Mesenchymal stem cells from amnion and amniotic fluid in the bovine

B Corradetti^{1,2}, A Meucci², D Bizzaro¹, F Cremonesi^{2,3} and A Lange Consiglio²

¹Department of Life and Environmental Sciences, Università Politecnica delle Marche, Ancona, Italy, ²Reproduction Unit, Large Animal Hospital, Università degli Studi di Milano, Via dell'Università 6, 26900 Lodi, Italy and

³Department of Veterinary Science for Animal Health, Production and Food Safety, Università degli Studi di Milano, Milano, Italy

Correspondence should be addressed to F Cremonesi at Reproduction Unit, Large Animal Hospital, Università degli Studi di Milano; Email: fausto.cremonesi@unimi.it

Abstract

Amnion and amniotic fluid (AF) are noncontroversial and inexhaustible sources of mesenchymal stem cells (MSCs) that can be harvested noninvasively at low cost. As in humans, also in veterinary field, presumptive stem cells derived from these tissues reveal as promising candidates for disease treatment, specifically for their plasticity, their reduced immunogenicity, and high anti-inflammatory potential. The aim of this work is to obtain and characterize, for the first time in bovine species, presumptive MSCs from the epithelial portion of the amnion (AECs) and from the AF (AF-MSCs) to be used for clinical applications. AECs display a polygonal morphology, whereas AF-MSCs exhibit a fibroblastic-like morphology only starting from the second passage, being heterogeneous during the primary culture. For both lines, the proliferative ability has been found constant over the ten passages studied and AECs show a statistically lower ($P < 0.05$) doubling time with respect to AF-MSCs. AECs express MSC-specific markers (*ITGB1* (CD29), *CD44*, *ALCAM* (CD166), *ENG* (CD105), and *NT5E* (CD73)) from P1 to P3; in AF-MSCs, only *ITGB1*, *CD44*, and *ALCAM* mRNAs are detected; *NT5E* is expressed from P2 and *ENG* has not been found at any passage. AF-MSCs and AECs are positive for the pluripotent markers (*POU5F1* (OCT4) and *MYC* (c-Myc)) and lack of the hematopoietic markers. When appropriately induced, both cell lines are capable of differentiating into ectodermal and mesodermal lineages. This study contributes to reinforce the emerging importance of these cells as ideal tools in veterinary medicine. A deeper evaluation of the immunological properties needs to be performed in order to better understand their role in cellular therapy. *Reproduction* (2013) **145** 391–400

Introduction

As in humans, also in veterinary medicine, mesenchymal stem cells (MSCs) have been harvested successfully from a wide range of tissues (Smith *et al.* 2003, Kern *et al.* 2006, Koerner *et al.* 2006, Arnhold *et al.* 2007, Giovannini *et al.* 2008, Vidal *et al.* 2008). The most characterized sources of MSCs are bone marrow (Fortier *et al.* 1998, Ringe *et al.* 2002, Mitchell *et al.* 2003, Smith *et al.* 2003, Kern *et al.* 2006, Vidal *et al.* 2006, Arnhold *et al.* 2007, Vidal *et al.* 2008) and the adipose tissue (Kern *et al.* 2006, Vidal *et al.* 2008); however, the procedures employed to isolate these tissues are still invasive and cells are usually obtained with low efficiency. Furthermore, their differentiative and proliferative potential has been reported to decrease with the age of the donor (Pittenger *et al.* 1999). The possibility to collect a large amount of cells, in an inexpensive and noninvasive way, and without being risky for the donor is of a great concern for regenerative

medicine and cellular therapy, especially if there is the chance to expand the cells *in vitro* and to cryogenically bank (Carlin *et al.* 2006, Cremonesi *et al.* 2008).

As such, gestational tissues including umbilical cord blood (Kern *et al.* 2006, Koch *et al.* 2007, Guest 2008, Reed & Johnson 2008, Bartholomew *et al.* 2009, Schuh *et al.* 2009, Seo *et al.* 2009, Raoufi *et al.* 2011), umbilical cord tissues (Mitchell *et al.* 2003, Carlin *et al.* 2006, Hoynowski *et al.* 2007, Cremonesi *et al.* 2008, Passeri *et al.* 2009, Corradetti *et al.* 2011, Lovati *et al.* 2011, Iacono *et al.* 2012a), amniotic tissues (Filioli Uranio *et al.* 2011, Iacono *et al.* 2012a, 2012b, Lange-Consiglio *et al.* 2012), and amniotic fluid (AF) (Chen *et al.* 2011, Dev *et al.* 2011, Lovati *et al.* 2011, Iacono *et al.* 2012a) have been recently suggested, also in veterinary medicine, as appealing candidates for the derivation of MSCs to use in cell biotechnology. The human lesson teaches that presumptive adult stem cells derived from gestational tissues retain highest proliferation capacity, longest telomere length, broadest

differentiation, and extensive proliferative potential when compared with cells obtained from adult tissues (Kogler *et al.* 2004, Kern *et al.* 2006). In particular, MSCs obtained from human amnion have been shown to retain immunomodulatory properties as well as to strongly inhibit T lymphocyte proliferation (Magatti *et al.* 2008) and to survive when transplanted in immunocompetent animals without inducing any tumorigenic effect *in vivo* (Avila *et al.* 2001, Kubo *et al.* 2001, Sankar & Muthusamy 2003, Yuge *et al.* 2004). As amnion-derived progenitor cells, MSCs from AF (AF-MSCs) are thought to be in an intermediate stage between embryonic stem cells and lineage-restricted adult stem cells (In 't Anker *et al.* 2004, Delo *et al.* 2006, De Coppi *et al.* 2007, Sessarego *et al.* 2008, Gucciardo *et al.* 2009), originating from several fetal tissues, including skin, digestive, respiratory, and urinary systems (Prusa & Hengstschlager 2002, You *et al.* 2009, Da Sacco *et al.* 2010). AF-MSCs are known to play a role in preventing rejection of the fetus and are thought to have low immunogenicity (Wang *et al.* 2006). In addition to this, no karyotypic abnormalities or transformation potential *in vitro* have been still associated with amnion- or AF-derived cells (Sessarego *et al.* 2008).

In veterinary field, the amniotic membrane has been employed for ocular surface reconstruction in dog (Arcelli *et al.* 2009) and horse (Ollivier *et al.* 2006, Plummer *et al.* 2009) due to its ability to promote re-epithelialization, to reduce fibrosis (Solomon *et al.* 2005), and to modulate angiogenesis (Dua *et al.* 2004).

Even if amnion has been widely used in clinical therapies, we have been the first to isolate and characterize presumptive MSCs derived from the equine amniotic membranes (both epithelial and mesenchymal portions) and to demonstrate their application in clinical regenerative medicine, in particular for the treatment of spontaneous tendon injuries in horses (Lange-Consiglio *et al.* 2012). However, for other species, the potential of these cells or those obtained from the AF to be used in cellular therapy needs to be fully determined, and further *in vitro* and *in vivo* investigations are needed.

Despite the importance of bovine species as model for *in vivo* studies, little is known about bovine MSCs. So far, they have been derived from umbilical cord blood (Raoufi *et al.* 2011) and bone marrow (Bosnakovski *et al.* 2005, 2006) and attempts to isolate cells from the amniotic membrane or from the AF have not been reported in the literature yet.

The aim of the present work is to isolate presumptive MSCs from the epithelial portion (Miki *et al.* 2005, 2007a) of the amnion (AECs) and from the AF and to characterize them in terms of morphology, specific mesenchymal or pluripotent markers, and proliferative and differentiative potential.

Results

Amnion collection and isolation of amniotic epithelial cells

Cells were selected purely on their ability to adhere to plastic. Isolated cells readily attached to plastic culture dishes. AECs displayed typical polygonal epithelial morphology, whereas AF-MSCs observed during primary culture consisted of two types of cells: one was similar to fibroblasts and others were flat and circular, resembling epithelial cells. Starting from the second passage, the AF-MSCs became homogeneous exhibiting fibroblast-like morphology. Representative images are shown in Fig. 1.

Proliferation assays

For both cell lines, AECs and AF-MSCs, the proliferative ability has been found constant over the ten passages studied with an average of 1.34 ± 0.30 and 2.06 ± 0.37 days for AECs and AF-MSCs respectively. Differences between AECs and AF-MSCs doubling times (expressed as days) were observed at P6, when AECs showed a statistically lower ($P < 0.05$) doubling time (1.08 ± 0.46) with respect to AF-MSCs (2.69 ± 0.32). Differences observed between AECs and AF-MSCs doubling times at other passages were not significant (Fig. 2).

The number of cell colonies formed was counted at P0 after seeding cells at different density/cm² as previously reported (Lange-Consiglio *et al.* 2012). Both AECs and AF-MSCs demonstrated a statistically significant increase in colony-forming unit (CFU) frequency with increasing cell-seeding densities. For each density of seeding (with the exception of the 100 cells/cm²), AECs showed a significantly higher CFU ($P < 0.05$) in comparison to AF-MSCs (Table 1).

RNA extraction and RT-PCR analysis

As shown by RT-PCR (Fig. 3), amnion-derived cells (AECs) expressed MSC-specific markers (*ITGB1* (CD29), *CD44*, *ALCAM* (CD166), *ENG* (CD105), and *NT5E* (CD73)) over the three passages studied (from P1 to P3). In cells isolated from AF, only *ITGB1*, *CD44*, and *ALCAM* mRNAs were detected from P1 to P3. Expression of *NT5E* was only observed from P2, whereas *ENG* was not detected at any stage. Cells from amnion and AF were positive for the pluripotency-associated markers studied (*POU5F1* (OCT4) and *MYC* (c-Myc)) and lacked of *CD34*, *CD14*, and *PTPRC* (CD45) over the passages studied. Major histocompatibility complex, class I (*MHC-I*) expression was demonstrated in each cell population, while *MHC-II* was expressed nor in AECs or in AF-MSCs.

Figure 1 Cell morphology. Monolayer of adherent polygonal epithelial AECs and spindle-shaped fibroblastic-like AF-MSCs. Magnification, 20 $\times$; scale bar, 20 μ m.

Multipotent differentiation

Molecular analysis of bovine adult tissues (fat, cartilage, and spinal cord) showed the expression of the specific genes evaluated (Fig. 4). After 3 weeks of culture in adipogenic medium, both amnion- and AF-derived cells developed lipid deposits as demonstrated by the Oil red O staining. Cells maintained in regular control medium did not stain positively (Fig. 4A). For AECs, differentiation was confirmed by peroxisome proliferator-activated receptor- γ (*PPARG*), adipocyte fatty acid-binding protein (*FABP4*), and leptin (*LEP*) expression, whereas AF-MSCs only expressed *FABP4* and *LEP* (Fig. 4B).

Chondrogenic differentiation has been identified by Alcian blue staining as an evident deposition of glycosaminoglycans in the matrix (Fig. 4A). In both cell types, the early marker for chondrogenesis *SOX9* has been detected with the consequent expression of aggrecan (*ACAN*). mRNAs for *COL2A1* were only detected, at a basal level, in AF-MSCs (Fig. 4B).

Three days were enough to induce neurogenic differentiation. Changes in cell morphology were evident as cells (both AECs and AF-MSCs) adopted a typical morphology of neural cells, with dendrite-like

processes (Fig. 4A). Both cell populations expressed nestin (*NES*), but in AECs, glial fibrillary acidic protein (*GFAP*) mRNAs were also detected (Fig. 4B).

Discussion

Extra-gestational tissues have been widely suggested to be the ideal sources of MSCs due to their accessibility, being usually discarded at birth (Parolini *et al.* 2008, 2009). Although amnion is a thin membrane composed of two different portions, the mesenchymal and the epithelial one, we decided to focus our attention on the derivation of progenitor cells from the epithelial area of placenta. In 2005, Miki *et al.* (2005) reported that cells obtained from the fetal side of placenta might retain a multipotent phenotype even after the differentiation from the epiblast. The aim of this study was to characterize, for the first time in bovine species, cells obtained from the amniotic epithelial membrane and the AF and to evaluate these tissues as potential candidates for the isolation of progenitor cells to employ in cellular therapies.

Our *in vitro* differentiation studies support the findings already reported in humans (In 't Anker *et al.* 2004, De Coppi *et al.* 2007) and in equine species (Lange-Consiglio *et al.* 2012): cells isolated from the amniotic epithelial portion, as well as those obtained from the AF, show high plasticity, being able to differentiate into multiple germ layers (mesoderm and ectoderm). In particular, according to data obtained from human amniotic epithelial cells (Miki *et al.* 2005, 2007a), we prove the ability of bovine amnion-derived cells to undergo astrocyte differentiation, as demonstrated by *GFAP* expression following neurogenic induction. On the contrary, in cells derived from AF, only the presence of *NES*, a marker expressed in neuronal precursor stem cells, has been detected, confirming the neurogenic induction occurred as previously observed in pig (Zheng *et al.* 2010). Both cell lines converted into a typical neuron-like morphology when appropriately induced.

When stimulated to differentiate toward the adipogenic lineage, both cell lines stained positively for Oil red O staining and expressed genes involved in lipid biosynthesis and storage. In induced AEC mRNAs for

Figure 2 Doubling time over ten passages during cell culture for AECs and AF-MSCs. Statistically significant difference was observed in doubling time means between AECs and AF-MSCs (* $P < 0.05$).

Table 1 CFU assay.

Density (cells/cm ²)	Total cells	CFU	One CFU each
AECs			
100	950	1.57 ± 0.62 ^{a,A}	605.10
250	2375	21.63 ± 3.15 ^{b,B}	109.80
500	4750	48 ± 1.43 ^{c,B}	98.96
1000	9500	56.01 ± 2.78 ^{c,A}	169.61
AF-MSCs			
100	950	1.07 ± 0 ^{a,A}	887.85
250	2375	7.53 ± 2.98 ^{a,A}	315.41
500	4750	26.95 ± 4.72 ^{b,A}	176.25
1000	9500	40.53 ± 3.99 ^{d,B}	234.39

Different small letters superscripts (a, b, and c) indicate statistically different comparisons ($P < 0.05$) between cell densities in each group (AECs or AF-MSCs). Different capital letter superscripts (A, B, and C) indicate statistically different comparisons ($P < 0.05$) between the same cell density among groups (AECs and AF-MSCs).

PPARG, which is crucial for the preadipocyte commitment (Totonoz *et al.* 1994), and *FABP4* and *LEP* that are regarded as intermediate and late markers of adipocyte differentiation were detected. AF-MSCs only expressed *FABP4* and *LEP* that are associated with the intermediate and late phases in developing adipocytes (Bernlohr *et al.* 1985), which may be led to a distinct developmental program characteristic of stem cells (Zuk *et al.* 2002). The potential of bovine MSCs to undergo chondrogenesis has been assessed by staining the cells with Alcian blue and confirmed using markers commonly associated with the chondrocyte phenotype: the early marker *SOX9*, collagen type 2 and *ACAN*, the most essential cartilage proteoglycan, and key markers of chondrocyte differentiation (Han & Lefebvre 2008). In AECs and AF-MSCs, the expression of *SOX9*, which has been reported to regulate the rate of chondrocyte differentiation by controlling the expression of a series of chondrocyte-specific genes including *ACAN* and *COL2A1* (Tchetina *et al.* 2003), was detected. However, AECs and AF-MSCs showed a different pattern of expression in response to the activation of *SOX9*: *ACAN* expression was demonstrated in both cell lines, whereas a basal level of *COL2A1* was only detected in AF-MSCs. The lack of expression of *COL2A1* might be led to the culture conditions employed in this study as chondrogenic differentiation of MSCs in monolayer culture appears to be dose dependent and time dependent in relation to the bioactive factors used (Bosnakovski *et al.* 2004, 2005).

Bovine AF-MSCs represent a heterogeneous population originating from the three germ layers, whose cells share an epithelial origin and are derived from either the developing embryo or the inner surface of the amniotic membrane (Pappa & Anagnou 2009). This was evident during the primary culture (at P0), when fibroblastic-like or flatter and circular cells were recognized. By the first passage, however, a selection of spindle-shaped cells occurred, according to what has been previously observed in equine species by our group (Lovati *et al.*

2011) or other researchers (Iacono *et al.* 2012a). Amniotic epithelial cells displayed the typical polygonal morphology reported for human (Miki *et al.* 2005, 2007a) and equine species (Lange-Consiglio *et al.* 2012).

As shown by proliferation assays, cells from the epithelial portion of amnion had high and comparable proliferative capacity until passage 6 (Soncini *et al.* 2007, Miki *et al.* 2010). At that time, robust proliferation was still observed; however, AF-MSCs showed a higher doubling time (2.69 ± 0.32) compared with AECs (1.08 ± 0.46). When AF-MSCs and AECs were seeded at different densities, they were able to form clones with frequency that, in both cases, increased with the cell-seeding density, suggesting that paracrine signaling between amnion- and AF-derived cells at P0 occurs (Sarugaser *et al.* 2005).

As suggested earlier, MSCs are the best-represented subpopulation within AF-SCs. To characterize this population, we evaluated the specific mesenchymal and pluripotent marker expression (Roubelakis *et al.* 2007, Soncini *et al.* 2007). Our data confirm this observation

Figure 3 RT-PCR analysis of mesenchymal (*ITGB1*, *CD44*, *ALCAM*, *NT5E*, and *ENG*), pluripotent (*POU5F1* and *MYC*), and hematopoietic (*CD34*, *CD14*, and *PTPRC*) specific gene expression on AECs and AF-MSCs from P1 to P3. *MHC-II* was not detected. *GAPDH* has been employed as reference gene.

Figure 4 Multipotent differentiation potential of AECs and AF-MSCs. (A) Immunostaining on induced AECs and AF-MSCs and their controls (AECs CTRL and AF-MSCs CTRL). Oil Red O, Alcian blue, and Nissle staining to assess adipogenic, chondrogenic, and neurogenic differentiation respectively. Magnification, 20 $\times$; scale bars, 20 μ m. (B) RT-PCR analysis for *PPARG*, *FABP4*, and *LEP* for adipogenesis; *SOX9*, *COL2A1*, and *ACAN* for chondrogenesis; *NES* and *GFAP* for neurogenesis. Gene expression for the positive control (fat, cartilage, and spinal cord), induced cells (1 and 3 for AF-MSCs and AECs respectively), and their controls (2 and 4 for AF-MSCs and AECs respectively).

showing that mRNAs for *CD44*, *ITGB1*, and *ALCAM* can be detected in AF-MSCs as well as *MHC-I*, whereas these cells lack of *MHC-II* and the hematopoietic markers *CD34*, *CD14*, and *PTPRC* (Kim *et al.* 2007). *NT5E* was only observed by passage 2 and *ENG* was not expressed at all. More importantly, cultured AF-MSCs expressed pluripotency markers such as the Octamer binding protein 3/4 (*OCT3/4*) and *MYC* (Pan *et al.* 2002, Tsai *et al.* 2004, Bossolasco *et al.* 2006, De Coppi *et al.* 2007, Roubelakis *et al.* 2007, 2011, Perin *et al.* 2010), which are known to maintain stem cell pluripotency and self-renewal (Varlakhanova *et al.* 2010). Similarly, AECs were positive for *CD44*, *ITGB1*, *NT5E*, *ENG*, *ALCAM*, and *MHC-I* and negative for *CD34*, *CD14*, *PTPRC*, and *MHC-II* expression (Miki *et al.* 2007b, 2010, Soncini *et al.* 2007, Marongiu *et al.* 2010, Manuelpillai *et al.* 2011). Further investigation demonstrated that AECs also express embryonic stem cell markers such as *POU5F1* and *MYC*.

Our findings suggest, for the first time, that bovine amnion and AF represent alternative sources of

progenitor cells to employ in cell-based therapies, in a similar manner to several other species (Chen *et al.* 2011, Lovati *et al.* 2011, Iacono *et al.* 2012a, 2012b, Lange-Consiglio *et al.* 2012). Further studies, including pre-clinical and a deeper evaluation of immunological properties, are needed for the *in vivo* applications in order to better understand their role in cellular therapy.

In conclusion, the present data contribute to reinforce the emerging importance of the amniotic and extra-embryonic tissues, making the cells derived from these tissues ideal tools in the veterinary regenerative medicine.

Materials and Methods

Materials

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

Amnion and AF collection

Samples were transported to the laboratory at 4 °C and processed within 8 h. Two AFs were collected into sterile syringes during the full-term delivery of different cattle as previously reported (Lovati *et al.* 2011). When it was possible, the AF was aspirated into a sterile syringe directly from the vulva protruded amniotic sac before its spontaneous rupture. Allanto-amniotic membranes were obtained at term of normal pregnancies and after vaginal delivery from three cattle. Portions of allanto-amnion were kept at 4 °C in PBS (EuroClone) with 100 U/ml penicillin–100 mg/ml streptomycin and amphotericin B and were processed within 12 h. The amniotic membrane was stripped from the overlying allantois and cut into small pieces (about 9 cm² each) before enzymatic digestion.

Isolation of amniotic epithelial cells

Isolation of amniotic epithelial cells was performed as previously reported (Lange-Consiglio *et al.* 2012) in horse. Briefly, amnion fragments (about 12 g for an extension of 630 cm²) were incubated for 9 min at 37 °C in PBS containing 2.4 U/ml dispase (Becton Dickinson, Milan, Italy). After a resting period (5–10 min) at room temperature in high-glucose DMEM (HG-DMEM; EuroClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine (Sigma), the fragments were digested twice with 0.05% (w/v) trypsin/EDTA for ~45 min at 37 °C each time. The amnion fragments were then removed, and mobilized cells were passed through a 100 mm cell strainer before being collected by centrifugation at 200 g for 10 min.

Isolation of MSCs from AF

Isolation of MSCs from AF was performed following the procedures previously described (Lovati *et al.* 2011), consisting of centrifuging the samples at 400 g for 10 min, removing the supernatant and collecting the pellet formed at the bottom of the tube. Before seeding the cells, the pellet was washed three times in PBS.

Cell culture

Cultures were established in HG-DMEM supplemented with 10% FBS, 10 ng/ml epidermal growth factor, 1% penicillin (100 U/ml)–streptomycin (100 mg/ml), 0.25 mg/ml amphotericin B, and 2 mM L-glutamine. The number of viable cells was counted by the trypan blue dye exclusion method, using a Burkert chamber. For maintenance of cultures, cells were plated at up to 1×10^5 cells/cm² and incubated at 38.5 °C in a humidified atmosphere (90%) with 5% CO₂. To remove non-adherent cells, the medium was replaced for the first time after 72 h and then changed either twice per week thereafter or according to the experiment requirements. Adherent cells were detached with 0.05% trypsin–EDTA (EuroClone) just before reaching confluence (80%) and then reseeded for culture maintenance. Passage 3 was the last time point included for characterization and differentiation studies.

Proliferation assays

Proliferation of AECs and AF-MSCs has been determined as previously reported (Corradetti *et al.* 2011, Lange-Consiglio *et al.* 2012). Doubling time for passages 1–10 has been assessed plating 9×10^3 cells into six-well tissue culture plates. Every 4 days, cells were trypsinized, counted, and replated at the same density. Mean doubling time was calculated from days 0 to 4. The mean of population doublings (PD) was obtained for each passage according to the formulae $CD = \log(N_c/N_0)/\log_2$ and $PD = CT/CD$, where CD represents cell doubling, N_c represents the number of cells at confluence, N_0 represents seeded cells, and CT represents the culture time. Data representative of three independent experiments were reported.

CFU assay

CFU assays were performed at P0 on freshly isolated cells at different densities (100, 250, 500, and 1000 cells/cm²). Cells were plated in six-well plates and cultured in 5% CO₂ and 90% humidity at 38.5 °C for 2 weeks in HG-DMEM-supplemented medium. Then, colonies were fixed with 4% formalin and stained with 1% methylene blue (Serva, Heidelberg, Germany) in 10 mM borate buffer, pH 8.8 (Fluka BioChemika, Buchs, Switzerland) at room temperature, and washed twice. Colonies formed by 16–20 nucleated cells were counted under a BX71 microscope (Olympus).

RNA extraction and RT-PCR analysis

Expression of specific MSC (*CD44*, *ITGB1*, *NT5E*, *ENG*, and *ALCAM*), pluripotent (*MYC* and *POU5F1*), and hematopoietic (*CD34*, *CD14*, and *PTPRC*) markers was investigated by RT-PCR analysis on undifferentiated cells. To evaluate whether cells could be well tolerated by the host once transplanted, expression of the *MHC-I* and *MHC-II* was assessed. Total RNA was extracted at P1 and P3 from bovine AECs and AF-MSCs using TRIzol reagent (Invitrogen), followed by DNase treatment according to the manufacturer's specifications. RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized from 500 ng total RNA, using the iScript retrotranscription kit (Bio-Rad Laboratories). Conventional PCR was performed in a 25 ml final volume with DreamTaq DNA Polymerase (Fermentas, St Leon Rot, Germany) 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.

For differentiation experiments, total RNA was extracted from undifferentiated (control cells) and from induced amnion- or AF-derived cells, and RT-PCR analysis was performed as described earlier. Bovine adult tissues (fat, cartilage, and spinal cord) were employed as positive controls for assessing the expression of *PPARG*, *FABP4* and *LEP* for adipogenesis, the Sry-related high-mobility group box transcription factor (*SOX9*), collagen type 2 α 1 (*COL2A1*) and *ACAN* for chondrogenesis, and *GFAP* and *NES* for neurogenesis. Bovine-specific

Table 2 Oligonucleotide sequences used for RT-PCR analysis.

Markers	Forward (5' → 3')	Reverse (5' → 3')	Annealing temperature (°C)	Product length (bp)
POU class 5 homeobox 1 (<i>POU5F1</i>)	CACACTAGGATATACCCAGGC	GGAGATATGCAAGGCAGAGA	60	177
<i>MYC</i>	GCGCCGCATTCGCGAAACTT	TGAGGGGCATCGCTGCAAGC	58	214
Major histocompatibility complex I (<i>MHC-I</i>)	GATCTCACTGACCTGGCA	CTGAGGAGGTTCCCATCTC	60	199
<i>MHC-II</i>	CCTCGCTTGCCTGAATTTGC	ACAGGTGCCGACTGATGC	53	299
<i>ALCAM</i>	GTATTIATTCGCTTCAGGTCCT	TCTACCAGGGAGCATTATAGTC	59	755
Ecto-5'-nucleotidase (<i>NT5E</i>)	AAGGTTCTGTGGTCCAGGCCT	TGCATTCTCGAAAGCGGCAGGA	68	260
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	ATGAGATCAAGAAGGTGGTG	CCAAATTCATTGTCGTACCAG	60	190
Integrin β 1 (<i>ITGB1</i>)	GTTGGTTCTGCAGTTACGATCAG	AACCAAACCCAATTCGGAAGTC	52	203
CD44 antigen (<i>CD44</i>)	AACAGTAGGAGAAGGTGTGG	TCATGAACCTGGTCTTGGGTC	61	166
Endoglin (<i>ENG</i>)	ACAAGTCTTGCGAAGACAGTC	GATGTCTGGAGAGTCAGCTC	61	182
<i>PTPRC</i> molecule (<i>CD45</i>)	CTTCTGGCATTCCGCTTTGC	AATGGAGGCTCACACGAAGG	58	350
CD14 molecule (<i>CD14</i>)	TCCGAAGCCTGACTGGTCTA	TGTCGGCTCCCTTGAGAAAC	56	104
<i>CD34</i>	CCTGAAGCTAAATGAGACCT	AACTTTCTGTCTGTTGGTC	58	173
Leptin (<i>LEP</i>)	CAATGACATCTCACACACGAG	CGGCCAGAGGTGGAGAAG	55	212
Adipocyte fatty acid-binding protein (<i>FABP4</i>)	CTGGCATGGCCAAACCCA	GTAATTGTACCAGAGCACC	55	182
Peroxisome proliferator-activated receptor (<i>PPARG</i>)	CGCACTGGAATTAGATGACAGC	CACAATCTGTCTGAGGTCTGTC	55	199
Sex-determining region Y-box 9 (<i>SOX9</i>)	CATGAAGATGACCGACGAG	CGTCTTCTCCGTGTCGGA	55	118
Collagen type 1, alpha I (<i>COL1A1</i>)	CGCGGATTGTGCTGCTTGC	AGGTCCCATCAGCCCCATTGGT	55	269
Aggrecan (<i>ACAN</i>)	CGCTGTCTCGCAAGTGATGG	CGGTTCAAGGATGCTGACACTC	60	175
Glial fibrillary acidic protein (<i>GFAP</i>)	GGCACCTTGAGGCAGAAGCTC	CTCCTGGAGCTCCCGCACCT	60	195
Nestin (<i>NES</i>)	ACCACTGAGCAGTTCAGCTGG	TTGCAGGTGTCTGCAGCCGT	55	187

oligonucleotide primers were designed using open source PerlPrimer Software v. 1.1.17 (Marshall 2004), based on available NCBI *Bos taurus* sequences or on Mammal multi-aligned sequences. Primers were designed across an exon–exon junction in order to avoid DNA amplification. Primers were used at 300 nM final concentrations. Their sequences and the conditions used to amplify each gene are shown in Table 2. *GAPDH* was employed as a reference gene.

Differentiation assays

Cells at P3 were seeded at a density of $3 \times 10^3/\text{cm}^2$ for all differentiation studies. Adipogenic, chondrogenic, and neurogenic differentiation were assessed as previously reported (Corradetti *et al.* 2011, Lange-Consiglio *et al.* 2012). Non-induced control cells were cultured for the same time in standard control medium (HG-DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B, and 2 mM L-glutamine). Adipogenesis using conventional Oil red O staining (0.1% in 60% isopropanol) demonstrated lipid droplets; chondrogenesis was demonstrated by Alcian blue staining (pH 2.5), and neurogenic by conventional Nissl staining (0.1% cresyl violet solution), which showed increasing ribosomes.

Statistical analysis

Statistical analysis was performed using GraphPad Instat 3.00 for Windows (GraphPad Software, La Jolla, CA, USA). Three replicates for each experiment (doubling times and CFU) were

performed and the results are reported as mean $\pm$ s.d. One-way ANOVA for multiple comparisons by Student–Newman–Keuls multiple comparison tests was used. CFU comparison among different cell plating densities inside each group (AECs or AF-MSCs) and between groups (AECs and AF-MSCs) of the same cell density were analyzed. $P < 0.05$ was considered as significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

- Arcelli R, Tibaldini P, Angeli G & Bellezza E 2009 Equine amniotic membrane transplantation in some ocular surface diseases in the dog and cat: a preliminary study. *Veterinary Research Communications* **33** (Suppl 1) 169–171. (doi:10.1007/s11259-009-9284-6)
- Arnhold SJ, Goletz I, Klein H, Stumpf G, Beluche LA, Rohde C, Addicks K & Litzke LF 2007 Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. *American Journal of Veterinary Research* **68** 1095–1105. (doi:10.2460/ajvr.68.10.1095)

- Avila M, Espana M, Moreno C & Pena C 2001 Reconstruction of ocular surface with heterologous limbal epithelium and amniotic membrane in a rabbit model. *Cornea* **20** 414–420. (doi:10.1097/00003226-200105000-00016)
- Bartholomew S, Owens SD, Ferraro GL, Carrade DD, Lara DJ, Librach FA, Borjesson DL & Galuppo LD 2009 Collection of equine cord blood and placental tissues in 40 thoroughbred mares. *Equine Veterinary Journal* **41** 724–728. (doi:10.2746/042516409X429446)
- Bernlohr DA, Doering TL, Kelly TJ & Lane MD 1985 Tissue specific expression of p422 protein, a putative lipid carrier in mouse adipocytes. *Biochemical and Biophysical Research Communications* **132** 850–855. (doi:10.1016/0006-291X(85)91209-4)
- Bosnakovski D, Mizuno M, Kim G, Ishiguro T, Okumura M, Iwanaga T, Kadosawa T & Fujinaga T 2004 Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. *Experimental Hematology* **32** 502–509. (doi:10.1016/j.exphem.2004.02.009)
- Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M & Fujinaga T 2005 Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells. *Cell and Tissue Research* **319** 243–253. (doi:10.1007/s00441-004-1012-5)
- Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M & Fujinaga T 2006 Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. *Biotechnology and Bioengineering* **93** 1152–1163. (doi:10.1002/bit.20828)
- Bossolasco P, Montemurro T, Cova L, Zangrossi S, Calzarossa C, Buatiotis S, Soligo D, Bosari S, Silani V, Delilieri GL et al. 2006 Molecular and phenotypic characterization of human amniotic fluid cells and their differentiation potential. *Cell Research* **16** 329–336. (doi:10.1038/sj.cr.7310043)
- 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. *Reproductive Biology and Endocrinology* **4** 8. (doi:10.1186/1477-7827-4-8)
- Chen J, Lu Z, Cheng D, Peng S & Wang H 2011 Isolation and characterization of porcine amniotic fluid-derived multipotent stem cells. *PLoS ONE* **6** e19964. (doi:10.1371/journal.pone.0019964)
- Corradetti B, Lange-Consiglio A, Barucca M, Cremonesi F & Bizzaro D 2011 Size-sieved subpopulations of mesenchymal stem cells from intervacular and perivascular equine umbilical cord matrix. *Cell Proliferation* **44** 330–342. (doi:10.1111/j.1365-2184.2011.00759.x)
- Cremonesi F, Violini S, Lange Consiglio A, Ramelli P, Ranzenigo G & Mariani P 2008 Isolation, *in vitro* culture and characterization of foal umbilical cord stem cells at birth. *Veterinary Research Communications* **32** (Suppl 1) S139–S142. (doi:10.1007/s11259-008-9116-0)
- Da Sacco S, De Filippo RE & Perin L 2007 Amniotic fluid as a source of pluripotent and multipotent stem cells for organ regeneration. *Current Opinion in Organ Transplantation* **183** 1193–1200. (doi:10.1097/MOT.0b013e3283424f6e)
- De Coppi P, Bartsch G Jr, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ et al. 2010 Isolation of amniotic stem cell lines with potential for therapy. *Nature Biotechnology* **25** 100–106. (doi:10.1038/nbt1274)
- Delo DM, De Coppi P, Bartsch G Jr & Atala A 2006 Amniotic fluid and placental stem cells. *Methods in Enzymology* **419** 426–438. (doi:10.1016/S0076-6879(06)19017-5)
- Dev K, Giri S, Kumar A, Yadav A, Singh B & Gautam S 2011 Derivation, characterization and differentiation of buffalo (*Bubalus bubalis*) amniotic fluid derived stem cells. *Reproduction in Domestic Animals* **47** 704–711. (doi:10.1111/j.1439-0531.2011.01947.x)
- Dua HS, Gomes JA, King AJ & Maharajan VS 2004 The amniotic membrane in ophthalmology. *Survey of Ophthalmology* **49** 51–77. (doi:10.1016/j.survophthal.2003.10.004)
- Filioli Uranio M, Valentini L, Lange-Consiglio A, Caira M, Guaricci AC, L'Abbate A, Catacchio CR, Ventura M, Cremonesi F & Dell'Aquila ME 2011 Isolation, proliferation, cytogenetic, and molecular characterization and *in vitro* differentiation potency of canine stem cells from foetal adnexa: a comparative study of amniotic fluid, amnion, and umbilical cord matrix. *Molecular Reproduction and Development* **78** 361–373. (doi:10.1002/mrd.21311)
- Fortier LA, Nixon AJ, Williams J & Cable CS 1998 Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *American Journal of Veterinary Research* **59** 1182–1187.
- Giovannini S, Brehm W, Mainil-Varlet P & Nestic D 2008 Multilineage differentiation potential of equine blood-derived fibroblast-like cells. *Differentiation* **76** 118–129. (doi:10.1111/j.1432-0436.2007.00207.x)
- Gucciardo L, Lories R, Ochsenbein-Kolble N, Done E, Zwijsen A & Deprest J 2009 Fetal mesenchymal stem cells: isolation, properties and potential use in perinatology and regenerative medicine. *BJOG: an International Journal of Obstetrics and Gynaecology* **116** 166–172. (doi:10.1111/j.1471-0528.2008.02005.x)
- Guest DJ 2008 Defining the expression of marker genes in equine mesenchymal stromal cells. *Stem Cells and Cloning: Advances and Applications* **1** 1–9. (doi:10.2147/SCCAA.S3824)
- Han Y & Lefebvre V 2008 L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. *Molecular and Cellular Biology* **28** 4999–5013. (doi:10.1128/MCB.00695-08)
- Hoynowski SM, Fry MM, Gardner BM, Leming MT, Tucker JR, Black L, Sand T & Mitchell KE 2007 Characterization and differentiation of equine umbilical cord-derived matrix cells. *Biochemical and Biophysical Research Communications* **362** 347–353. (doi:10.1016/j.bbrc.2007.07.182)
- Iacono E, Brunori L, Pirrone A, Pagliaro PP, Ricci F, Tazzari PL & Merlo B 2012a Isolation, characterization and differentiation of mesenchymal stem cells from amniotic fluid, umbilical cord blood and Wharton's jelly in the horse. *Reproduction* **143** 455–468. (doi:10.1530/REP-10-0408)
- Iacono E, Cunto M, Zambelli D, Ricci F, Tazzari PL & Merlo B 2012b Could fetal fluid and membranes be an alternative source for mesenchymal stem cells (MSCs) in the feline species? A preliminary study *Veterinary Research Communications* **36** 107–118. (doi:10.1007/s11259-012-9520-3)
- In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE & Kanhai HH 2004 Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* **22** 1338–1345. (doi:10.1634/stemcells.2004-0058)
- Kern S, Eichler H, Stoeve J, Kluter H & Bieback K 2006 Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24** 1294–1301. (doi:10.1634/stemcells.2005-0342)
- Kim J, Lee Y, Kim H, Hwang KJ, Kwon HC, Kim SK, Cho DJ, Kang SG & You J 2007 Human amniotic fluid-derived stem cells have characteristics of multipotent stem cells. *Cell Proliferation* **40** 75–90. (doi:10.1111/j.1365-2184.2007.00414.x)
- Koch TG, Heerkens T, Thomsen PD & Betts DH 2007 Isolation of mesenchymal stem cells from equine umbilical cord blood. *BMC Biotechnology* **7** 26. (doi:10.1186/1472-6750-7-26)
- Koerner J, Nestic 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. (doi:10.1634/stemcells.2005-0264)
- Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C et al. 2004 A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *Journal of Experimental Medicine* **200** 123–135. (doi:10.1084/jem.20040440)
- Kubo M, Sonoda Y, Muramatsu R & Usui M 2001 Immunogenicity of human amniotic membrane in experimental xenotransplantation. *Investigative Ophthalmology & Visual Science* **42** 1539–1546.
- Lange-Consiglio A, Corradetti B, Bizzaro D, Magatti M, Ressel L, Tassan S, Parolini O & Cremonesi F 2012 Characterization and potential applications of progenitor-like cells isolated from horse amniotic membrane. *Journal of Tissue Engineering and Regenerative Medicine* **6** 622–635. (doi:10.1002/term.465)
- Lovati AB, Corradetti B, Lange Consiglio A, Recordati C, Bonacina E, Bizzaro D & Cremonesi F 2011 Comparison of equine bone marrow-, umbilical cord matrix and amniotic fluid-derived progenitor cells. *Veterinary Research Communications* **35** 103–121. (doi:10.1007/s11259-010-9457-3)

- Magatti M, De Munari S, Vertua E, Gibelli L, Wengler GS & Parolini O 2008 Human amnion mesenchyme harbors cells with allogeneic T-cell suppression and stimulation capabilities. *Stem Cells* **26** 182–192. (doi:10.1634/stemcells.2007-0491)
- Manuelpillai U, Moodley Y, Borlongan CV & Parolini O 2011 Amniotic membrane and amniotic cells: potential therapeutic tools to combat tissue inflammation and fibrosis? *Placenta* **32** (Suppl 4) S320–S325. (doi:10.1016/j.placenta.2011.04.010)
- Marongiu F, Gramignoli R, Sun Q, Tahan V, Miki T, Dorko K, Ellis E & Strom SC 2010 Isolation of amniotic mesenchymal stem cells. *Current Protocols in Stem Cell Biology* (chapter 1 unit 1E 5). (doi:10.1002/9780470151808.sc01e05s12)
- Marshall OJ 2004 PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* **20** 2471–2472.
- Miki T, Lehmann T, Cai H, Stolz DB & Strom SC 2005 Stem cell characteristics of amniotic epithelial cells. *Stem Cells* **23** 1549–1559. (doi:10.1634/stemcells.2004-0357)
- Miki T, Marongiu F, Ellis E & Strom SC 2007a Isolation of amniotic epithelial stem cells. *Current Protocols in Stem Cell Biology* (chapter 1 unit 1E 3). (doi:10.1002/9780470151808)
- Miki T, Mitamura K, Ross MA, Stolz DB & Strom SC 2007b Identification of stem cell marker-positive cells by immunofluorescence in term human amnion. *Journal of Reproductive Immunology* **75** 91–96. (doi:10.1016/j.jri.2007.03.017)
- Miki T, Marongiu F, Dorko K, Ellis EC & Strom SC 2010 Isolation of amniotic epithelial stem cells. *Current Protocols in Stem Cell Biology* (chapter 1 unit 1E 3). (doi:10.1002/9780470151808)
- Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T *et al.* 2003 Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells* **21** 50–60. (doi:10.1634/stemcells.21-1-50)
- Ollivier FJ, Kallberg ME, Plummer CE, Barrie KP, O'Reilly S, Taylor DP, Gelatt KN & Brooks DE 2006 Amniotic membrane transplantation for corneal surface reconstruction after excision of corneolimbic squamous cell carcinomas in nine horses. *Veterinary Ophthalmology* **9** 404–413. (doi:10.1111/j.1463-5224.2006.00480.x)
- Pan GJ, Chang ZY, Scholer HR & Pei D 2002 Stem cell pluripotency and transcription factor Oct4. *Cell Research* **12** 321–329. (doi:10.1038/sj.cr.7290134)
- Pappa KI & Anagnou NP 2009 Novel sources of fetal stem cells: where do they fit on the developmental continuum? *Regenerative Medicine* **4** 423–433. (doi:10.2217/rme.09.12)
- Parolini O, Alviano F, Bagnara GP, Bilic G, Buhning HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N *et al.* 2008 Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* **26** 300–311. (doi:10.1634/stemcells.2007-0594)
- Parolini O, Soncini M, Evangelista M & Schmidt D 2009 Amniotic membrane and amniotic fluid-derived cells: potential tools for regenerative medicine? *Regenerative Medicine* **4** 275–291. (doi:10.2217/17460751.4.2.275)
- Passeri S, Nocchi F, Lamanna R, Lapi S, Miragliotta V, Giannesi E, Abramo F, Stornelli MR, Matarazzo M, Plenteda D *et al.* 2009 Isolation and expansion of equine umbilical cord-derived matrix cells (EUCMCs). *Cell Biology International* **33** 100–105. (doi:10.1016/j.cellbi.2008.10.012)
- Perin L, Sedrakyan S, Giuliani S, Da Sacco S, Carraro G, Shiri L, Lemley KV, Rosol M, Wu S, Atala A *et al.* 2010 Protective effect of human amniotic fluid stem cells in an immunodeficient mouse model of acute tubular necrosis. *PLoS ONE* **5** e9357. (doi:10.1371/journal.pone.0009357)
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S & Marshak DR 1999 Multilineage potential of adult human mesenchymal stem cells. *Science* **284** 143–147. (doi:10.1126/science.284.5411.143)
- Plummer CE, Ollivier F, Kallberg M, Brooks D, Barrie K, Utter M & Gelatt K 2009 The use of amniotic membrane transplantation for ocular surface reconstruction: a review and series of 58 equine clinical cases (2002–2008). *Veterinary Ophthalmology* **12** (Suppl 1) 17–24. (doi:10.1111/j.1463-5224.2009.00741.x)
- Prusa AR & Hengstschrager M 2002 Amniotic fluid cells and human stem cell research: a new connection. *Medical Science Monitor* **8** RA253–RA257.
- Raoufi MF, Tajik P, Dehghan MM, Eini F & Barin A 2011 Isolation and differentiation of mesenchymal stem cells from bovine umbilical cord blood. *Reproduction in Domestic Animals* **46** 95–99. (doi:10.1111/j.1439-0531.2010.01594.x)
- 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. *Journal of Cellular Physiology* **215** 329–336. (doi:10.1002/jcp.21312)
- Ringe J, Kaps C, Schmitt B, Buscher K, Bartel J, Smolian H, Schultz O, Burmester GR, Haupl T & Sittlinger M 2002 Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. *Cell and Tissue Research* **307** 321–327. (doi:10.1007/s00441-002-0525-z)
- Roubelakis MG, Pappa KI, Bitsika V, Zagoura D, Vlahou A, Papadaki HA, Antsaklis A & Anagnou NP 2007 Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. *Stem Cells and Development* **16** 931–952. (doi:10.1089/scd.2007.0036)
- Roubelakis MG, Bitsika V, Zagoura D, Trohatou O, Pappa KI, Makridakis M, Antsaklis A, Vlahou A & Anagnou NP 2011 *In vitro* and *in vivo* properties of distinct populations of amniotic fluid mesenchymal progenitor cells. *Journal of Cellular and Molecular Medicine* **15** 1896–1913. (doi:10.1111/j.1582-4934.2010.01180.x)
- Sankar V & Muthusamy R 2003 Role of human amniotic epithelial cell transplantation in spinal cord injury repair research. *Neuroscience* **118** 11–17. (doi:10.1016/S0306-4522(02)00929-6)
- 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. (doi:10.1634/stemcells.2004-0166)
- Schuh EM, Friedman MS, Carrade DD, Li J, Heeke D, Oyserman SM, Galuppo LD, Lara DJ, Walker NJ, Ferraro GL *et al.* 2009 Identification of variables that optimize isolation and culture of multipotent mesenchymal stem cells from equine umbilical-cord blood. *American Journal of Veterinary Research* **70** 1526–1535. (doi:10.2460/ajvr.70.12.1526)
- Seo MS, Jeong YH, Park JR, Park SB, Rho KH, Kim HS, Yu KR, Lee SH, Jung JW, Lee YS *et al.* 2009 Isolation and characterization of canine umbilical cord blood-derived mesenchymal stem cells. *Journal of Veterinary Science* **10** 181–187. (doi:10.4142/jvs.2009.10.3.181)
- Sessarego N, Parodi A, Podesta M, Benvenuto F, Moggi M, Raviolo V, Litanian M, Kunkl A, Ferlazzo G, Bricarelli FD *et al.* 2008 Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application. *Haematologia* **93** 339–346. (doi:10.3324/haematol.11869)
- Smith RK, Korda M, Blunn GW & Goodship AE 2003 Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. *Equine Veterinary Journal* **35** 99–102. (doi:10.2746/042516403775467388)
- Solomon A, Wajngarten M, Alviano F, Anteby I, Elchalal U, Pe'er J & Levi-Schaffer F 2005 Suppression of inflammatory and fibrotic responses in allergic inflammation by the amniotic membrane stromal matrix. *Clinical and Experimental Allergy* **35** 941–948. (doi:10.1111/j.1365-2222.2005.02285.x)
- Soncini M, Vertua E, Gibelli L, Zorzi F, Denegri M, Albertini A, Wengler GS & Parolini O 2007 Isolation and characterization of mesenchymal cells from human fetal membranes. *Journal of Tissue Engineering and Regenerative Medicine* **1** 296–305. (doi:10.1002/term.40)
- Tchetina E, Mwale F & Poole AR 2003 Distinct phases of coordinated early and late gene expression in growth plate chondrocytes in relationship to cell proliferation, matrix assembly, remodeling, and cell differentiation. *Journal of Bone and Mineral Research* **18** 844–851. (doi:10.1359/jbmr.2003.18.5.844)
- Totonoz P, Hu E, Graves RA, Budvari AI & Spiegelman BM 1994 mPPAR γ 2: tissue-specific regulator of an adipocyte enhancer. *Genes and Development* **8** 1224–1234. (doi:10.1101/gad.8.10.1224)
- 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. *Human Reproduction* **19** 1450–1456. (doi:10.1093/humrep/deh279)
- Varlakhanova NV, Cotterman RF, deVries WN, Morgan J, Donahue LR, Murray S, Knowles BB & Knoepfler PS 2010 Myc maintains embryonic stem cell pluripotency and self-renewal. *Differentiation* **80** 9–19. (doi:10.1016/j.diff.2010.05.001)

- Vidal MA, Kilroy GE, Johnson JR, Lopez MJ, Moore RM & Gimble JM 2006 Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. *Veterinary Surgery* **35** 601–610. (doi:10.1111/j.1532-950X.2006.00197.x)
- Vidal MA, Robinson SO, Lopez MJ, Paulsen DB, Borkhsenius O, Johnson JR, Moore RM & Gimble JM 2008 Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow. *Veterinary Surgery* **37** 713–724. (doi:10.1111/j.1532-950X.2008.00462.x)
- Wang M, Yoshida H, Kawashima H, Ishizaki M, Takahashi TA & Hori J 2006 Immunogenicity and antigenicity of allogenic amniotic epithelial transplants grafted to the cornea, conjunctiva, and anterior chamber. *Investigative Ophthalmology & Visual Science* **47** 1522–1532. (doi:10.1167/iops.05-0787)
- You Q, Tong X, Guan Y, Zhang D, Huang M, Zhang Y & Zheng J 2009 The biological characteristics of human third trimester amniotic fluid stem cells. *Journal of International Medical Research* **37** 105–112.
- Yuge I, Takumi Y, Koyabu K, Hashimoto S, Takashima S, Fukuyama T, Nikaido T & Usami S 2004 Transplanted human amniotic epithelial cells express connexin 26 and Na–K-adenosine triphosphatase in the inner ear. *Transplantation* **77** 1452–1454. (doi:10.1097/00007890-200405150-00023)
- Zheng YM, Zhao XE & An ZX 2010 Neurogenic differentiation of EGFP gene transfected amniotic fluid-derived stem cells from pigs at intermediate and late gestational ages. *Reproduction in Domestic Animals* **45** e78–e82. (doi:10.1111/j.1439-0531.2009.01568.x)
- Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P & Hedrick MH 2002 Human adipose tissue is a source of multipotent stem cells. *Molecular Biology of the Cell* **13** 4279–4295. (doi:10.1091/mbc.E02-02-0105)

Received 2 November 2012

First decision 3 December 2012

Accepted 12 February 2013