STEM CELLS AND DEVELOPMENT
Volume 27, Number 22, 2018

DOI: 10.1089/scd.2018.0114

Identification and Characterization of a Stem Cell-Like Population in Bovine Milk: A Potential New Source for Regenerative Medicine in Veterinary

Caterina Pipino,[1,*] Domitilla Mandatori,[2,*] Flavia Buccella,[3] Paola Lanuti,[2] Alessandra Preziuso,[1] Federica Castellani,[3] Lisa Grotta,[3] Pamela Di Tomo,[2] Sonia Marchetti,[3] Natalia Di Pietro,[2] Angelo Cichelli,[1] Assunta Pandolfi,[1,*] and Giuseppe Martino[3,*]

Milk is a complex fluid required for development, nutrition and immunological protection to the newborn offspring. Interestingly, latest finding proved the presence of novel stem cell population in human milk with multilineage differentiation potential. Given that little is known about cellular milk content in other mammalian species such as bovine, the purpose of our study was to isolate and characterize a potential stem cell-like population in bovine milk. In detail, we first analyzed the phenotype of the isolated cells able to grow in plastic adherence and then their capability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. Bovine milk stem cells (bMSCs) resulted plastic adherent and showed a heterogeneous population with epithelial and spindle-shaped cells. Successively, their immunophenotype indicated that bovine milk cells were positive for the typical epithelial markers E-cadherin, cytokeratin-14, cytokeratin-18, and smooth muscle actin. Notably, a subset (30%–40%), constantly observed in purified milk cells, showed the typical mesenchymal surface antigens CD90, CD73, and CD105. Furthermore, the same percentage of bMSCs expressing CD90, CD73, and CD105 presented the stemness markers SOX2 and OCT4 translocated in their nuclei. Finally, our data showed that bMSCs were able to differentiate into osteoblasts, chondroblasts, and adipocytes. In addition, the flow cytometry analysis revealed the presence of a subpopulation of events characterized by typical extracellular vesicles (EVs, size 0.1–1 μm), which did not contain nuclei and were positive for the same markers identified on the surface of bMSCs (CD73, CD90, and CD105), and thus might be considered milk cell-derived EVs. In conclusion, our data suggest that bovine milk is an easily available source of multipotent stem cells able to differentiate into multiple cell lineages. These features can open new possibilities for development biology and regenerative medicine in veterinary area to improving animal health.

Keywords: bovine milk, stem cell-like population, veterinary regenerative medicine

Introduction

Milk, produced in the mammary glands of all mammals, is the first food necessary for protection, nutrition, and development of newborn offspring [1,2]. However, the functions of milk are highly conserved, its composition is dynamic and variable, and differs substantially not only between but also within mammalian species [3]. Many factors may be associated with this variation, including maternal diet, the environment, and potentially genetic factors [4,5]. Despite these variations, milk is a complex fluid containing common molecules, including proteins, easily digestible fats, carbohydrates, vitamins, immunomodulatory factors, as well as viable cells [6]. Although so far the majority of studies have focused on bioactive molecules in milk, little is known about milk cell properties and origin [7]. It is only recently that researchers have begun studying the nature and features of milk cells. Particularly, it was found that milk contains a heterogeneous cellular composition characterized by epithelial cells (lactocytes and myoepithelial cells) other than leukocytes and immune cells [7,8]. In addition, very interesting findings suggest the presence of stem cells in human milk [9]. Particularly, stem and differentiated cells from the lactating epithelium could enter in milk through cell

Departments of [1]Medical, Oral and Biotechnological Sciences, and [2]Medicine and Aging Science, ''G. d'Annunzio'' University Chieti-Pescara, Centro di Scienze dell'Invecchiamento e Medicina Traslazionale (Ce.S.I.-MeT), StemTeCh Group, Chieti, Italy.
[3]Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy.
*These authors contributed equally to this work.

migration and/or as a consequence of the mechanical shear forces during breastfeeding [2]. One should point out that, based on the capability of the mammary gland epithelium to expand and regress during adult life, the existence of mammary stem cells (MaSCs) was hypothesized since 1970s [10]. A small number of MaSCs exists in a quiescent state in this organ and it is only activated during pregnancy and breastfeeding to proliferate and differentiate by hormonal signals [11,12]. Interestingly, multipotent MaSCs able to self-renew and differentiate in the two main mammary epithelial cells were found in both mammary gland [13–16] and human milk [17–19].

Subsequently, Hassiotou et al. [2] have identified, for the first time, a novel stem cell population in human milk, named human breastmilk stem cells (hBSCs) expressing embryonic stem cell (ESC)-associated gene, including POU class 5 homeobox 1 (OCT4), sex-determining region Y-box 2 (SOX2), Nanog homeobox, and Kruppel-like factor 4 (KLF4), with multilineage differentiation potential.

Following the discovery of hBSCs, which highlighted breastmilk as a novel non-invasive source of multipotent stem cells potentially useful for regenerative medicine, nowadays the attention is focused on the study of milk composition in other mammalian species such as the bovine.

In this context, Martignani et al. [20] have demonstrated the presence of bovine MaSCs in the mammary gland able to regenerate cells secreting milk when transplanted in mice, and Cravero et al. [21] have demonstrated that adult stem cells reside in the bovine mammary gland and possess regenerative potential. Moreover, an interesting study recently describes bovine milk as a source of extracellular vesicles (EVs) released by cells into the extracellular environment and able to improve in vitro the osteoblast differentiation of human mesenchymal stem cells [22]. This finding would be of great importance, since EVs represent a reasonable cell-free approach alternative to cell therapy in tissue regeneration or as potential biomarkers and delivery systems for active molecules [23,24].

Thus, the purpose of our study was to isolate and characterize a potential stem cell-like population from bovine milk stem cells (bMSCs). In detail, we evaluated the plastic adherence when maintained in vitro, the expression of specific epithelial and mesenchymal markers, and the capability to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [25]. Moreover, we were able to identify EVs from bMSCs showing positive expression for the same markers of bMSCs.

Materials and Methods

Bovine milk sample collection

Bovine milk samples (15 samples, 500 mL/sample) were collected from animals during standard milking procedures. All samples were collected at mid-lactation (day 90–120).

In detail, milk was collected in farm of dairy cows routinely milked for milk processing and distribution, and this did not require an ethics statement. Moreover, the milk collection from the dairy cows is normed by specific conventions subscribed between the Faculty of Veterinary Medicine of Teramo and the ASL, organ of the Ministry of Health vocated to the control and safety of dairy farms. In fact, in each dairy farm, the collection of milk is controlled by an inspector, which is a Veterinary in charge of following the regularity of all the procedures.

Bovine milk samples were transported immediately to the laboratory under aseptic condition and preserved for maximum 12 h at room temperature (RT) before being processed. Of note, milk collection was made using a specific protocol to reduce the microbial contamination. Briefly, after washing and disinfection of the bovine mammary gland with ethanol 70%, the first milk jets were discarded and milk samples were collected in sterile bottles added with 1% penicillin/streptomycin and 1% amphotericin B (Sigma-Aldrich, St. Louis, MI).

Bovine milk cell isolation and culture

Bovine milk was diluted with sterile phosphate-buffered saline (PBS) (Sigma-Aldrich) in ratio 1:1 to minimize lipids on pellet formation and then centrifuged at 1,400 rpm for 15 min at RT. In detail, the supernatant and the fat layer were discarded and the cellular pellet washed twice in wash solution composed of sterile sodium chloride with 1% penicillin/streptomycin and 1% amphotericin B. Finally, the cells were suspended in a standard medium composed of Minimum Essential Medium Alpha (α-MEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco-Life Technologies, Monza, Italy), 1% penicillin/streptomycin, and 1% L-glutamine, and grown under a controlled atmosphere (5% CO_2 and 37°C). The medium was changed every 3 days and after ~7–10 days, the cultures had reached 70%–80% confluence. The cells were expanded at 1:3 dilution using Trypsin-EDTA (2.5X) (Sigma-Aldrich). All experiments were performed using cells between passages 3–6.

Flow cytometry

Cell staining and acquisition for flow cytometry. Isolated bovine milk cells (15 samples) were stained for their phenotype analysis. Briefly, the staining for surface antigens was performed by incubating 5×10^5 cells/sample with primary antibody against CD73 (1:50, polyclonal rabbit anti-CD73 antibody; Antibodies-online), CD90 (1:50, polyclonal rabbit anti-CD90 antibody; Antibodies-online), CD105 (1:200, monoclonal anti-CD105 antibody; ThermoFisher Scientific), and E-cadherin (1:50, monoclonal anti-E-cadherin antibody; Cell Signaling Technology) for 30 min on ice, followed by staining the samples with the appropriate Alexa488-labeled secondary antibody (1:1,500; Jackson ImmunoResearch Laboratories) for 30 min on ice.

The staining of intracellular antigens was carried out as previously described [26]. Briefly, a previous step of membrane fixation and permeabilization using FACS Lysing and Permeabilizing Solution (BD Bioscience, Milano, Italy) was performed. SOX2 (1:100, monoclonal rabbit anti-SOX2; Cell Signaling Technology), OCT4 (1:50, polyclonal anti-OCT4 antibody; Abnova), cytokeratin-14 (CK14; 1:10, polyclonal rabbit anti-CK14; My Biosource), cytokeratin-18 (CK18) fluorescein isothiocyanate (FITC)-conjugated (1:10, monoclonal anti-CK18 antibody; Antibodies-online), cytokeratin-19 (CK19; 1:100, polyclonal rat anti-KRT19; Acris), or smooth muscle actin (SMA; 1:100, rabbit anti-SMA; My Biosource) was added to each tube together

with DRAQ5 (0.2:100), used as nuclear marker. The samples were incubated for 30 min on ice, followed by staining with the appropriate Alexa488-labeled secondary antibody (1:1,500; Jackson ImmunoResearch Laboratories) for 30 min on ice.

1×10^4 events/sample were acquired by flow cytometry (FACS Canto II or FACSVerse; BD Biosciences). Samples were analyzed by FACSuite v 1.0.5 (BD Biosciences) software.

Extracellular vesicles (EVs) staining and acquisition for flow cytometry. The identification and analysis of bMSC-derived EVs were carried out as previously described [26]. Briefly, samples were stained by the same above described antibodies used for bMSC phenotype. After 30 min of staining (4°C in the dark), 1×10^6 events/sample were acquired by ImageStream (40× objective; AMNIS). The threshold was placed on the FITC channel, while, to avoid EVs loss, no threshold on morphological parameters were applied. EV morphological properties were confirmed by running Megamix Plus-Side Scatter (SSC) and Forward Scatter beads (Biocytex, Marseille, France) at the same photomultiplier voltages used for EVs detection.

Flow cytometry and ImageStream data analysis. All samples were analyzed by FACSuite v 1.0.5 software (BD Biosciences). To evaluate not specific fluorescence, secondary antibody-alone controls were used.

Data were expressed as percentage of positivity, and as mean fluorescence intensity (MFI) ratio. The MFI ratio was calculated by dividing the MFI of positive events by the MFI of negative events (MFI of secondary antibody). Each antibody/reagent was titrated (eight point titration) under assay conditions. Instrument performances and data reproducibility were sustained and checked by the Cytometer Setup and Tracking Module (BD Biosciences).

Imaging flow cytometry (ImageStream by using IDEAS software; AMNIS) was used to determine cytoplasm-nucleus translocation of OCT4 and SOX2. Secondary antibody alone controls were used to correctly set the gating strategy.

Multipotency assays

Bovine milk cells were plated at the density of $\sim$3,000/cm^2 in six-well plates to evaluate the multipotency capability, following the protocol already used for mesenchymal stem cells from other sources [27–29].

For osteogenic differentiation bovine milk cells were treated for up to 21 days with an osteogenic medium (OM) containing Dulbecco's modified Eagle's medium low glucose (DMEM-LG), 0.05 mM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma-Aldrich) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. The OM was replaced every 3 days and calcium deposits were finally visualized and quantified by Alizarin Red S staining.

For chondrogenic differentiation, bovine milk cells were treated for up to 21 days with a chondrogenic medium (CM) composed of DMEM high glucose (DMEM-HG) supplemented with 1% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 100 nM dexamethasone, 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X; Invitrogen), 50 μg/mL ascorbate-2 phosphate, 1 mM sodium pyruvate (Sigma-Aldrich), and 10 ng/mL of TGF-β1 (PeproTech EC Ltd., London, UK). The CM was replaced every 3 days and glycosaminoglycan (GAG) production was assessed by Alcian Blue staining.

To induce adipogenic differentiation, bovine milk cells were cultured with an adipogenic medium (AM) composed of DMEM-HG supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 100 μM indomethacin (all from Sigma-Aldrich), and 10 μg/mL insulin (Eli Lilly, HumanLoh 100 U/mL; Nederland). The AM was replaced every 3 days for 4 weeks. Cells were then stained with Oil Red O to identify the presence of lipid droplets.

Alizarin Red S staining. Osteogenic differentiation of bovine milk cells was assessed by Alizarin Red staining (Sigma-Aldrich) using an established protocol [28,30]. In detail, after two washes with PBS, cells were fixed in 10% formaldehyde and then stained with 40 mM of Alizarin Red S solution (pH 4.2) for 20 min at RT to detect and quantify the calcium deposits.

Alcian Blue staining. To identify the GAG, the Alcian Blue staining (Sigma-Aldrich) was performed as already established [29]. Briefly, cells were fixed in cold methanol for 5 min and then incubated in 1% Alcian Blue (pH 2.5) solution for 30 min at RT. After rinsing three washes with deionized water for 2 min, accumulation cells were incubated in 1% sodium dodecyl sulfate solution for 30 min to quantify GAG. Absorbance of the solubilized solution was measured at 605 nm using a microplate spectrophotometer (Spectramax SM190).

Oil Red O staining. Bovine milk cells were washed twice with PBS and fixed with formaldehyde for 10 min at RT. The fixed cells were incubated for 1 h in freshly diluted Oil Red O solution (Sigma-Aldrich), prepared by mixing six parts of Oil Red O stock solution (0.5% Oil Red O in isopropanol) and four parts of distilled water.

RNA isolation and quantitative reverse transcription-PCR

The total RNA was isolated from bovine milk using Trizol® reagent (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA concentration and quality were evaluated using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific).

For reverse transcription, the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) was used.

The equivalent of 0.1 μg of cDNA was used for the reactions of quantitative reverse transcription-PCR (qRT-PCR) carried out using TaqMan Universal Master Mix II (Thermofisher Scientific) and commercially available TaqMan Gene Expression Assay: bovine alkaline phosphatase (*ALP*, Bt03244508_m1); bovine osteopontin (*OPN*, Bt03213107_m1); bovine collagen type I alpha I (*COL1A1*, Bt01463861_g1); bovine collagen type II alpha I (*COL2A1*, Bt03251861_m1); bovine peroxisome proliferator-activated receptor gamma (*PPAR-γ*, Bt03217547_m1); bovine fatty acid binding protein 4 (*FABP4*, Bt03213820_m1); and bovine glyceraldehyde-3-phosphate dehydrogenase (Bt03210913_g1). The gene expression levels were analyzed with ABI Prism 7900 Sequence Detection System (ThermoFisher Scientific). The relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed by the unpaired Student's

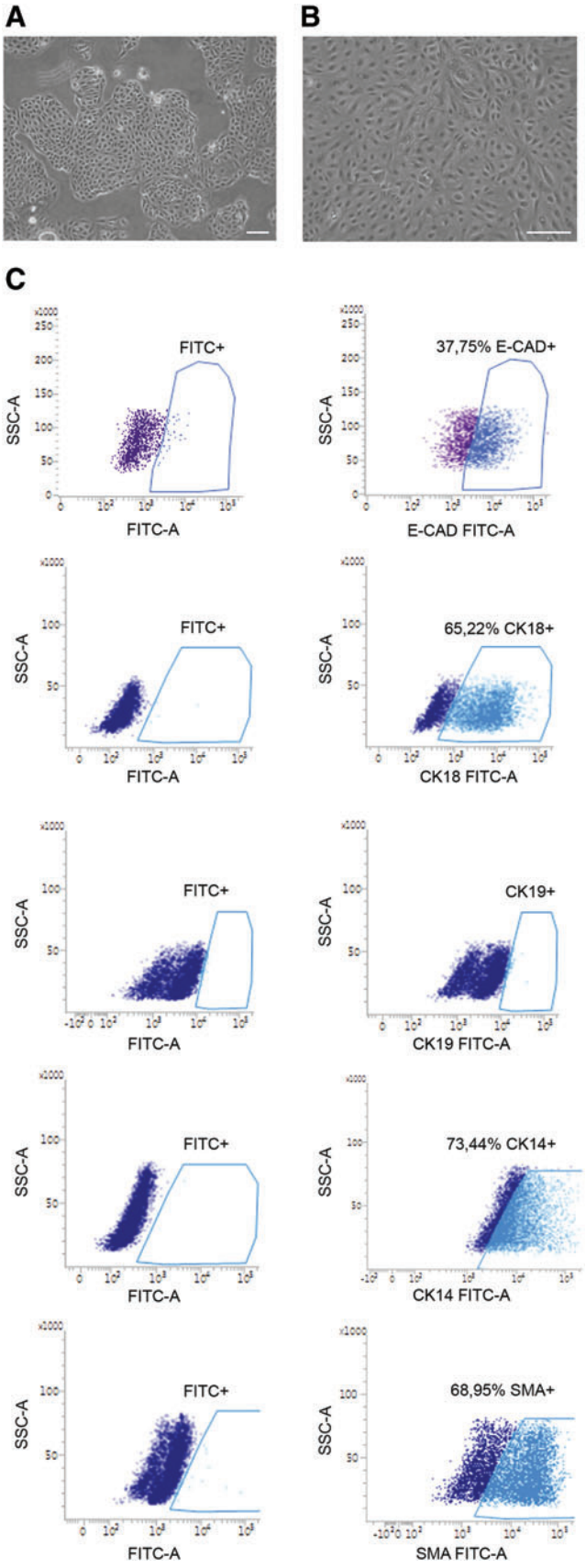

FIG. 1. Morphological analysis and phenotype of the bovine milk cell population. Representative phase-contrast images of cells derived from bovine milk with **(A)** epithelial- (initially culture) and **(B)** fibroblast-like morphology (third passage). Scale bar: 100 µm. **(C)** Representative dot plots show the expression of E-cadherin (FITC-conjugated), CK18 (FITC-conjugated), CK19, CK14, and SMA. Gates ($FITC^+$) were drawn on the basis of the respective secondary antibody-alone sample. CK14, cytokeratin-14; CK18, cytokeratin-18; CK19, cytokeratin-19; FITC, fluorescein isothiocyanate; SMA, smooth muscle actin. Color images available online at www.liebertpub.com/scd

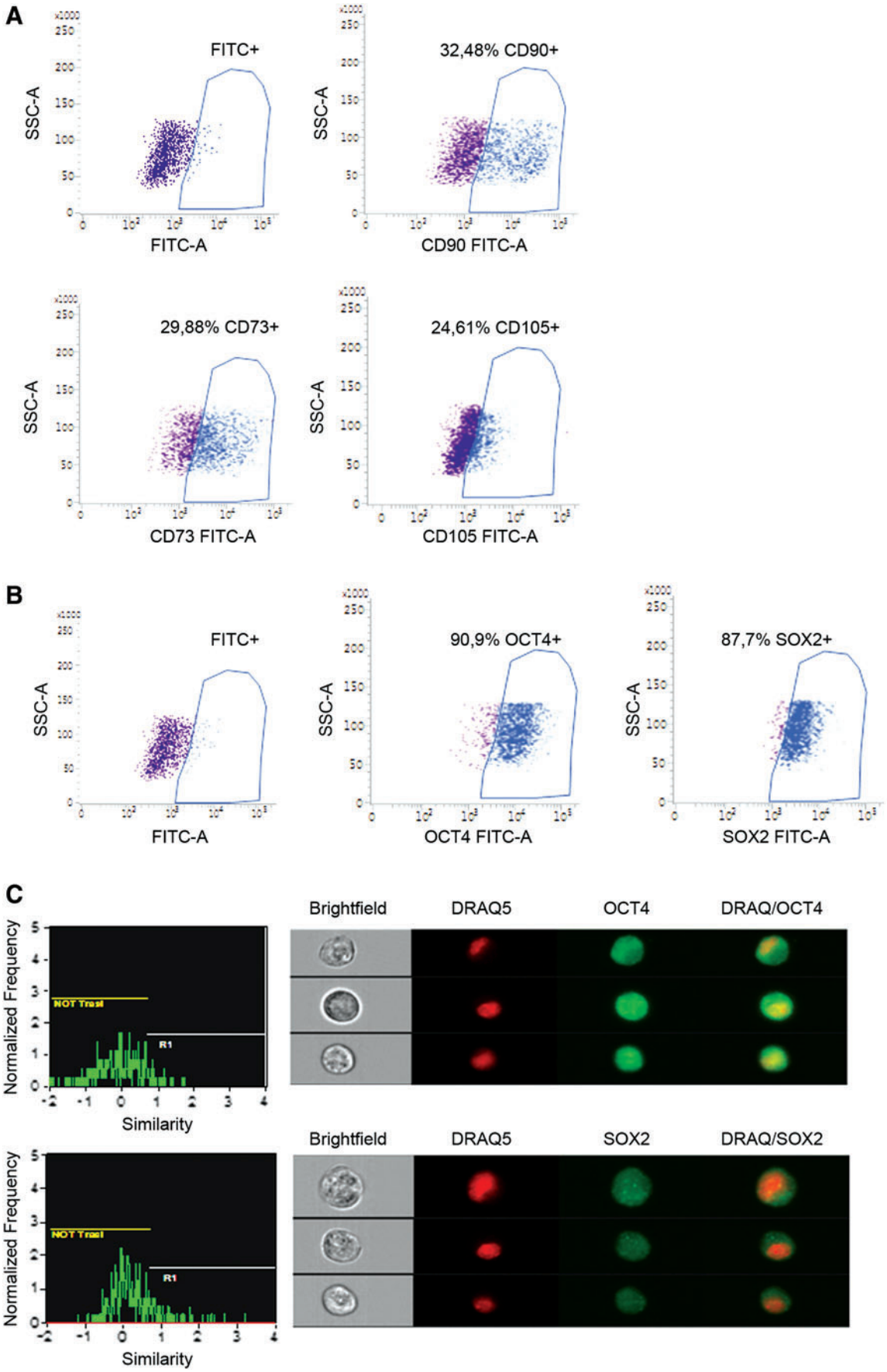

FIG. 2. Stem cell-like phenotype of the bovine milk cell population. **(A)** Representative dot plots show the surface expression of CD90 (FITC conjugated), CD73. and CD105. **(B)** Representative dot plots show the intracellular expression of FITC-conjugated OCT4 and SOX2. Gates ($FITC^+$) were drawn on the basis of the respective secondary antibody-alone sample. **(C)** Representative single-cell images of OCT4 and SOX2 nuclear localization, obtained by ImageStream. Color images available online at www.liebertpub.com/scd

t-test. All experiments were performed employing 15 cellular strains obtained from 15 bovine milk samples. Each experiment was performed using at least three different cellular strains ($n=3$) and in technical duplicate or triplicate. A P value <0.05 was considered statistically significant.

Results

Bovine milk cell phenotype

Cells from bovine milk samples ($n=15$) were harvested as widely described in Materials and methods section. Phase-contrast light microscopy images (Fig. 1A) revealed that adherent bovine milk cells showed initially a mainly round/polyhedral epithelial-like morphology (E-like morphology). Moreover, following two additional passages, bovine milk cells also exhibited (Fig. 1B) a subpopulation with an elongated fibroblast-like morphology (F-like morphology; star-shaped with long cytoplasmic processes) typical of mesenchymal stem cells.

In agreement with these results, flow cytometry analysis (Fig. 1C) confirmed that 57%–60% of bovine milk live cell fraction (identified on the basis of their scatter parameters) at passage 3 expressed the typical epithelial markers, including E-cadherin, CK18, CK14, and SMA. On the other hand, CK19 resulted negative. Interestingly, a positive cellular fraction (30%–40%) for the typical mesenchymal surface antigens CD90, CD73, and CD105 (Fig. 2A) was found. Moreover, this subset was also positive for the intracellular stemness markers OCT4 and SOX2 (Fig. 2B), mainly localized into the nucleus (Fig. 2C), as confirmed by ImageStream AMNIS analysis.

Altogether, these evidence suggest that bovine milk contains a stem cell-like population named bMSCs.

Multilineage differentiation potential of bMSCs

bMSCs were analyzed for their capability to commit into osteogenic, chondrogenic, and adipogenic lineages induced by specific differentiation media.

As shown in Fig. 3, after 21 days of osteogenic differentiation, bMSCs changed their morphology with formation of cellular aggregates and calcified nodules as revealed by Alizarin Red S staining (Fig. 3A). A significant increase of mineral matrix deposition was also observed (Fig. 3B). The bMSCs osteogenic ability was further validated by analyzing

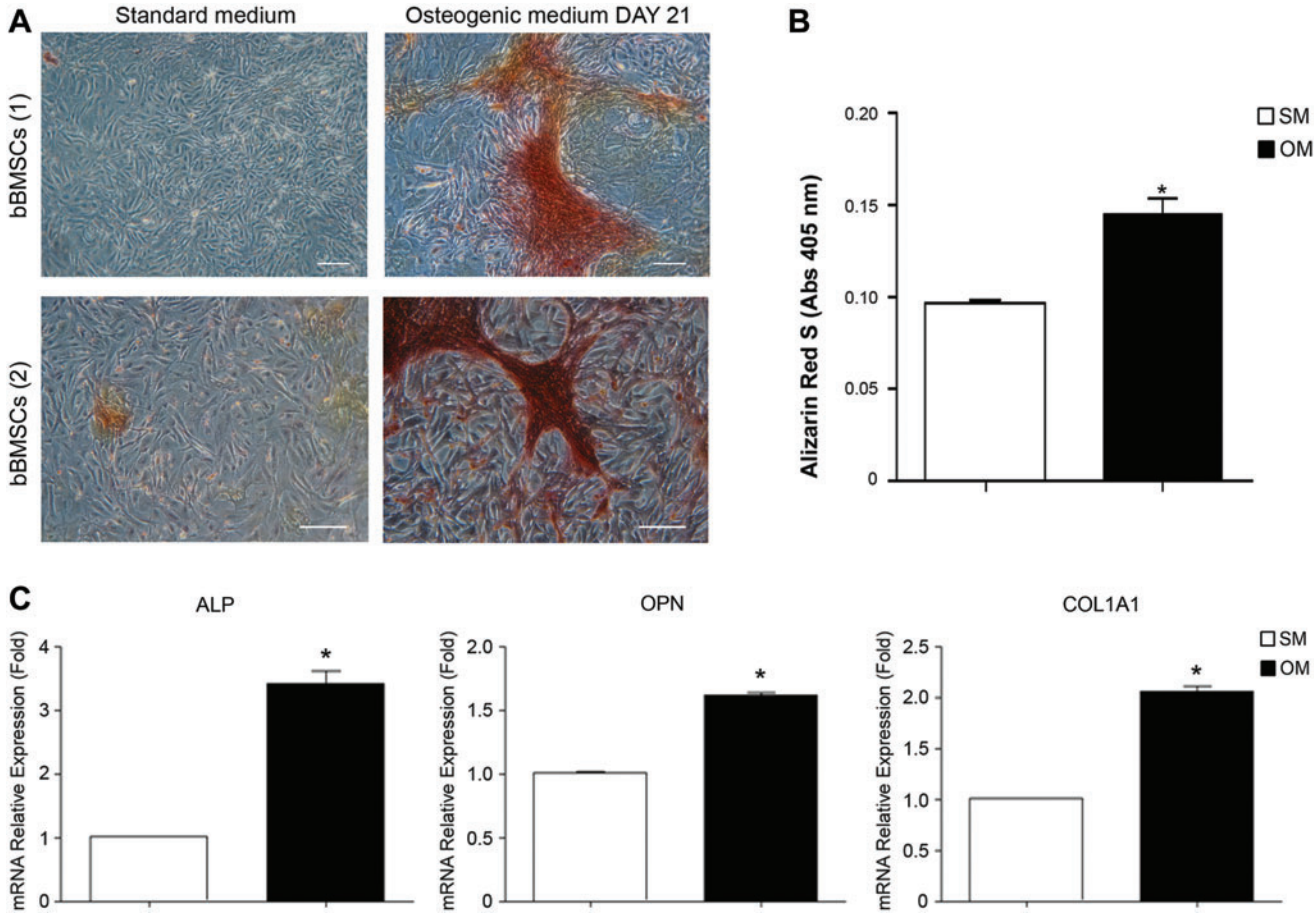

FIG. 3. Osteogenic differentiation of bMSCs. **(A)** Representative images and **(B)** quantification of Alizarin Red staining carried out after 21 days of osteogenic differentiation. **(C)** qRT-PCR analysis of the typical osteogenic markers *ALP*, *OPN*, and *COL1A1* shows increased mRNA levels in differentiated bMSCs compared to undifferentiated cells. For all experiments, results are expressed as mean ± SD of at least three independent experiments ($n=3$) (*$P<0.05$ vs. SM). *ALP*, alkaline phosphatase; bMSCs, bovine milk stem cells; *COL1A1*, collagen type II alpha I; mRNA, messenger RNA; OM, osteogenic medium; *OPN*, osteopontin; qRT-PCR, quantitative reverse transcription-PCR; SD, standard deviation; SM, standard medium. Color images available online at www.liebertpub.com/scd

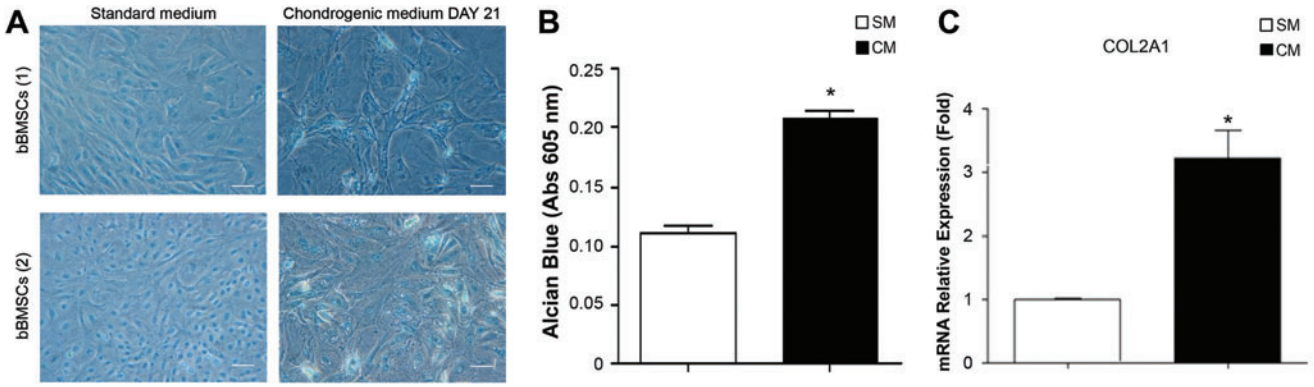

FIG. 4. Chondrogenic differentiation of bMSCs. **(A)** Alcian Blue staining of two different bMSC samples (1 and 2) following 21 days of chondrogenic differentiation. **(B)** Relative quantification of Alcian Blue staining. **(C)** qRT-PCR analysis of the specific chondrogenic marker *COL2A1* shows increased mRNA level in differentiated bMSCs compared to undifferentiated cells. For all experiments, results are mean ± SD of at least three independent experiments ($n = 3$) (*$P < 0.05$ vs. SM). Color images available online at www.liebertpub.com/scd

the expression of typical osteogenic markers. Following 21 days of osteogenic induction, the bMSCs showed significant upregulation of *ALP*, *OPN*, and *COL1A1* messenger RNA (mRNA) expression level (Fig. 3C).

Under chondrogenic conditions for 21 days, an increase of GAGs was revealed as shown by representative images (Fig. 4A) and quantification of Alcian Blue staining (Fig. 4B). The chondrogenic differentiation was also confirmed by increased *COL2A1* mRNA expression level, a typical chondrogenic marker (Fig. 4C).

Moreover, following 28 days of adipogenic treatment, bMSCs showed a morphological change and formation of

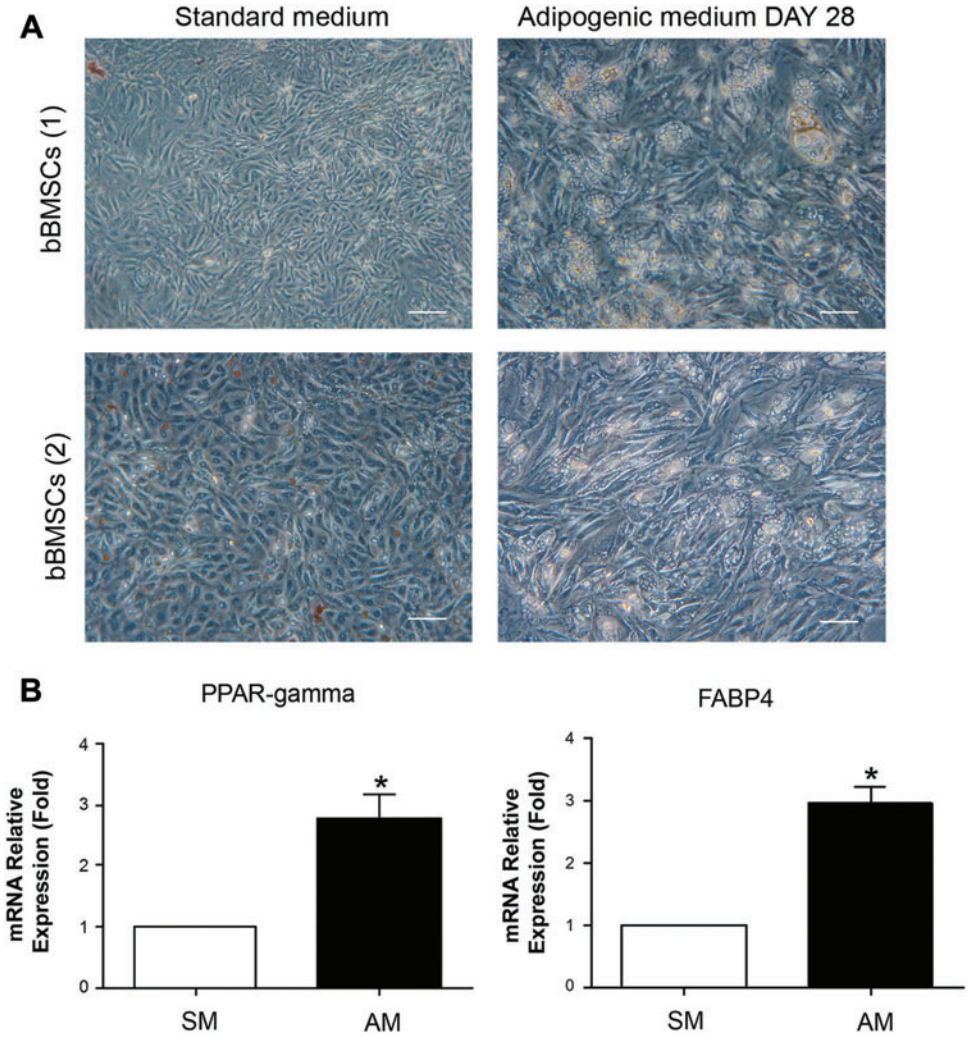

FIG. 5. Adipogenic differentiation of bMSCs. **(A)** Lipid droplet accumulation in two different representative experiments of bMSCs (1 and 2) on day 28 of adipogenic differentiation. **(B)** qRT-PCR analysis of the adipogenic markers *PPAR-γ* and *FABP4* shows increased mRNA levels in differentiated bMSCs compared to undifferentiated cells. Results are mean ± SD of at least three independent experiments ($n = 3$) (*$P < 0.05$ vs. SM). *FABP4*, fatty acid binding protein 4; *PPAR-γ*, peroxisome proliferator-activated receptor gamma. Color images available online at www.liebertpub.com/scd

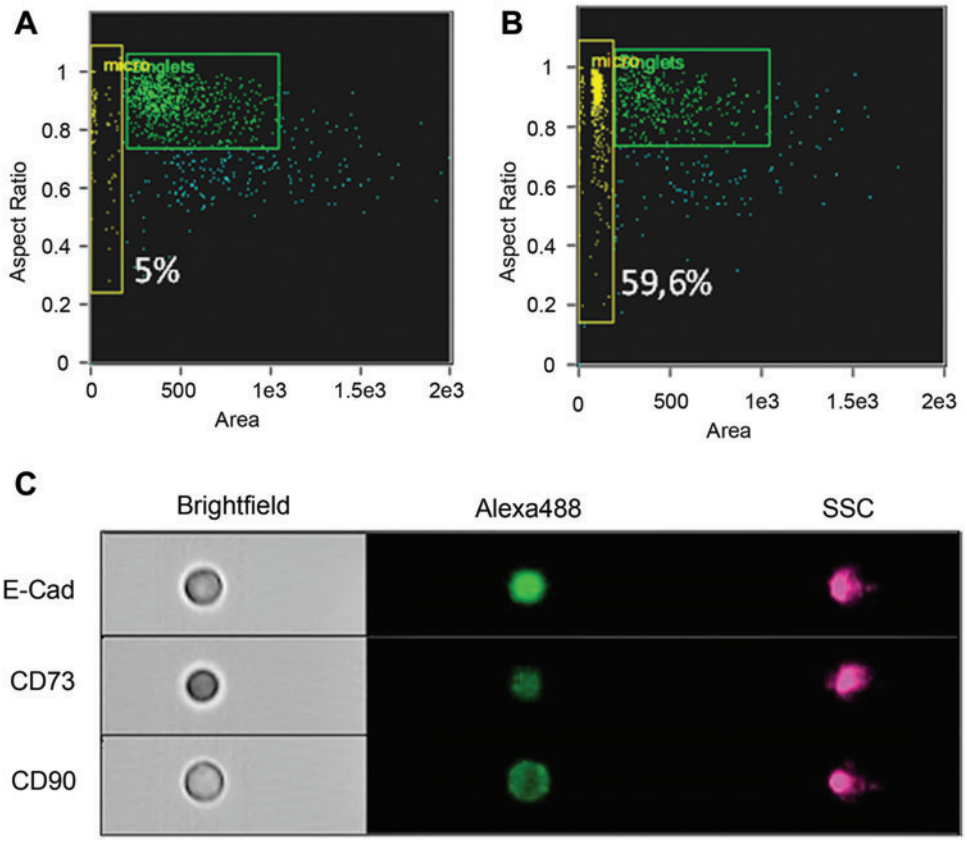

FIG. 6. Milk cell-derived extracellular vesicles. **(A, B)** ImageStream analysis of morphological parameters for two different milk cell clones. A subset of events characterized by scatter values (extracellular vesicle gate) lower than that identified for cells (singlets gate) has been identified in all analyzed samples. **(C)** Events in the extracellular vesicle gate display round shapes (bright field images) and express E-cadherin, CD73, and CD90 (*green staining*). Gates were placed on the basis of the respective secondary antibody-alone sample. Color images available online at www.liebertpub.com/scd

multiple vacuoles (Fig. 5A) associated with a significant increase in *PPAR-γ* and *FABP4* mRNA levels (Fig. 5B). Nevertheless, although a morphological change was evident, no significant difference was found after Oil Red O staining (Fig. 5A), indicating that bMSCs did not reach a fully differentiated stage of maturity, due to a possible blockage in adipocyte maturation.

Identification of milk cell-derived EVs

The flow cytometry analysis of bovine milk cells revealed the presence of a subpopulation of events, characterized by scatter parameters values lower than that measured for cells falling in the 0.1–1 μm region size, as measured by MegaMix plus bead running. The percentage of this subset of events changed depending on cell clone (5%–60%), as demonstrated by the ImageStream analysis (Fig. 6A, B). As shown in Fig. 6C, such events do not contain nuclei and result positive for the same markers identified on the surface of milk cells (E-cadherin, CD73, and CD90); thus, they may be considered milk cell-derived EVs.

Discussion

The emerging field of regenerative medicine has intensified the demand for novel advantageous sources of stem cells. Some studies have described the presence of stem/progenitor cells in the mammary gland [31–34] and more recently in breastmilk [2]. Overall, milk is undoubtedly a dynamic and heterogeneous natural source of cells that has gained interest in the last years [2,35]. Interestingly, Hassiotou et al. [2] have demonstrated that human breastmilk, cultured in pluripotent stem cell conditions, expresses typical markers found in ESCs. Moreover, multipotent stem cells with potential use in regenerative medicine have been isolated from human milk [2,9,17,18,35–37]. These findings, although the somatic cells from cow milk may present a composition very different from the human one [38], support the hypothesis that similar stem cells may exist also in bovine milk.

To date, official methods for the isolation and characterization of stem cells from bovine milk do not exist. Thus, this study is intended to establish how to efficiently obtain, expand and characterize them. Interestingly, in this study, we have shown that cells from bovine milk can be easily isolated through a noninvasive method and their proliferation can be maintained up to many passages.

In particular, the microscope analysis revealed that cells isolated from bovine milk are able to grow in plastic adherence and represent a heterogeneous population containing both epithelial- and fibroblast-like cells (Fig. 1). Indeed, according to Baratta et al. [23], the isolated cells mainly showed an epithelial-like morphology, thus indicating a presumable origin of the majority of cells from the lactating epithelium. However, in agreement with findings of Kaingade et al. in human cells [39], after two additional passages, the bovine milk cells also showed a subpopulation with typical fibroblast-like mesenchymal stem cell morphology, characterized by star shape with long cytoplasmic processes [40]. Although concern was initially expressed about the possibility of finding bovine-specific antibodies, we were able to characterize the cells by their expression of

a specific panel of markers. From the analysis of their immunophenotype, bovine milk cells resulted positive for the epithelial markers E-cadherin and CK18 (Fig. 1C), thus confirming the microscope analysis (Fig. 1A). Moreover, our cells collected at mid-lactation were positive for CK14 and SMA (Fig. 1C) [23,41]. However, bovine milk cells resulted negative for CK19, therefore suggesting their composition based on the two epithelial cell populations originating from luminal and myoepithelial (basal) compartments [6,42–44].

These findings, in agreement with those previously described by Baratta et al. [23] in the alive milk cell population, represent original data concerning the composition of somatic cells in cow's milk. As reported by Boutinaud and Jammes [38], epithelial cells are the principal cells in human secretion, while macrophages represent the predominant cell type (35%–79%) in cow's milk, acting as sentinels against the mastitis caused by pathogens. However, although the analysis of somatic cells in bovine milk denotes an interesting point to investigate, herein we had the purpose to isolate and characterize a stem cell-like population potentially useful in veterinary regenerative medicine. Hence, for this study, we did not use directly the bovine milk, but its isolated cells cultured up to the third passage. It is therefore reasonable to assume that the epithelial cell percentage we found in the isolated cultured cells is higher compare to the one found in whole milk [38], probably due to the in vitro plastic adherence and passages in the culture of cells.

Consequently, the use of isolated cultured cells could justify the discrepancy with previous controversial results found in whole milk [45,46] and the absence of immune cells that, besides confirming the state of health of the tissue, may have been lost during in vitro culture passages. Of note, our data are in line with studies on cells isolated from human milk cultured for several passages [6,9], showing initially heterogeneous population and with homogeneous mesenchymal stem cells upon several passages, possible due to epithelial-mesenchymal transition (EMT). Hence, we can postulate that in our investigation, the mesenchymal stem cells may grow through EMT, in which they express both epithelial and mesenchymal markers.

Furthermore, for the first time, a cellular fraction (30%–40%) positive for the typical mesenchymal and stem cell markers CD90, CD73, CD105, OCT4, and SOX2 (Fig. 2) was also found and the same amount of cells staining positive for the intracellular stemness markers OCT4 and SOX2 also presented their nuclear localization, indicating their functionality (Fig. 2C). Notably, in agreement with a previous work [8,17], these results indicate that bovine milk could represent a source of a putative stem cell-like population that, in this study, we named bMSCs. These findings are further supported by interesting data on human milk cells demonstrating the presence of cells with positive expression of hematopoietic, mesenchymal, and neuroepithelial markers such as CD133, Stro-1, and nestin, respectively [8].

Based on our previous data obtained using heterogenous (E- and F-like morphology) human amniotic fluid mesenchymal stem cells [29], we decided to culture the entire cell population obtained from bovine milk, thus avoiding any cell manipulations, like cell sorting for a specific marker. In detail, we evaluated their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. Interestingly, bMSCs displayed a high differentiation capability toward osteogenic and chondrogenic lineages. Regarding the adipogenic differentiation, although bMSCs did not show a specific Oil Red O staining, we found morphological changes such as the appearance of large vacuoles. In addition, the expression of the adipose-specific genes *PPAR-γ* and *FABP4* was significantly induced throughout adipogenic differentiation. So far, the adipogenic commitment of milk stem cells has been demonstrated only from cells isolated from human breastmilk [9]. In particular, Hassiotou and co-authors have demonstrated that these cells were able to differentiate into adipocytes if cultured in pluripotent stem cell conditions [2]. The poor Oil Red O staining obtained in this study is possible due to the heterogeneous population not able to reach a full adipogenic commitment. Therefore, it is possible that a selection of a more pluripotent stem cell population from bovine milk is needed to achieve a full terminal adipogenic differentiation. However, as previously cited, in this study, we decided to culture the entire milk cell population without any selection and manipulation, to characterize the cells derived from the native environment. A minimal manipulation is of clinical importance for potential application in veterinary tissue regeneration [29,47].

Today, it is well known that the stem cell capability to promote tissue regeneration is explained also through the release of EVs [48]. In this regard, a previous study demonstrated the presence of EVs in bovine colostrum and mature milk [49]. As their potential role in health and disease is drawing increasing attention, in this study, we evidenced EVs derived from bMSCs, which interestingly displayed the same antigenic pattern of the respective parental cells (E-cadherin, CD73, and CD90). These data clearly suggest the potential use of EVs for in vitro and in vivo application in functional studies. Their isolation in veterinary may aid diagnosis in the future, providing a new clinical tool to treat diseases and potential biomarkers for quality control of milk. An in-depth study of EVs will help to indentify particular subtypes of interest and explore their specific roles in cell to cell communication and in milk production.

Overall, we have demonstrated, for the first time, that a stem cell-like population can be isolated from bovine milk, further validating previous studies in the human counterpart [2,6,8,9]. This outcome opens new avenues for the veterinary use of milk as an innovative, noninvasive source of multipotent stem cells, which may have a potential role in regenerative medicine in veterinary field. In particular, milk stem/progenitor cells could be used to treat specific diseases or in the repair of damaged tissues, such as postmastitis mammary tissue injury [50], which affect milk production and quality. In addition, bMSCs and their derived EVs may be also used as a diagnostic tool to monitor and evaluate aging and functionality of the breast that can be compromised by infectious processes.

In conclusion, this study could potentially offer techniques to restore milk production, to manipulate lactation yield, and for regenerative medicine with minimal risks of rejection and side effects.

Acknowledgments

The authors thank Prof. Roberta Piva for her valuable suggestions during the study. They also thank Giuseppina Bologna for technical assistance and Stem Cell Research (SCR) Italy for the Award 2017 to D.M.

Author Disclosure Statement

No competing financial interests exist.

References

1. Hinde K and JB German. (2012). Food in an evolutionary context: insights from mother's milk. J Sci Food Agric 92: 2219–2223.
2. Hassiotou F, A Beltran, E Chetwynd, AM Stuebe, AJ Twigger, P Metzger, N Trengove, CT Lai, L Filgueira, P Blancafort and PE Hartmann. (2012). Breastmilk is a novel source of stem cells with multilineage differentiation potential. Stem Cells 30:2164–2174.
3. Khan S, AR Hepworth, DK Prime, CT Lai, NJ Trengove and PE Hartmann. (2013). Variation in fat, lactose, and protein composition in breast milk over 24 hours: associations with infant feeding patterns. J Hum Lact 29:81–89.
4. Hassiotou F, AR Hepworth, TM Williams, AJ Twigger, S Perrella, CT Lai, L Filgueira, DT Geddes and PE Hartmann. (2013). Breastmilk cell and fat contents respond similarly to removal of breastmilk by the infant. PLoS One 8:e78232.
5. Butte NF, C Garza, JE Stuff, EO Smith and BL Nichols. (1984). Effect of maternal diet and body composition on lactational performance. Am J Clin Nutr 39:296–306.
6. Hassiotou F, DT Geddes and PE Hartmann. (2013). Cells in human milk: state of the science. J Hum Lact 29:171–182.
7. Hassiotou F and PE Hartmann. (2014). At the dawn of a new discovery: the potential of breast milk stem cells. Adv Nutr 5:770–778.
8. Fan Y, YS Chong, MA Choolani, MD Cregan and JK Chan. (2010). Unravelling the mystery of stem/progenitor cells in human breast milk. PLoS One 5:e14421.
9. Patki S, S Kadam, V Chandra and R Bhonde. (2010). Human breast milk is a rich source of multipotent mesenchymal stem cells. Hum Cell 23:35–40.
10. Taylor-Papadimitriou J, M Shearer and MG Stoker. (1977). Growth requirements of human mammary epithelial cells in culture. Int J Cancer 20:903–908.
11. Pang WW and PE Hartmann. (2007). Initiation of human lactation: secretory differentiation and secretory activation. J Mammary Gland Biol Neoplasia 12:211–221.
12. Asselin-Labat ML, F Vaillant, JM Sheridan, B Pal, D Wu, ER Simpson, H Yasuda, GK Smyth, TJ Martin, GJ Lindeman and JE Visvader. (2010). Control of mammary stem cell function by steroid hormone signalling. Nature 465:798–802.
13. Dontu G, WM Abdallah, JM Foley, KW Jackson, MF Clarke, MJ Kawamura and MS Wicha. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17:1253–1270.
14. Shackleton M, F Vaillant, KJ Simpson, J Stingl, GK Smyth, ML Asselin-Labat, L Wu, GJ Lindeman and JE Visvader. (2006). Generation of a functional mammary gland from a single stem cell. Nature 439:84–88.
15. Villadsen R, AJ Fridriksdottir, L Rønnov-Jessen, T Gudjonsson, F Rank, MA LaBarge, MJ Bissell and OW Petersen. (2007). Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol 177:87–101.
16. Visvader JE. (2009). Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes Dev 23:2563–2577.
17. Cregan MD, Y Fan, A Appelbee, ML Brown, B Klopcic, J Koppen, LR Mitoulas, KM Piper, MA Choolani, YS Chong and PE Hartmann. (2007). Identification of nestin-positive putative mammary stem cells in human breastmilk. Cell Tissue Res 329:129–136.
18. Thomas E, N Zeps, M Cregan, P Hartmann and T Martin. (2011). 14-3-3sigma (sigma) regulates proliferation and differentiation of multipotent p63-positive cells isolated from human breastmilk. Cell Cycle 10:278–284.
19. Thomas E, T Lee-Pullen, P Rigby, P Hartmann, J Xu and N Zeps. (2012). Receptor activator of NF-κB ligand promotes proliferation of a putative mammary stem cell unique to the lactating epithelium. Stem Cells 30:1255–1264.
20. Martignani E, P Eirew, P Accornero, CJ Eaves and M Baratta. (2010). Human milk protein production in xenografts of genetically engineered bovine mammary epithelial stem cells. PLoS One 5:e13372.
21. Cravero D, E Martignani, S Miretti, E Macchi, P Accornero and M Baratta. (2014). Bovine mammary epithelial cells retain stem-like phenotype in long-term cultures. Res Vet Sci 97:367–375.
22. Oliveira MC, OJ Arntz, EN Blaney Davidson, PL van Lent, MI Koenders, PM van der Kraan, WB van den Berg, AV Ferreira and FA van de Loo. (2016). Milk extracellular vesicles accelerate osteoblastogenesis but impair bone matrix formation. J Nutr Biochem 30:74–84.
23. Baratta M, MG Volpe, D Nucera, G Gabai, N Guzzo, M Fustini and E Martignani. (2015). Differential expression of living mammary epithelial cell subpopulations in milk during lactation in dairy cows. J Dairy Sci 98:6897–6904.
24. Baratta M, S Miretti, P Accornero, G Galeati, A Formigoni, G Gabai, D Nucera and E Martignani. (2017). CD49f+ mammary epithelial cells decrease in milk from dairy cows stressed by overstocking during the dry period. J Dairy Res 84:414–417.
25. Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, Dj Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317.
26. Di Tomo P, P Lanuti, N Di Pietro, MPA Baldassarre, M Marchisio, A Pandolfi, A Consoli and G Formoso. (2017). Liraglutide mitigates TNF-alpha induced pro-atherogenic changes and microvesicle release in HUVEC from diabetic women. Diabetes Metab Res Rev 33.
27. Di Tomo P, C Pipino, P Lanuti, C Morabito, L Pierdomenico, V Sirolli, M Bonomini, S Miscia, MA Mariggiò, et al. (2013). Calcium sensing receptor expression in ovine amniotic fluid mesenchymal stem cells and the potential role of R-568 during osteogenic differentiation. PLoS One 8: e73816.
28. Pipino C, P Di Tomo, D Mandatori, E Cianci, P Lanuti, MB Cutrona, L Penolazzi, L Pierdomenico, E Lambertini, et al. (2014). Calcium sensing receptor activation by calcimimetic R-568 in human amniotic fluid mesenchymal stem cells: correlation with osteogenic differentiation. Stem Cells Dev 23:2959–2971.

29. Pipino C, L Pierdomenico, P Di Tomo, F Di Giuseppe, E Cianci, I D'Alimonte, C Morabito, L Centurione, I Antonucci, et al. (2015). Molecular and phenotypic characterization of human amniotic fluid-derived cells: a morphological and proteomic approach. Stem Cells Dev 24:1415–1428.
30. Gregory CA, WG Gunn, A Peister and DJ Prockop. (2004). An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. Anal Biochem 329:77–84.
31. Motyl T, JB Bierła, M Kozłowski, M Gajewska, B Gajkowska and M Koronkiewicz. (2011). Identification, quantification and transcriptional profile of potential stem cells in bovine mammary gland. Livest Sci 136:136–149.
32. Capuco AV. (2007). Identification of putative bovine mammary epithelial stem cells by their retention of labeled DNA strands. Exp Biol Med (Maywood) 232:1381–1390.
33. Capuco AV, CM Evock-Clover, A Minuti and DL Wood. (2009). In vivo expansion of the mammary stem/progenitor cell population by xanthosine infusion. Exp Biol Med (Maywood) 234:475–482.
34. Holland MS, MH Tai, JE Trosko, LD Griffin, JA Stasko, NC Cheville and RE Holland. (2003). Isolation and differentiation of bovine mammary gland progenitor cell populations. Am J Vet Res 64:396–403.
35. Twigger AJ, AR Hepworth, CT Lai, E Chetwynd, AM Stuebe, P Blancafort, PE Hartmann, DT Geddes and F Kakulas. (2015). Gene expression in breastmilk cells is associated with maternal and infant characteristics. Sci Rep 5:12933.
36. Kaingade PM, I Somasundaram, AB Nikam, SA Sarang and JS Patel. (2016). Breastmilk-derived mesenchymal stem cells in vitro are likely to be mediated through epithelial-mesenchymal transition. Breastfeed Med 11:152.
37. Kakulas F, DT Geddes and PE Hartmann. (2016). Breastmilk is unlikely to be a source of mesenchymal stem cells. Breastfeed Med 11:150–151.
38. Boutinaud M and H Jammes. (2002). Potential uses of milk epithelial cells: a review. Reprod Nutr Dev 42:133–147.
39. Kaingade PM, I Somasundaram, AB Nikam, SA Sarang and JS Patel. (2016). Assessment of growth factors secreted by human breastmilk mesenchymal stem cells. Breastfeed Med 11:26–31.
40. Rauner G and I Barash. (2012). Cell hierarchy and lineage commitment in the bovine mammary gland. PLoS One 7: e30113.
41. Martignani E, D Cravero, S Miretti, P Accornero and M Baratta. (2014). Bovine mammary stem cells: new perspective for dairy science. Vet Q 34:52–58.
42. Park HJ, WY Lee, HY Jeong and H Song. (2016). Regeneration of bovine mammary gland in immunodeficient mice by transplantation of bovine mammary epithelial cells mixed with matrigel. Int J Stem Cells 9:186–191.
43. Martignani E, D Cravero, S Miretti, P Accornero and M Baratta. (2015). Clonogenic assay allows for selection of a primitive mammary epithelial cell population in bovine. Exp Cell Res 338:245–250.
44. Rauner G and I Barash. (2016). Enrichment for repopulating cells and identification of differentiation markers in the bovine mammary gland. J Mammary Gland Biol Neoplasia 21:41–49.
45. Sarikaya H, C Werner-Misof, M Atzkern and RM Bruckmaier. (2005). Distribution of leucocyte populations, and milk composition, in milk fractions of healthy quarters in dairy cows. J Dairy Res 72:486–492.
46. Rainard P and C Riollet. (2006). Innate immunity of the bovine mammary gland. Vet Res 37:369–400.
47. Roubelakis MG, V Bitsika, D Zagoura, O Trohatou, KI Pappa, M Makridakis, A Antsaklis, A Vlahou and NP Anagnou. (2011). In vitro and in vivo properties of distinct populations of amniotic fluid mesenchymal progenitor cells. J Cell Mol Med 15:1896–1913.
48. Katsuda T, N Kosaka, F Takeshita and T Ochiya. (2013). The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. Proteomics 13:1637–1653.
49. Hata T, K Murakami, H Nakatani, Y Yamamoto, T Matsuda and N Aoki. (2010). Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs. Biochem Biophys Res Commun 396:528–533.
50. Sharma N and DK Jeong. (2013). Stem cell research: a novel boulevard towards improved bovine mastitis management. Int J Biol Sci 9:818–829.

Address correspondence to:
Prof. Assunta Pandolfi
Department of Medical, Oral and Biotechnological Sciences
''G. d'Annunzio'' University Chieti-Pescara
Centro di Scienze dell'Invecchiamento e Medicina Traslazionale (Ce.S.I.-MeT)
StemTeCh Group
Chieti 66100
Italy

E-mail: assunta.pandolfi@unich.it

Received for publication May 31, 2018
Accepted after revision August 23, 2018
Prepublished on Liebert Instant Online August 24, 2018