Mesenchymal stem cells from amnion and amniotic fluid in the bovine

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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.

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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, Ring 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).

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. 2001, Sankar & Muthusamy 2003, Yuge et al. 2001, Delo et al. 2004, Kern 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. 2007, AECs and from the AF have not been 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).

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. The 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).

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.
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 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 MSCs from amnion and amniotic fluid in bovine 393

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Figure 1 Cell morphology. Monolayer of adherent polygonal epithelial AECs and spindle-shaped fibroblastic-like AF-MSCs. Magnification, 20×; scale bar, 20 μm.

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).
PPARG, which is crucial for the preadipocyte commitment (Toto noz 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.
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 allantoids 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 nm 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–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 Burker chamber. For maintenance of cultures, cells were plated at up to 1 × 10⁶ 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 × 10³ 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 (Nc/N0)/log2 and PD = CT/CD, where CD represents cell doubling, Nc represents the number of cells at confluence, No 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 PPARγ, FABP4 and LEP for adipogenesis, the Sry-related high-mobility group box transcription factor (SOX9), collagen type 2 (COL2A1) and ACAN for chondrogenesis, and GFAP and NES for neurogenesis. Bovine-specific
Table 2 Oligonucleotide sequences used for RT-PCR analysis.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
<th>Annealing temperature (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POU class 5 homesbox 1 (POU5F1)</td>
<td>CACACTAGGATAACCTACCAAGGCAAGC</td>
<td>GGAGATATGCAGAAGGCGAGAGAGAAGGCAAGCTG</td>
<td>60</td>
<td>177</td>
</tr>
<tr>
<td>MYC</td>
<td>GGCCCGGATCCCGGAAACCTGTTGCCAAGGCAAGCAGCG</td>
<td>TGGAGGGGATCCGTCGCAAGCAGCAG</td>
<td>58</td>
<td>214</td>
</tr>
<tr>
<td>Major histocompatibility complex 1 (MHC-I)</td>
<td>CATCTCTGCAATGATACACCAGGGACCATTTATAGTCT</td>
<td>CTTCTACCAGGGAGCATTTATAGTC</td>
<td>60</td>
<td>199</td>
</tr>
<tr>
<td>MHC-II</td>
<td>CACCTGTTGGTGCATTTGGGCAAGGCAAGC</td>
<td>TGAGGGCAGGAGGACCTGATGGGC</td>
<td>53</td>
<td>299</td>
</tr>
<tr>
<td>ALCAM</td>
<td>CTTTATATCTCGGCTTTCGCTTTCGCTTTC</td>
<td>ACAGGTGGCAGCTGATGC</td>
<td>59</td>
<td>755</td>
</tr>
<tr>
<td>Ecto-5'-nucleotidase (NT5E)</td>
<td>AAGGATCTGTGTCGTCGAGCCCTGCTT</td>
<td>CGGTTCAGGGATGCTGACACTC</td>
<td>68</td>
<td>260</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>ATGAGATCAAGAAGGTC</td>
<td>GCAATATTGTCTGCAAGCAG</td>
<td>60</td>
<td>190</td>
</tr>
</tbody>
</table>

Integrin β1 (ITGB1) | GTCAGTTCTCTGAGTTAGTGTGCTAG | ACAAACCAAAATTCACTGGAAGT | 52 | 203                |
| CD44 antigen (CD44) | AACAGATAGGAGGAGGTGTTGCCAG | TCTGAACTCTGTCCGCAAGT | 61 | 166                |
| Endoglin (ENG)     | ACAAGTCTGCAAGAAGGCAAGCAGCT | CATGCTCTGAGAGGACTGCT | 61 | 182                |
| PTPRC molecule (CD45) | TCTTGGCTGCTTCTGGTTCGCTT | AATGGGCACTGCAAGG | 58 | 350                |
| CD14 molecule (CD14) | TCCTGAGGGCTCTGCTTTCGCTT | TGTCGGCTCCTGATGAAAC | 56 | 104                |
| CD34               | CCTGAAGCTAATGACCTCAGCT | AACTTTCCTGTCGGTGTGCT | 58 | 173                |
| Leptin (LEP)       | CAGGCTACATCACAAGACAGGAG | CAGGCGAGGCTAGGGAAG | 55 | 212                |
| Adipocyte fatty acid-binding protein (FABP4) | CTGGGATGGGCAAAACCGA | GTACTTGTACGAGACCGG | 55 | 182                |
| Peroxisome proliferator-activated receptor (PPARG) | CCGCAGGATTAGTATGACG | CAAATCTGCTCTGAGGTCTG | 55 | 199                |
| Sex-determining region Y-box 9 (SOX9) | CATGAAGATGACCCGAGAGCAG | CTGCTTCTCCGCTGCG | 55 | 118                |
| Collagen type 1, alpha 1 (COL1A1) | CGCCGAGTTTGGTCTGTGGT | AGGTCCCATCAGCAGG | 55 | 269                |
| Aggrecan (ACAN)    | CGGCTCTGCTGCAAGAAGGTAATGG | CTGGCTAGGGCATGCTAG | 55 | 269                |
| Glial fibrillary acidic protein (GFAP) | GGACCAAGGAGGAAAGGCTC | CTCCCTGAGGCTCCGCACT | 60 | 195                |
| Nestin (NES)       | ACCACTGAGAGCTCCAGGTGG | TCGGACGGTTCTGACGGCTG | 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 × 10^5/cm² 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 ± 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.

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