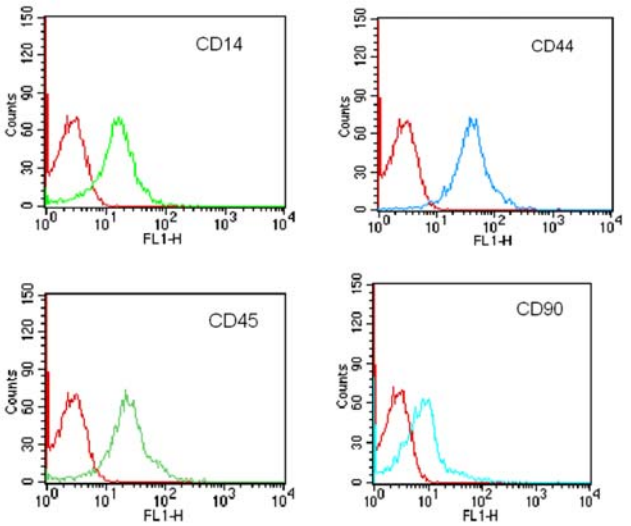

Figure 6 **Flow cytometry of EAMSCs**

2.6. Differentiation protocols

To ascertain the differentiation ability of EAMSCs, P3 cells grown in α-MEM were plated at 5000 cells/cm^2 on 4-well chamber slides (Sigma) and incubated in α-MEM for 10 days.

Differentiation protocols were performed as follows:

Osteogenesis: Cultures were incubated in α-MEM supplemented with 10% FCS and 10% HS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 20 mM 2-glycerophosphate (Sigma), 100 nM dexamethasone (Sigma) and 250 μM ascorbate 2-phosphate (Sigma) for 3 weeks. Cells were fixed with a 10% buffered formalin solution (Sigma) for 20 min at room temperature and stained with Alizarin Red (pH 4.1; Sigma) for 20 min at room temperature.

Adipogenesis: Cultures were incubated in α-MEM that was supplemented with 10% FCS and 10% HS, 100 units/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 5 μg/ml insulin (Lilly), 50 μM indomethacin (Sigma), 1 μM dexamethasone (Sigma) and 0.5 μM isobutylmethylxanthine (Sigma) for 2 weeks. Cells were fixed with 10% formalin for 20 min at room temperature and stained with 0.5% Oil Red O (Sigma) in methanol (Sigma) for 20 min at room temperature.

Chondrogenesis: Cultures were incubated for 3 weeks in CBM (chondrocyte basal medium; Cambrex Bio Science, Walkersville, MD, U.S.A.). Cells were fixed with 10% formalin for 20 min at room temperature and stained with Alcian Blue solution (pH 2.5; Sigma) for 20 min at room temperature. Cell nuclei were counterstained with Weigert's iron haematoxylin.

To test the effect of EGF supplementation on differentiation ability, cells grown in α-MEM+EGF were plated at 5000 cells/cm^2

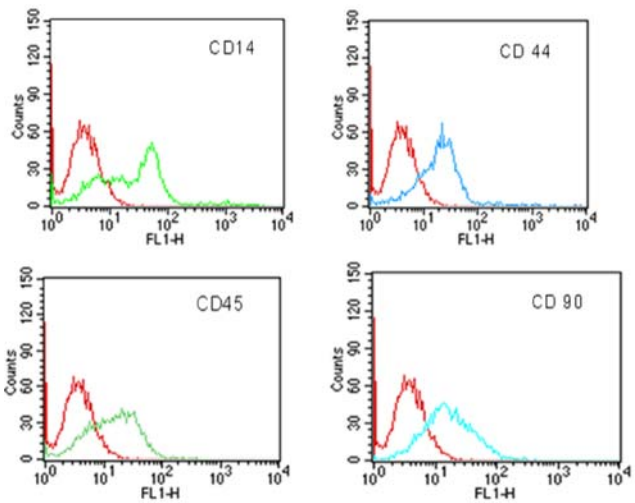

Figure 7 **Flow cytometry of leucocytes extracted from a sample of equine peripheral blood**

on 4-well chamber slides and incubated in α-MEM+EGF for 10 days. Subsequently, adipogenic, osteogenic and chondrogenic differentiations were attempted by using the same protocols mentioned above with the supplementation of the media with EGF (10 ng/ml).

2.7. FACS and immunochemical analyses

EAMSCs at P3 were analysed for epitope expression using a FACScan cytofluorimeter (Becton Dickinson, San Jose, CA, U.S.A.). Cells were detached by tripsinization, centrifuged, resuspended in PBS (Euroclone) at 1×10^6 cells/ml and processed according to the manufacturer's recommendations. Analyses were performed using anti-MHC I (Serotec, Oxford, UK), anti-MHC II (Serotec), anti-CD14 (Serotec), anti-CD45 (Serotec), anti-CD44 (Chemicon, Temecula, CA, U.S.A.), anti-β-1-integrin (Chemicon) and anti-CD90 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) mouse monoclonal antibodies. As secondary antibody, an FITC goat anti-mouse IgG (Santa Cruz Biotechnology) was used. A minimum of 10000 events were acquired for each sample.

Amnion was included in paraffin and then sectioned; the sections were deparaffinized, permeabilized with methanol for 2 min at room temperature and washed three times with PBS.

H/E (haematoxylin and eosin) and Azan Mallory techniques were used for histochemical study.

c-Kit (marker of cell proliferation and differentiation; BioLegend, San Diego, CA, U.S.A.), CD105 (marker of endothelial cells proliferation associated with angiogenesis; Abcam, Cambridge, U.K.) and Oct-4 (antigen involved in self-renewal of embryonic stem cells or in the maintenance of stemness; Abcam) were used as primary antibodies and incubated for 1 h at room temperature.

Slides were then incubated with a biotinylated policlonal secondary antibody (Vectastain®; Vector Laboratories, Burlingame, CA, U.S.A.) for 10 min at room temperature, followed by a streptavidin-peroxidase (Biospa Division, Milano, Italia) incubation for 10 min at room temperature. After each step, slides were rinsed three times in PBS. NovaRED (Vector® NovaRED™; Vector Laboratories) was used as the chromogenic revealing system and the reaction was monitored by light microscopy.

3. Results

3.1. Isolation and culture of EAMSCs

On average $1.2 \pm 0.6 \times 10^7$ ($n=4$) nucleated cells were extracted from amnion and $8 \pm 3 \times 10^5$ ($n=4$) MSCs were isolated at P0. In the first step of the culture, two types of cells were isolated: a spindle-shaped type and a round shape type which disappeared in the early passages. The cells reached confluence after 8 days in P0 (Figure 1). Mesenchymal cells cultured with α-MEM 10-10 reached senescence after ten passages with a CPD of 27.96 ± 8.7.

Clones of cells identifying CFU were stained (Figure 2).

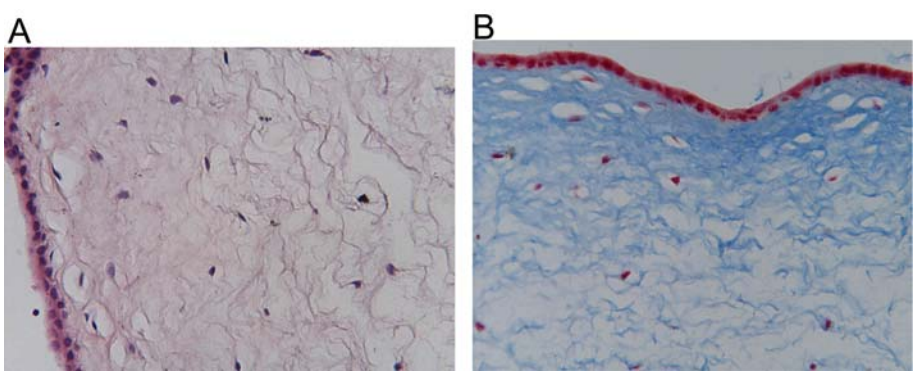

Figure 8 **Amnion sections stained with H/E (A) and Azan Mallory (B) (magnification: ×25)**

3.2. Growth kinetics of EAMSCs

α-MEM supplemented with 10% FCS and 10% HS was an excellent culture medium in terms of expansion and cell passage number ($P=0.005$). In addition, EGF added to α-MEM resulted in a significant increase in PD parameter for each step ($P=0.005$; Figure 3). In addition (Figure 3), DT was calculated for each medium (with or without supplementation of EGF) and each step showed changes in growth rates in relation to culture medium and step number analysed (Figure 4), but the high S.D. values do not allow a proper statistical analysis.

3.3. Analysis of differentiation of EAMSCs

EAMSCs differentiated in chondrogenic, adipogenic and osteogenic types.

Adipogenic differentiation: Rounded cells containing lipid vesicles in the cytoplasm positive with Oil Red O staining were observed (Figure 5); the amount of these granules increased over time.

Chondrogenic differentiation: Glycosaminoglycans in the matrix were observed after Alcian Blue staining.

Osteogenic differentiation: From the fifth day, a rapid mineralization of bone matrix and formation of nodules was observed. The bone nodules, which appeared red by Alizarin Red staining, were associated with the accumulation of fibroblastoid cells close to each other and their size was larger when the culture medium was supplemented with EGF.

3.4. FACS, histochemical and immunohistochemical analyses

EAMSCs showed the following phenotype: CD90+, CD44+, CD14+ and CD45+ (Figure 6).

Cytofluorimetric analysis of leucocytes isolated from equine peripheral blood confirmed the reliability of the antibodies employed (Figure 7).

Amnion sections showed the presence of fibroblastoid cells in the connective tissue (Figure 8).

Immunohistochemical staining showed a positivity for c-Kit, CD105 and Oct-4 antigens (Figure 9).

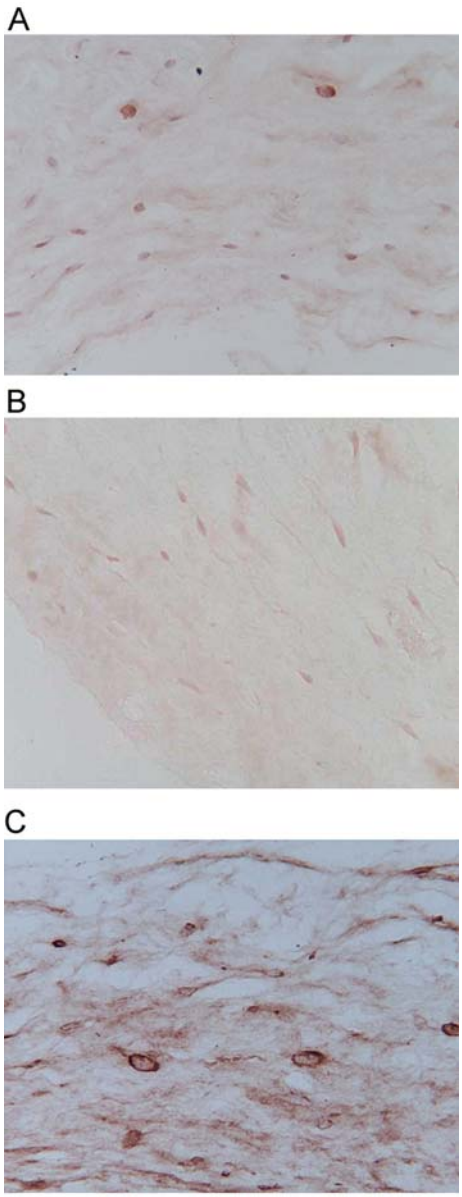

Figure 9 **c-Kit antigen (A), CD105 antigen (B) and Oct-4 antigen (C) (magnification: ×25)**

4. Discussion

The aim of the present paper was to study the EAMSCs for their possible use in cell therapy because of non-invasive techniques for the cell extraction: indeed, it is easy to sample amnion immediately after birth because the colt is released from the chorion and the mare delivers the rest of the placenta later.

Bacterial and fungal contamination represented the main problem to be settled, due to the environment where the procedure of amnion sampling was performed. For this purpose we used a sampling protocol employed in our previous study (Passeri et al., 2009). The positive correlation between the number of mononuclear cells ($1.6 \pm 0.6 \times 10^7$) and the number of CFU (136.7 ± 39.4) is in agreement with previously reported data (Meirelles Lda and Nardi, 2003). This could represent useful information for the optimization of cell isolation.

The culture medium α-MEM (supplemented with 10% FCS and 10% HS) with the addition of EGF increased the number of cells obtained up to senescence. This result is supported by our previous experience (Passeri et al., 2009) and by several studies on human, mouse and pig MSCs (Krampera et al., 2005; Tamama et al., 2006). When grown in specific culture media, EAMSCs differentiated into adipogenic, chondrogenic and osteogenic lineages. The effects of the addition of EGF in the culture medium did not affect their ability to differentiate, as reported by Passeri et al. (2009) for equine umbilical cord. EGF showed no effect on chondrogenic and

adipogenic differentiation. The supplementation of the osteogenic medium with EGF led to the formation of bone nodules whose size is influenced by EGF. The positive results obtained by immunohistochemistry to the c-Kit, CD105 and Oct-4 confirm the presence of MSCs in amnion, as reported for human tissue (Insausti et al., 2010). This study develops a technique for the sampling, isolation and expansion of EAMSCs. In addition, flow cytometry and immunohistochemistry showed the potential of these stem cells, although with some differences from the MSCs isolated from other species and other tissues. Their differentiation into chondrogenic, adipogenic and osteogenic lineages confirms the role of MSCs. Furthermore, this study could form the basis for the establishment a database for collecting and preserving stem cells used in the field of equine regenerative medicine.

Author contributions

P. Urciuoli, F. Nocchi, R. Lamanna and S. Passeri carried out the EAMSCs isolation and differentiation. M. Iorio designed and performed the FACS analysis. Alessandra Coli, E. Giannessi, S. Passeri and M. R. Stornelli designed and performed the immunohistochemical study. Alessandra Coli, S. Passeri, S. Lapi and P. Urciuoli contributed to writing and revising the manuscript. Alessandra Coli, S. Passeri, E. Giannessi, M. R. Stornelli and F. Scatena conceived the entire experimental design and provided funding.

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