

Isolation and in Vitro Characterization of Bovine Amniotic Fluid Derived Stem Cells at Different Trimesters of Pregnancy

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Abstract Amniotic fluid (AF) is a source of multipotent mesenchymal stem cells (MSCs), very promising cells for tissue engineering in clinical application. The aim of this work was to isolate and characterize cells isolated from bovine AF as alternative sources of primitive multipotent stem cells in a species that could be a large-animal model for biomedical and biotechnology researches. Samples were recovered, at slaughterhouse, from 39 pregnant cows at different trimesters of pregnancy and cells were cultured in vitro. At passages (P) 3 and 7 differentiation towards chondrogenic, osteogenic and adipogenic lineages was induced. Flow cytometry analysis for CD90, CD105, CD73, CD44, CD34, CD45 and CD14 was performed, immunocytochemistry (ICC) for Oct4, SSEA4, α -SMA, Vimentin, N- and E- Cadherin and CK and qPCR analysis for OCT4, NANOG and SOX2 were carried out. The cell yield was significantly higher in the first trimester compared to the second and the third one ($P < 0.05$). Cells were isolated from 25/39 samples and cell population appeared heterogeneous. Two main cell types were identified in samples from all trimesters: round- (RS) and spindle-shaped (SS) cells. 17/25 samples showed both populations (mixed, MX). Both cell types showed MSC-markers and differentiation capability with some variability related to the passages. The SS-population also expressed low levels of stemness markers such as NANOG and SSEA4 but not OCT4. Bovine AF shows a heterogeneous cell population

containing also MSCs, multipotent cells that represent an intermediate stage between embryonic stem cells and adult ones.

Keywords Amniotic fluid · Bovine · Mesenchymal stem cells · Differentiation · Pluripotency

Introduction

The amniotic fluid (AF) represents a protective liquid layer that surrounds the foetus during its development. It gives mechanical support and nutrients for foetal growth [1, 2]. It is mainly composed by water and the production of AF is determined by excretion of foetal urine and oral, nasal, tracheal and pulmonary fluids, so AF overall composition changes with gestational ages [1, 3, 4]. Most importantly, it was discovered that AF contains a heterogeneous mixture of stem cell populations (AFSCs) deriving from both the foetus and the surrounding amniotic membrane [1, 3, 5]. In humans, AFSCs can be classified into three types of plastic-adherent cells depending on their morphology and their growth characteristics: epitheloid cells (E-type cells), that appear at the beginning of the culture, decrease during the first passages and are supposed to derive from foetal skin and urine; amniotic fluid specific cells or round-shaped cells (AF-type or RS cells), which appears at the beginning of the culture and persist throughout the passages; fibroblast-like cells or spindle-shaped cells (F-type or SS cells), which originate from connective tissue and dermal fibroblasts and derive from mesenchymal tissues [3, 4, 6, 7]. In the majority of studies on human AF, a prevalence of the spindle-shaped population was found [8–11]. Some research groups [12–14] found both populations persisting through passages, while Roubelakis and co-workers [15] reported the prevalence of round-shaped (RS) population over the spindle-shaped (SS). In veterinary medicine the

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predominant AFSCs population found is represented by the spindle-shaped one in different species: horse [16, 17], buffalo [18], pig [19, 20], sheep [21, 22], dog [23, 24], cat [25] and cattle [26, 27]. Only Sartore and colleagues [19] isolated from pig AF both spindle-shaped cells and round amniocytes. SS cells represent the AF subpopulation of mesenchymal stem cells (MSCs), multipotent stem cells which express specific cell-surface antigen markers, in detail CD73, CD90 and CD105, and which lack the markers CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. MSCs can be also stimulated *in vitro* to produce osteoblasts, chondrocytes and adipocytes, cells of the mesodermal lineage [3, 28]. AFSCs are also known to express embryonic stem cells markers, such as Oct3-4, SSEA3-4, Nanog, Sox2 [3, 29] as well as endothelial (von Willebrandt factor, CD144) and mesenchymal (alpha smooth muscle actin α -SMA, vimentin, N-cadherin) markers [4, 5, 7, 19, 21]. This is the reason why these cells are considered an intermediate stage between embryonic and adult stem cells and this feature makes them good candidates for clinical applications [8, 10, 30, 31]. Another feature that makes MSCs good candidates for potential therapeutic use is their migration potential and the expression of adhesion molecules in common with leukocytes like CD44, CD24, CD29 and CD18 [32, 33]. AFSCs represents a source of stem cells that can replace the use of embryonic ones (ESCs) and could be obtained in higher yield compared to stem cells from adult organs [3]. Effectively, AF allows to avoid the limitation of access in sources related to bone marrow MSCs because AF, like foetal adnexa, represents waste at the partum, it is easy to obtain and represents an accessible and not risky source [34, 35]. It is also important to underline that human AF-MSCs have not shown tumorigenicity [10], and they showed immunomodulatory properties [36].

The bovine is an important agricultural species with significant commercial value and it is also an attractive large animal model for biomedical and biotechnology research. Bovine pregnancy lasts about 280 days, like the human one, so the cow represents a good model to understand differences in cells composition among trimesters. The development of large animal experimental models, including cattle, may open alternative strategies for investigating MSCs physiology and potential application for human and veterinary regenerative medicine. For this reason, it could be interesting to identify a cell population from bovine amniotic fluid that would display a high proliferative ability and a longer lifespan than a normal differentiated cell line, in order to allow possible applications in regenerative medicine but also gene targeting experiments and a successful use for somatic cell nuclear transfer for biotechnology applications based on genetic engineering. The aim of this study was to investigate and characterize bovine AFSCs (bAFSCs) populations by analysing phenotype, gene expression profile, growth curves and differentiation potential

and evaluating the AF cells yield in different gestational periods.

Materials and Methods

Materials

All chemical were obtained from Sigma-Aldrich (St. Louis, MO, USA), plastic dishes and tubes from Sarstedt Inc. (Newton, NC, USA), unless otherwise indicated.

Samples Collection and Cell Isolation

Bovine amniotic fluid samples were harvested at the slaughterhouse from pregnant uteri of 39 Holstein cows at different gestational ages. Bovine pregnancy was divided into 3 trimesters, as done in humans [37], and gestational periods were estimated by measuring crown-rump length of the foetuses [38]. AF was collected into 50 ml sterile syringes (IMI, Padua, Italy) and transported to the laboratory at 4 °C within 1 h.

AF samples were then diluted with Dulbecco's Phosphate Buffer Solution DPBS (1:1, v/v) containing 100 IU/ml penicillin and 100 mg/ml streptomycin and centrifuged (Heraeus Megafuge 1.0R; rotor: Heraeus #2704; ThermoFisher Scientific Inc.) for 15 min at 470 g. Pellets were suspended in 5 ml of culture medium (DMEM/M199) containing DMEM and TCM199 (1:1), 10 % (v/v) FBS (Life Technologies), 100 IU/ml penicillin and 100 mg/ml streptomycin and centrifuged for 15 min at 470 g. Supernatant was discarded and cells were re-suspended in 1 ml of culture medium and counted in a Neubauer chamber.

Cell Culture and Population Doublings

Bovine AFSCs were seeded in 25 cm² flasks at the density of 5×10^3 cells/cm². Cells were cultured in DMEM/M199 in a humidified atmosphere with 5 % CO₂ at 38.5 °C. Different cell populations found at P0 were classified on the basis of their morphology: spindle-shaped cells (SS), round-shaped (RS), fibroblastoid (fi) and large and flat (LF). Samples which presented both SS population and RS population were defined as mixed samples (MX). Different morphologies are reported in the Fig. 1. When cultures reached about 80–90 % confluence, cells were dissociated with 0.25 % (w/v) trypsin solution, counted and re-seeded to the subsequent passage, named "Passage 1" or P1.

Cell-doubling time (DT) and cell-doubling numbers (CD) were calculated through the following formulae [39]:

$$CD = \ln(N_f/N_i)/\ln(2) \quad (1)$$

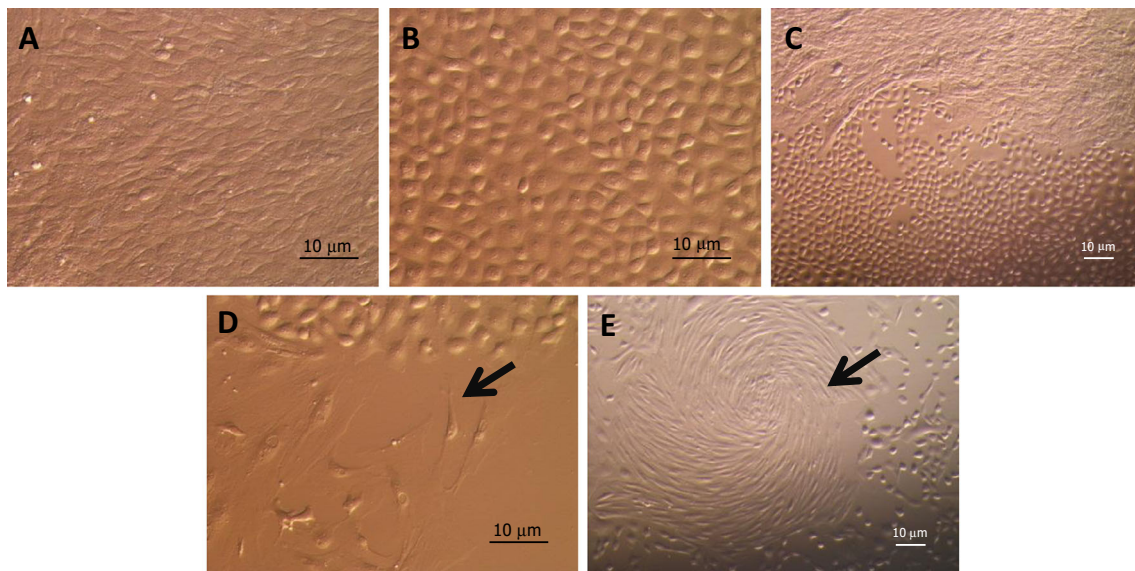


Fig. 1 Different types of adherent AF-cells isolated at P0: spindle-shaped (SS) cells (**a**), rounded (RS) cells (**b**), large and flat (LF) cells (**d**, *black arrow*) and fibroblastoid (fi) cells (**e**, *black arrow*). In the most part of the

samples (17/25) an heterogeneous population composed of SS and RS cells, named mixed (MX) population, was found (**c**). Bars=10 µm

where N_f is the final number of cells and N_i the initial number of cells;

$$DT = CT/CD \quad (2)$$

where CT is the cell culture time.

Growth curves of different cell populations persisting through passages (SS, RS and MX) were compared to understand if DT and CD differ in relation to the cell type.

Adhesion and Migration Assays

In order to define differences between SS and RS populations, a spheroid formation-and-migration test was performed to evaluate differences in adhesion and migration. Cells from each sample type were cultured in ‘hanging drops’ (5,000 cells/drop) for 24 h, until spheroid formation was observed. Spheroid areas were determined, then spheroids were cultured under standard conditions to allow their adhesion and the migration of cells from the spheroids themselves [32]. The migration rate was calculated after 24 h of incubation, comparing images taken immediately before and after this incubation period. The pre-adhesion dimension of the spheroids gave also information about the adhesion ability of the cells: spheroid area and adhesion capability are inversely proportional.

The migration capability of both population types was also evaluated by a scratch assay (also known as Wound-Healing assay), as previously described [40]. Briefly, at 80–90 % confluence the cell monolayer was scraped using a p1000 pipet tip. After washing twice with PBS, the dish was

incubated for 24 h at 38.5 °C and 5 % CO₂ in a humidified atmosphere. Images were acquired both immediately after the tip-scratch (time 0) and after the incubation period (last time point or time 1), and the distances of each scratch closure were calculated by ImageJ software. The migration percentages were calculated using the following formula:

$$[(\text{distance at time 0} - \text{distance at time 1}) * 100] / \text{distance at time 0}$$

In Vitro Differentiation

Bovine AFSCs were expanded in culture at P3 and then their ability to differentiate into mesenchymal lineages was tested. Each experiment consisted of a treated group, cultured in specific inducing medium, and a control group, maintained in standard culture conditions. For differentiation cells were seeded at 5×10^3 cells/cm² in 6-well plates.

1. Adipogenic differentiation

In order to test their ability to differentiate into adipocytes, bAFSCs were cultured in DMEM/M199 plus rabbit serum (15 %), dexamethasone (1 µM), 3-isobutyl-1-methylxanthine (0.5 mM), ITS (Insulin Transferrin Selenite, 10 µg/ml), and indomethacin (0.2 µM). After 3 days of culture, 3-isobutyl-1-methylxanthine was removed and after 3 additional days dexamethasone was also removed and cells were cultured for additional 15 days [16].

To test adipogenic differentiation, cells were stained with Oil Red O. Briefly, cells were washed with PBS and then fixed with 2 % paraformaldehyde for 30 min at room

temperature. The paraformaldehyde was removed and cells were washed with 60 % isopropanol. Cells were finally covered with Oil Red O solution (0.3 % in 60 % isopropanol) to make lipid vacuoles to appear red. After 30 min at room temperature, cells were rinsed with distilled water.

2. Osteogenic differentiation

Osteogenic differentiation consisted of culturing the cells in culture medium supplemented with dexamethasone (0.1 μ M), beta-glycerophosphate (10 mM) and ascorbic acid-2-phosphate (50 μ M) [41]. The cells were cultured in a humidified atmosphere of 5 % CO₂ at 38.5 °C and the differentiation medium was changed every 3 days for 21 days. Under these culture conditions, the cells are expected to produce a mineralized matrix. To reveal it, cells were fixed with 10 % formalin for 1 h at room temperature and then were stained with the Von Kossa staining. Briefly, cells were washed with PBS and then fixed with 10 % formalin for 1 h at room temperature. The formalin was removed and cells were washed five times with distilled water. One ml of 5 % (w/v) silver nitrate was added and cells were exposed to yellow light for 45 min. The wells were washed five times with distilled water. Calcium phosphate deposits stained black.

3. Chondrogenic differentiation

For the purpose to stimulate the bAFSCs to differentiate into the chondrogenic lineage, they were cultured in DMEM/M199 supplemented with FBS (1 %), ITS (6.25 μ g/ml), dexamethasone (0.1 μ M), ascorbic acid-2-phosphate (50 nM) and human transforming growth factor (hTGF)- β 1 (10 ng/ml), as previously described [8, 16, 24, 25]. The cells were cultured in a humidified atmosphere of 5 % CO₂ at 38.5 °C and the medium was changed every 3 days for 3 weeks after cell seeding. To assess chondrogenic differentiation, cells were fixed with 10 % formalin for 1 h at room temperature, then stained with Alcian Blue solution (1 % in 3 % acetic acid, pH 2.5) for 15 min at room temperature. Alcian Blue stains polysaccharides like glycosaminoglycans.

Flow Cytometry

Bovine AFSCs of different gestational trimester at passages 3 (P3) and at P7 were immunophenotyped by flow cytometric analysis. Cells were fixed and permeabilised at the concentration of about 10⁶ cells/ml using Reagent 1 of Intraprep Kit (Beckman Coulter, Miami, FL), according to manufacturer's instructions. As previously reported, the International Society for Cytotherapy declared that human MSCs must be CD73, CD90 and CD105 positive and CD14, CD34 and CD45 negative [28]. Flow cytometry analysis was performed using anti-human antibodies routinely used. Cells were labelled with

the following monoclonal antibodies: CD105, CD45, CD90, CD44, CD34, CD14 and CD73 (all from Beckman Coulter, Fullerton, CA). To verify cross-reactivity, circulating bovine lymphocytes were used as control.

Immunocytochemistry

Cultured SS, RS and MX samples at P3 and at P7 were washed with DPBS, fixed with 4 % paraformaldehyde for 20 min at room temperature and then washed in phosphate buffer (PB). Cells were blocked in goat serum 10 % (Sigma) for 1 h and incubated overnight with primary antibodies against Oct4 (Abcam, ab18976) 1:50, SSEA4 (Hybridoma Bank, MC-813-70) 1:50, and alpha-SMA (Gene Tex) 1:500. Cells were washed in PB2 (Phosphate Buffer with 0.2 % of bovine albumin and 0.05 % of saponin) and incubated with anti-mouse- or anti-rabbit- FITC conjugated secondary antibodies for 1 h. After that, nuclei were labelled using Hoechst 33342. The excess of secondary antibody and Hoechst was removed by 3 washes with PB2. Bovine blastocysts and bovine fibroblasts were used respectively as positive and negative control. In order to define a profile of SS and RS cells, the expression of Vimentin, N-cadherin (mesenchymal marker), E-cadherin and Cytokeratin (epithelial markers) was analysed. The following primary antibodies were used: Vimentin clone 9 (DAKO M0725) 1:100, N-Cadherin (Biorbyt orb11100) 1:100, E-Cadherin (Cell Signaling Technologies #3195) 1:200, pan-Cytokeratin (Chemicon international, Millipore, CBL234) 1:250. With the purpose to test the cross-reactivity of our antibodies, the following types of cells were used as positive controls: bovine fibroblasts for Vimentin, bovine epithelial cells for pan-Cytocheratin, and bovine embryos for E-cadherin and N-Cadherin. Images were obtained with a Nikon Eclipse E400 microscope at a 40 \times magnification using the software Nikon NIS-Elements. Percentages of positive cells were obtained evaluating ratio between positive cells and total nuclei.

Molecular Characterization

In order to evaluate differences in markers expression between the two types of bAF-cells, qRT-PCR for the pluripotency genes OCT4, NANOG and SOX2 was performed on SS and RS cells both at P3 and at P7. Gene expression in the samples was normalized on bovine blastocysts, used as positive control, while bovine adult fibroblasts were used as negative control. To test cell differentiation, the following set of genes was evaluated on SS and RS differentiated samples both at P3 and P7: bovine PPAR γ for adipogenic differentiation, collagen type I (coll I) and Osteopontin (OPN) for osteogenic differentiation and collagen type I and type II (coll II) for chondrogenesis. In all the reaction 18S was used as house-keeping reference gene and the differentiated samples were

normalized on the respective undifferentiated P3. The specific set of primers used is listed in Table 1.

For RNA extraction, cells were washed with PBS, dissociated using 0.25 % trypsin solution and the pellets were snap-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy mini kit (Qiagen, 74104) following the manufacturer's instructions and it was reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, 170–8891). Reactions were conducted for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. RT-products (cDNAs) were used directly in PCR reactions. Real-time PCR reactions were performed in triplicate using iQ SYBR Green Supermix and a MyiQ Real-Time PCR Detection System. Data were analyzed with the iQ Optical System Software (Bio-Rad) with the DDct method.

Statistical Analysis

CDs, DTs, cells yield, mean AF volume harvested, spheroids areas and percentages of migration are expressed as mean \pm standard deviation. Statistical analyses were performed using IBM SPSS Statistics 21 (IBM Corporation, Milan, Italy). Data were analysed using one-way ANOVA or a Student's *t*-test (when comparing SS and RS populations only). For statistical analyses, the unique sample with the spindle-shaped morphology was cultured in 2 replicates. Significance has been assessed for $P < 0.05$.

Results

Cell Yield and Morphology

It was possible to isolate cells in 25/39 samples (64 %). In 7/39 samples, harvested at the same time, it was not possible to isolate cells because of a bacterial contamination, probably due to the non-sterile conditions of the slaughterhouse. In other 7/39 samples, harvested from cows at the third trimester of gestation (4/7) or at the end of the second trimester (3/7), cells did not grow. Of the 25 samples where cells were

isolated, 3 specimens were from the first trimester (0–93 days), 19 from the second one (94–187 days) and 3 were from the third one (188-term of pregnancy).

Mean volume of amniotic fluid collected was 26.7 ± 20.8 ml in the first trimester, 41.1 ± 13.9 ml in the second one and 34.0 ± 5.3 ml in the third one. Mean number of cell was 3533.3 ± 1747.4 cells/ml in the first trimester, 1035.0 ± 1260.9 cells/ml in the second trimester and 722.2 ± 502.3 cells/ml in the third one. The cell yield was significantly higher in the first trimester compared to the second and the third ones ($P < 0.05$).

At passage 0, it was possible to identify 4 different cell subpopulations in samples from all three trimesters: spindle-shaped cells, round-shaped cells, fibroblastoid cells and large and flat cells (Fig. 1). The last 2 types (fi and LF) of cells were lost after the first passage and they probably derived from foetal skin and urine. In 1/25 sample the spindle-shaped population predominated (SS sample) whereas in 7/25 samples (28 %) the round morphology prevailed (RS samples). In the rest of the samples (17/25, 68 %) both population were present, where the percentages of SS cells ranged from 10 to 40 % (mixed samples, MX). In these samples, spindle-shaped cells were only present until the third or fourth passage. This heterogeneity was found in samples from all three trimesters.

Cell Culture and Growth Curves

Undifferentiated cells were cultured until the eighth passage and total cell doubling number (CDs) and population-doubling times (DTs) were calculated. At P8, total CDs were statistically higher ($P < 0.05$) in the SS sample (24.9 ± 0.8) than in the MX samples (18.6 ± 2.5) and in the RS ones (17.3 ± 4.8) (Fig. 2). Mean doubling time was statistically lower ($P < 0.05$) for SS cells (38.4 ± 12.0 h) than MX samples (67.1 ± 25.0 h) and RS samples (82.9 ± 38.6 h).

Migration and Adhesion Assays

Both SS and RS cells formed spheroids when cultured in hanging drops. Average areas of the spheroids were 33138.1

Table 1 Sequence of primers used for qPCR analysis

Gene	Primer sequence FW	Primer sequence RV	Amplicon (bp)	Ref.
b18S	5'-GAATAACGCCGCCGCATCG-3'	5'-CGGACCAGAGCGAAAGCATTG-3'	133	[42]
boCT4	5'- TGCAGCAAATTAGCCACATC -3',	5'- AATCCTCACGTTGGGAGTTG-3'	123	[43]
bNANOG	5'-GTCCCGGTCAAGAAACAAAA-3'	5'-TGCATTGCTGGAGACTGAG-3'	107	[43]
bSOX2	5'-ACAGTTGCAAACGTGCAAAG-3'	5'-AGACCACGGAGATGGTTTTG-3'	114	[43]
bPPAR γ	5'-CGCACTGGAATTAGATGACAGC-3'	5'-CACAACTGTCTGAGGTCTGTC-3'	214	[43]
bOPN	5'-CCAATGAAAGCCCTGAG-3'	5'-TCCTCCTCTGTGGCATC-3'	310	[27]
bColl I	5'-AGAAGCATGTCTGGGTAGGAG-3'	5'-AGGATAGGCAGGCGAGATR-3'	358	[27]
bColl II	5'- ATCCATTGCAAACCCAAAGG -3'	5'- CCAGTTCAGGTCTCTTAGAG -3'	147	[44]

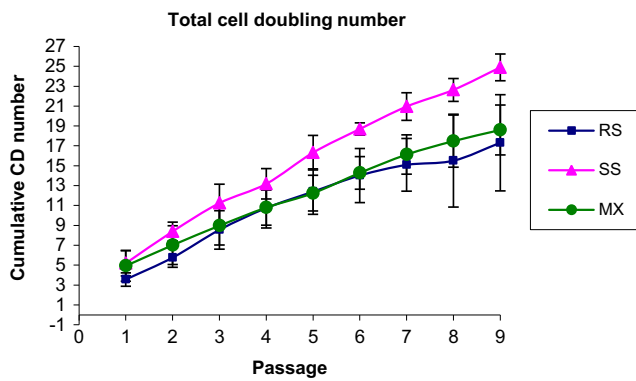


Fig. 2 Cell doubling numbers of SS-, RS- and MX- populations. At P8, total CDs were statistically higher ($P < 0.05$) in the SS sample than in the MX and in the RS ones

$\pm 1401.6 \mu\text{m}^2$ for the SS and $45014.8 \pm 1165.8 \mu\text{m}^2$ for the RS populations respectively. Spheroids from SS cells were significantly smaller than from RS cells ($P < 0.05$), indicating a higher adhesion capability. Average percentages of migration were significantly higher ($P < 0.05$) for spheroids originated from SS cells ($94.9 \pm 3.0\%$) than for the spheroids from RS samples ($76.2 \pm 4.5\%$). The faster migration of the spindle-shaped population was also confirmed by the scratch test. Average percentage of migration was significantly higher ($P < 0.05$) in SS ($96.9 \pm 2.5\%$) compared to RS ($79.9 \pm 2.2\%$).

Molecular Characterization of SS and RS Undifferentiated Cells

OCT4 expression was not detected both in SS and in RS (Fig. 3c), there was instead a very weak expression of NANOG in all the samples (Fig. 3b). Fibroblasts were negative both for OCT4 and for NANOG. SOX2 was not found in the RS population, while a weak expression was detected in the SS at both passages, but also in the bovine fibroblasts (Fig. 3a).

Fig. 3 qRT-PCR for the pluripotency genes OCT4, NANOG and SOX2. Evaluation of the expression in the two main cell type (SS- and RS-cells) at P3 and P7. Expression was normalized to bovine blastocysts, used as positive control

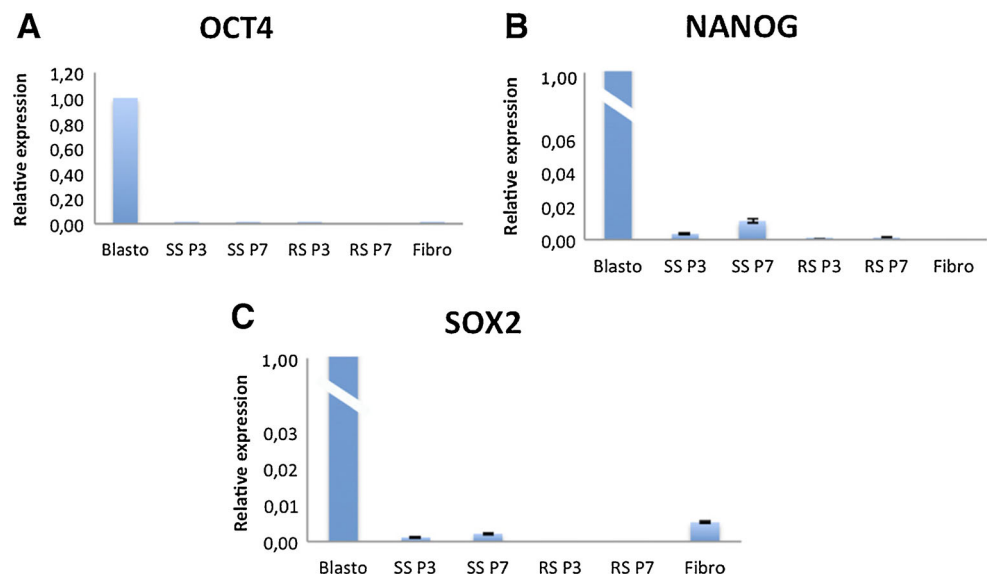
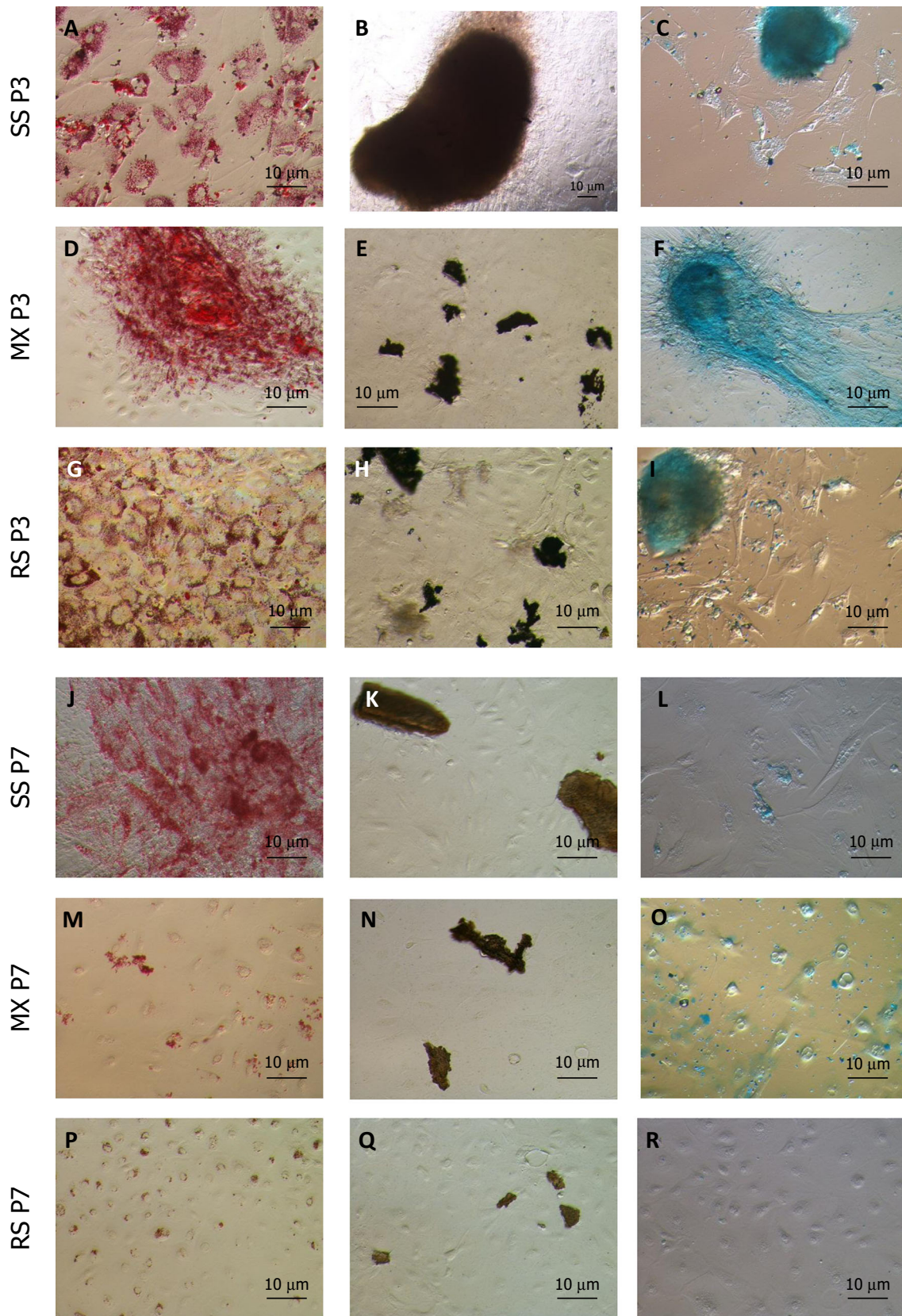


Fig. 4 Bovine AF samples were cultured over 3 weeks both at the third passage (from a to i) and at the seventh one (from j to r) under adipogenic (a, d, g, j, m, p), chondrogenic (c, f, i, l, o, r) and osteogenic (b, e, h, k, n, q) medium. Adipogenic induction was verified staining intra-cellular lipid droplets with Oil Red O, extracellular calcium deposition produced by cells under osteogenic induction was stained by von Kossa and chondrogenic differentiation was proved staining glycosaminoglycans of the cartilage matrix by Alcian Blue. All samples (SS-, MX- and RS- ones) differentiated well at P3 into all three lineages. At P7 only the SS sample kept a good differentiation into adipogenic and osteogenic lineages. RS- and MX- samples differentiated less into these two lineages whereas they did not show chondrogenic differentiation. Bars=10 μm

In Vitro Differentiation

All three types of cells (SS, RS and MX) were cultured in adipogenic, osteogenic and chondrogenic condition. To better characterize the proliferating SS and RS subpopulations, real time PCR was performed both for P3 and P7 undifferentiated and differentiated. Generally, SS cells showed a higher differentiation potential compared to RS cells and in MX samples was evident how SS subpopulation had a higher adipogenic and chondrogenic differentiation potential (Fig. 4d, f).

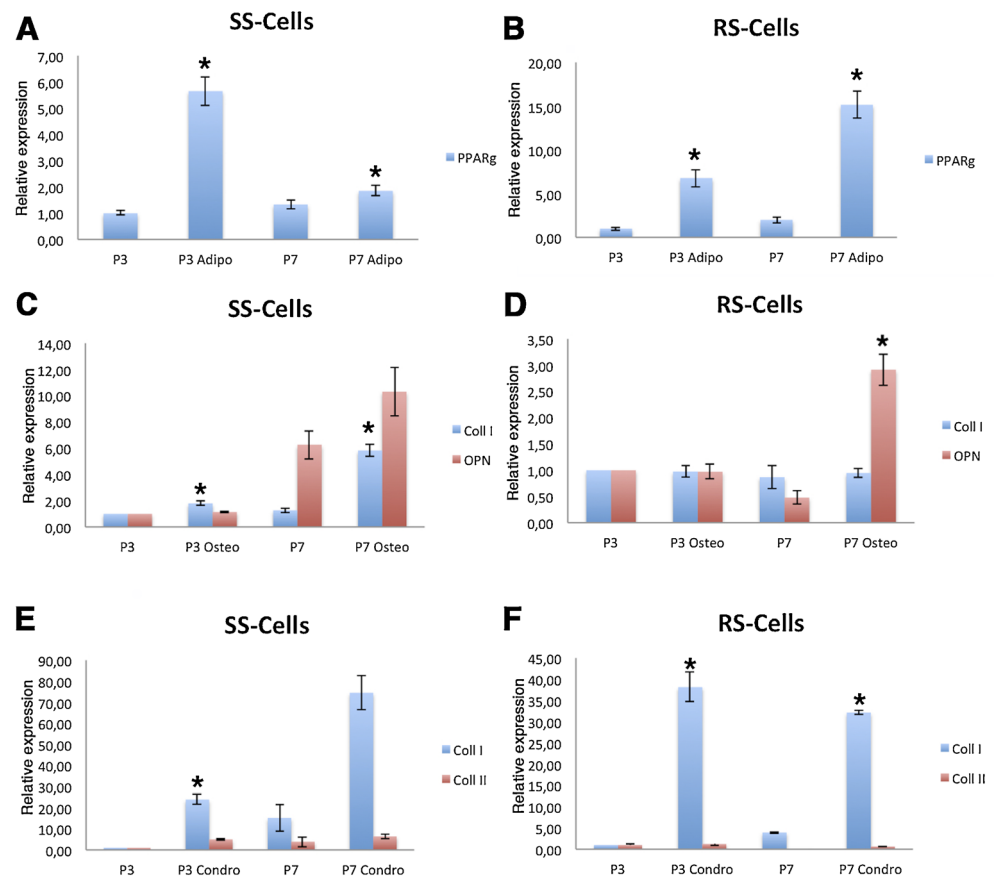
In particular, in adipogenic induced cell cultures, after 1 week the cells started to change morphology to a more rounded configuration. After 21 days, cells were round and showed evident lipid droplets within the cytoplasm, indicated by Oil Red O staining. At P3 (Fig. 4a, d, g), samples of all 3 types (SS, MX and RS) showed an adipogenic differentiation displayed by an intense Oil Red O staining. At P7 (Fig. 4m, j, p) SS cells showed a high accumulation of lipids in the cytoplasm, while MX and RS cells were still stained but with less intensity. Real Time PCR however indicated a significative ($P < 0.05$) upregulation of PPAR γ mRNA in both passages of SS and RS cells (Fig. 5a, b). All cell types grown in osteogenic medium both at P3 and P7 showed deposition of



matrix-like substance that appeared positive for Von Kossa staining (Fig. 4b, e, h). Gene expression analysis indicated an

upregulation of collagen type I and osteopontin in both SS passages and in RS at P7, while no changes were observed in

Fig. 5 Relative expression of markers related to differentiation lineages in both populations (RS and SS) at both passages (P3 and P7). In detail, PPAR γ for the adipogenic differentiation (**a, b**), Collagene type I and Osteopontin for the chondrogenic differentiation (**c, d**) and Collagene type I and II for the osteogenic one (**e, f**). The differentiated samples were normalized on the respective undifferentiated at P3



RS at P3 (Fig. 5c, d). Cells cultured in chondrogenic medium showed, after 3 weeks, depositions of glycosaminoglicans, which were stained by Alcian Blue. As for adipogenic differentiation, also in chondrogenic one it was evident that all three populations showed a higher ability to differentiate under the specific culture conditions at P3 (Fig. 4c, f, i), while at P7 (Fig. 4l, o, r) this potential was reduced in terms of glycosaminoglicans accumulation. Real-time PCR indicated a significant ($P < 0.05$) upregulation of collagen type I in SS cells at both passages and in RS cells only at P3 (Fig. 5e, f). Also collagen II expression was evaluated but none of the samples showed a significant upregulation, even if an increasing trend was found in the differentiated ones. Negative controls for all the 3 differentiation lineages were cultured in regular culture medium and stained with the 3 different methods. No positive stained cells were found (data not shown). As additional control, differentiation into the three lineages described before was induced on bovine fibroblasts and no positive cells were found (data not shown).

Immunophenotypic Characterization by Flow Cytometry

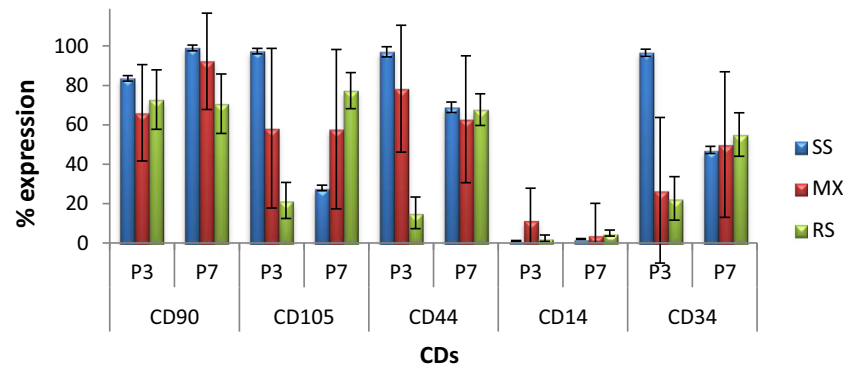
All cell types were positive for CD44 and CD105, with an increasing of their expression at P7 for all cell populations except for SS one (Fig. 6). All cell types also showed cross-

reaction with CD90 and the expression of this marker increased at P7 (Fig. 6). Overlay histograms of these flow cytometric analyses are reported in Fig. 7. It was not possible to evaluate the expression of the markers CD45 and CD73 in the bAFSCs because these CDs were negative also for lymphocytes (data not shown). Surprisingly, all cell populations showed expression of CD34 both at P3 and at P7, and the percentages reached about 50 % in all samples at P7 (Fig. 6). It is important to underline that also bovine fibroblasts and MSCs from bovine adipose tissue at first passages showed CD34 expression at flow cytometry while bovine lymphocytes did not expressed this marker (data not shown).

Phenotypic Analysis by Immunocytochemistry

Immunocytochemical staining of bAFSCs showed heterogeneity in the expression of SSEA4, an antigen commonly used as a marker for undifferentiated cells, and in the expression of alpha-SMA, a mesodermal marker. All cell lines did not showed expression of Oct4. Only samples with spindle-shaped cells showed expression of SSEA4 both at P3 (8.0 %) and at P7 (22.0 %), with an increasing of the expression through passages (Fig. 8a, b). Mixed populations (MX) and RS lines did not express this marker (data not shown). Alpha-SMA was expressed in all lines in different percentages

Fig. 6 SS-, MX- and RS-samples analysed for different antigens expression (CD90, CD105, CD44, CD34, CD14) by FACS analysis both at P3 and at P7



at P3: 9.4 % in SS (Fig. 8c), 0.9 % in RS (Fig. 8e) and 14.8 % in MX samples (Fig. 8g). In MX population is evident that percentages of positive cells are related to the presence of the spindle shaped cells (Fig. 8f, g, h). At P7 only SS sample maintained and increased the expression of this mesodermal marker, assessing it at 27.5 % (Fig. 8d). Both RS and SS cells did not express cytokeratin (Fig. 8m, n) and E-Cadherin was expressed in few RS cells (Fig. 8q) but not in SS cells (Fig. 8p). Vimentin was expressed by SS cells (Fig. 8i) while RS cells expressed this mesenchymal marker in a very low percentage, around 8 % (Fig. 8j). N-Cadherin was clearly expressed in the spindle shaped population (Fig. 8k) while in the round shaped one the expression was weaker and not present in all cells (Fig. 8l).

Discussion

Stem cells isolated from amniotic fluid represent an important source of cells with characteristics between pluripotent and adult stem cells [3, 8]. In the present study samples of AF have been easily collected at slaughterhouse from pregnant cows at

different trimesters of pregnancy and nucleated plastic-adherent cells have been isolated, and subsequently cultured and characterized, from the most part of the samples processed. Difficulties in isolation were found only for AF samples harvested at the end of the second trimester (about 180 days of gestation) or at the third, probably due to the relative small volume of sampling compared to the increasing amount of AF at these stages of pregnancy [45]. Furthermore, cells yield was almost threefold higher from samples collected in the first trimester than in the second and third, confirming that the ratio cells/ml decreases during pregnancy with the higher production of AF. On the other hand, differences about subpopulations isolated were not found among bAFSCs collected at different trimesters and changes in markers expression are to be associated with different cell populations. In fact, depending on the morphology, a heterogeneous population was found, as previously reported both for human amniotic fluid [4, 12, 13, 46] and in the veterinary field [19]. The two main populations isolated that persisted through passages were the spindle-shaped (SS) and the round-shaped (RS), even if at passage 0 there were also two other populations, as found by Bottai and colleagues [13] in human AF samples, classified as large and flat cells and fibroblastoid cells, that were lost after

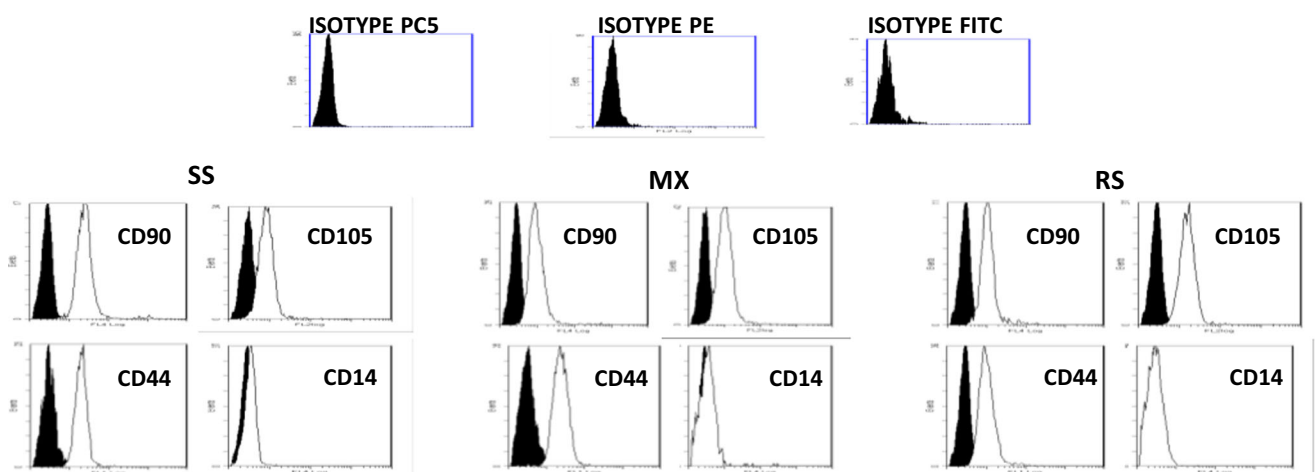


Fig. 7 Overlay histograms of cytometry analysis. In black isotypic controls are represented. Empty histograms represent the analysis with monoclonal antibodies on cell culture

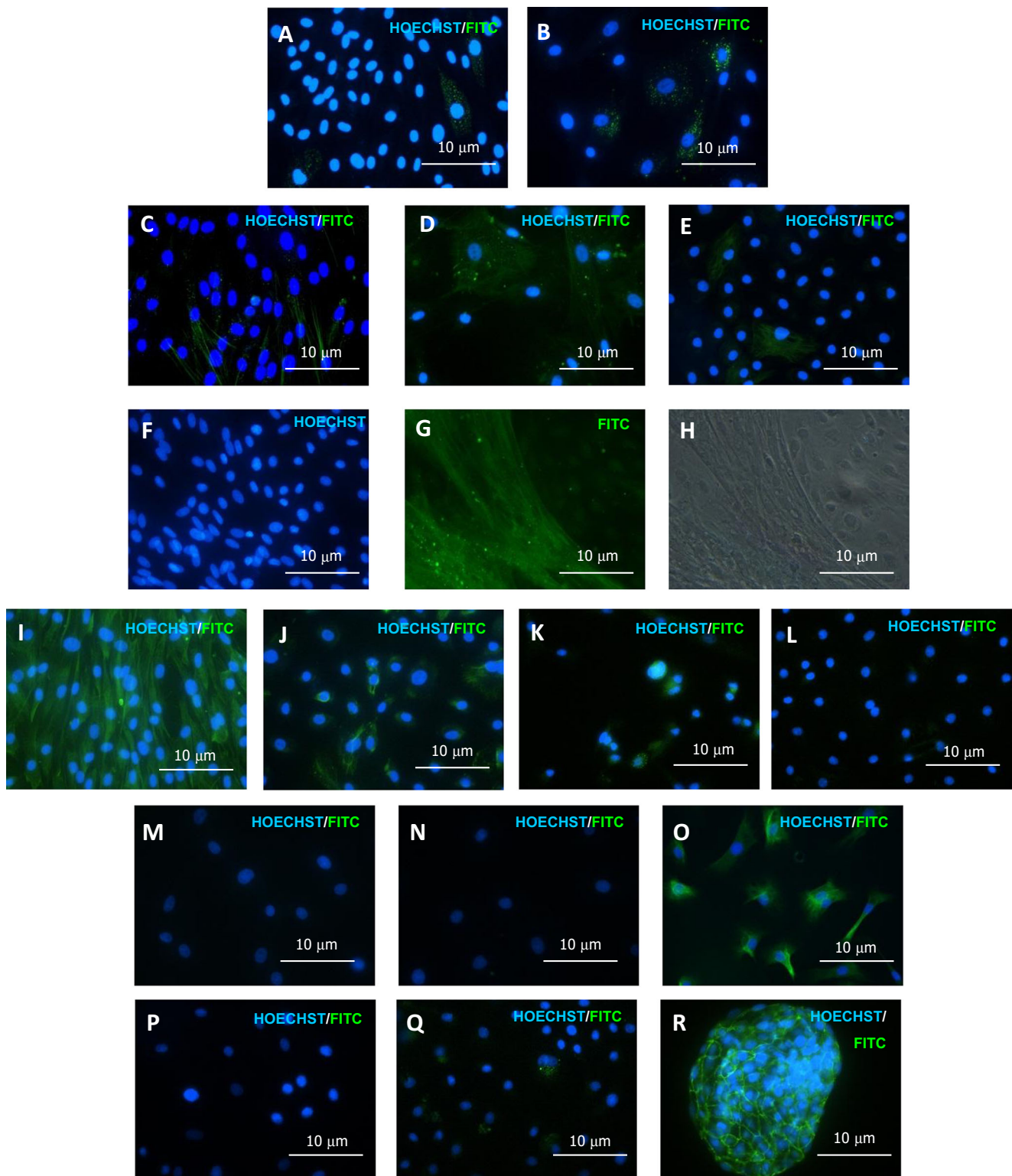


Fig. 8 Expression of the embryonic stem cell marker SSEA4 in the SS-sample at P3 (**a**) and at P7 (**b**). Expression of the mesenchymal marker alpha-SMA in the SS-sample (**c**), in a RS-sample (**e**) and in a MX-sample (**f**: Hoechst staining; **g**: FITC staining). It is clear how in the MX-samples the cells positive to the FITC staining (**g**) are the spindle-shaped cells (**h**). The **f**, **g** and **h** represent the same field. Expression of the mesenchymal marker Vimentin in the SS-population (**i**) and in the RS cells (**j**). Absence

of expression of the epithelial marker Cytokeratin both for RS cells (**n**) and for SS ones (**m**). Bovine epithelial cells were used as positive control (**o**). Expression of the mesenchymal marker N-Cadherin in the SS-sample (**k**) and in a RS one (**l**). Absence of expression of E-Cadherin both in spindle shaped cells (**p**) and in round shaped ones (**q**). All images, except for **f**, **g** and **h**, represent the merge of Hoechst and antibody stainings. Bars=10 µm

the first-second passage. The best-represented subpopulation in our bovine-AF samples was the round-shaped. This is in contrast with results obtained by two research groups [26, 27] that found less heterogeneity among samples and a prevalence of the spindle-shaped subpopulation.

As shown by proliferation assay, SS cells showed a lower mean doubling time (1.6 ± 0.5 days) compared to RS ones (3.5 ± 1.6 days). MX samples, which are composed by both populations until the third-fourth passage, had an intermediate average DT value (2.8 ± 1.0 days). This result suggests that the spindle-shaped subpopulation showed a rapid expansion in vitro, compared to the RS one. Moreover, SS subpopulations also showed a higher cell doubling number at P8 than the other 2 types of samples.

SS cells also migrated significantly faster and showed a statistically higher migration ability, compared to RS ones, as demonstrated by the migration assay. Migration ability is an important feature of MSCs because of its fundamental significance for systemic application [32]. The expression of adhesion molecules and the homing to injured environments are also important characteristics of MSCs [33]. Migration assay showed a significant higher adhesion capability of the SS population than the round shaped one, as confirmed by higher expression at P3 of CD44, a glycoprotein which has a role in MSCs migration [16]. At P7 all samples expressed this marker, highlighting an expression of CD44 through passages.

In vitro differentiation assay supports results obtained with human AFSCs [47] and animal AFSCs [16–18, 25, 26]. The choice of the three differentiation protocols (adipogenic, osteogenic and chondrogenic ones) was established on the basis of the values listed by the International Society of Stem Cells [28]. When adipogenic and osteogenic differentiation was induced, SS population showed at both passages a higher differentiation potential compared to RS capability in terms of accumulation of cytoplasmic lipids and deposition of glycosaminoglycans, respectively. At mRNA level, significant upregulation of specific differentiation markers was found for both populations, except for the expression of collagen type I in differentiated RS-cells at P3, indicating a selection through passages of cells able to differentiate toward the osteogenic lineage. Both cell types showed chondrogenic differentiation with production of cartilage matrix at P3 but not at P7. Observing differentiation potential of the mixed sample (MX) at P3, it is possible to see how SS cells differentiated better than the RS type, although there were not differences between the two cell types at mRNA level. Roubelakis and colleagues [4] also found that human AF (hAF)-SS population showed a better chondrogenic and osteogenic differentiation potential compared to hAF-RS-population one. An examination of the immunophenotype was performed observing the expression of different surface MSCs markers. Since there are no bovine-specific antibodies

for flow cytometry analyses, we used human ones, after checking for cross-reactions. Among our samples, all cell types had an increase in the expression of the MSC marker CD90 (Thy-1) from P3 to P7 and SS sample had a higher expression compared to RS and MX subpopulations at both passages. The other MSCs marker, CD105 (SH2 or endoglin or TGF β 1/3 receptor), increased its expression through passages, with the exception of the SS sample. All cell types were negative for the haematopoietic progenitor CD14. On the other hand, the lack of reactivity of bovine cells with the marker CD73 (NT5E, ecto-5'-nucleotidase) and bovine lymphocytes with CD45 (pan-leukocyte marker) maybe indicate that human antibodies did not cross-react with the corresponding bovine epitopes. It is important to underline that we found a percentage of positive cells for CD34 (marker for endothelial cells and hematopoietic progenitors cells) in all samples. At P3 this marker was expressed in high percentages by SS sample while in low percentages in RS and MX populations. At P7 there were about 50 % of CD34⁺ cells in all samples. Bovine MSCs isolated from the adipose tissue and bovine fibroblasts were also positive for this marker (unpublished data) whereas bovine lymphocytes revealed a CD34⁻ profile, as expected. Since a human antibody anti-CD34 was used, we could not exclude an aspecific cross-reaction with bovine adult cells. Anyway, the CD34 antigen is expressed on hematopoietic progenitor cells of all lineages as well as the most pluripotential stem cells. CD34 antigen expression is highest on the most primitive stem cells and is gradually lost as lineage committed progenitors differentiate [48]. Moreover, Corradetti and co-workers [26] did not find this marker at RT-PCR analyses, so further investigation is necessary.

It was proved that there is an amniotic fluid subpopulation that exhibit gene expression associated with pluripotency [3, 6, 34, 49] so investigation for markers such as OCT4, NANOG, SOX2 and SSEA4 were performed. By ICC it was discovered that SS population increased the expression of SSEA4 through passages, so it is probably due to a positive selection of a SSEA4⁺ population. Molecular analysis showed that only SS cells weakly expressed NANOG whereas OCT4 and SOX2 were not expressed by both subpopulations. The lack of expression of the embryonic marker OCT4 was also supported by ICC analyses. This is in contrast with previous studies performed on bAFSCs [26, 27]. This result is also in contrast with phenotypic characterization of AF-MSCs made by numerous groups in human medicine [8, 10, 29, 49–51] and in the veterinary field [17, 18, 20, 24] but recent studies on human AFSCs did also not find a population positive for Oct4 [14].

ICC investigations also showed different expression of alpha-SMA, a protein expressed by cells towards the mesodermal lineage. At P3 this marker was expressed by SS sample and by MX samples, where it was clear that this

marker was expressed only by cells with a spindle-shaped morphology. At P7 only the SS sample showed an alpha-SMA⁺ profile, MX specimens did not express the protein and this is probably related to the absence of spindle-shaped cells over the fourth passage. The spindle-shaped population also expressed other typical mesenchymal markers such as Vimentin and N-Cadherin, as found by other researchers [8, 13, 49, 52], while RS-cells expressed these markers in a very low percentages. Furthermore, SS cells did not express epithelial markers like Cytokeratin and E-Cadherin, and RS-cells expressed only E-Cadherin in a very low percentage, revealing that they were not epithelial cells.

In conclusion, both subpopulations isolated at early passages, SS and RS, showed expression of mesenchymal markers (CD90, CD105, CD44), a multilineage differentiation into mesenchymal lineages (adipogenic, osteogenic and chondrogenic ones) and average doubling times (DTs) comparable to human ones [13] and DTs of AFSCs from other species [16, 24, 25]. SS cells also showed a low expression of stemness markers such as Nanog and SSEA4, which are expressed over P3. Thus it is likely that SS cells are the presumptive AF mesenchymal subpopulation because they showed stem cells characteristics in addition to mesodermal markers, as for other species [19] and for humans [8]. Although RS cells showed some mesenchymal surface markers and had a good differentiation potential at early passages, the SS population is the ascribable to the mesenchymal one. The lack (Cytokeratin) or the very low expression (E-Cadherin) of epithelial markers in the RS population allows to exclude that these rounded cells are epithelial ones, and the low expression of mesenchymal markers (N-Cadherin, Vimentin), whose expression increases during the Epithelial to Mesenchymal transition, or EMT [53], collocate these cells in an intermediate stage. From our results we can assert that bovine AF shows two subpopulations and they differ in their phenotypic characteristics such as marker expression, proliferation rate, adhesion and migration properties and differentiation potential. The isolation of different populations does not depend on the trimesters of pregnancy and differences are only ascribable to different cell types.

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