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ABSTRACT

Backgroud: we previously demonstrated that adult stem cells reside in the bovine mammary gland and possess an intrinsic regenerative potential. The detection and characterization of bovine mammary stem cells in vitro may give a better understanding of the cyclic characteristic of mammary gland development. The aim of this study is to further investigate on the features retain by mammary stem cell-like detected in long term culture.

Results: flow cytometry analysis showed different subpopulations of mammary epithelial cells emerging according to the timing of cell culture. $CD49f^+$-cells significantly increased along the culture ($p<0.01$) and similar trend was observed, even if less regular, for $CD29^+$ cells. No difference along the culture was observed for CD24 positive cells. Finally ALDH1 positive cell population greatly enhances ($p<0.05$) after 35 days of culture. Specific cell differentiation markers as cytokeratin 18 (CK18) and cytokeratin 14 (CK14) were expressed all along the experimental period. After 35 days of culture a subset of cells still retained regenerative capabilities was observed *in vivo* xenotransplants. These cells were able to form organized pseudo-alveoli when transplanted in immunodeficient mice as shown by the expression of CK14, CK18, p63 (a mammary basal cell layer marker) and Epithelial Cell Adhesion Molecule (EpCAM). Progenitor content was also analyzed and quantified in vitro through colony-forming cell (CFC) assays along the experimental period.

Conclusions: these results demonstrate the presence of multipotent cell subpopulation with intrinsic regenerative potential in *in vitro* system with presumable maintenance and expansion of primitive population of adult mammary stem cells. The possibility to investigate mammary stem cells in long term culture open interesting perspective in the study of the biology of mammary stem cell regulation in regenerative medicine.

Keywords: CD49, ALDH1, stem cell, cell differentiation, bovine, mammary gland

BACKGROUND

Adult stem cells are presumed to exist during the entire life cycle of mammary glands, where they are required to expand the cell populations during pregnancy and sustain cell turnover to replace senescent cells. Stem cells are generally defined as cells displaying a self-renewal capacity either with or without differentiation, depending on the type of division. Symmetric division of stem cells produces two identical stem cells, resulting in the expansion of the stem cell population, whereas an asymmetric division will result in a new stem cell and a progenitor cell of a more committed lineage. These adult stem cells are generally considered long-lived, mostly quiescent, slow cycling cells that generate new stem cell, hereby maintaining the stem cell pool [1].

In order to study the functional properties of stem cells, it is necessary to identify and prospectively purify them, a task that has proven technically difficult because of the low numbers of stem cells in the tissue of origin, and the lack of universal morphologic traits for stem cells [2]. Most stem-cell enrichment protocols rely on immunosorting, and use sets of antibodies against cell-surface proteins. Current methods for detecting bovine mammary progenitors require the preparation of viable single-cell suspensions and their assessment in suitable *in vitro* or *in vivo* assays to detect the growth and differentiation properties of the input cells at a clonal density [3]. However, the efforts to purify adult stem cells from the bovine mammary gland have been hindered by the lack of cell-surface markers specific for undifferentiated or differentiated mammary cells [4] even if suitable in vitro and in vivo assays for testing stem cell properties have been proposed by our laboratory [5].

In rodents and human, primary cultures of mammary epithelial cells underwent a limited replication and rapidly differentiated in a process regulated by hormonal factors, extracellular matrix, and cell–cell interactions [6-9]. We have recently demonstrated that it is possible to maintain bovine epithelial cells with stem-like properties in long-term culture under appropriate culture conditions that maintain a multipotent cell subpopulation with intrinsic regenerative potential [10], but no further data are available to understand the mechanism involved in regulation of bovine mammary stem niche if those are the remaining part of stem cells already present in the culture since the beginning of culture

or if it is possible to a renewal of the pool during the culture time. This fact has a kind of interest because it may prove the expansion and enrichment of a stem cell subpopulation *in vitro.* In human it has previously demonstrated that non-adherent mammospheres are enriched in cells with functional characteristics of stem/progenitor cells that may be a feasible method to isolate and characterize mammary stem cells [11], however the loss of paracrine context with other cell types do not explain the causes which allow the presence and maintenance of the mammary stem cell niche [12].

The aim of this work was to analyze the surface antigens expression and functional features of cell populations in a long-term mammary cells culture that maintains the regenerative properties. The ability to sustain in long-term culture a stem cell niche should give interesting perspectives for the study of their proliferation and differentiation and a new tool for their manipulation.

RESULTS

Cell culture

Along the experimental period of cell culture from day 1 to day 35 it was possible to observe a cell population with heterogeneous morphology. Small and polygonal cells with a "cobblestone-like" morphology, small spindle-shaped cells and larger cells with an extended cytoplasm and an irregular cell surface were all evident. Immunocytochemistry analysis revealed that both CK18 positive and CK14 positive cells were detectable throughout the experimental period. The differences for CK14 and CK18 expression along the experimental period were calculated at each passage in cytospin preparation, (in supplementary data Fig. A we have reported a representative samples of $CK18^+$ and $CK14^+$ cell populations at P0 and P3). Fig.1 shows the cell frequency in percentage: $CK14^+$ cells increased significantly from P0 (26.1±12.9 %,) to P3 (P5 64.9±12.9%) ($p<0.05$) then it decreased. The highest level of $CK18^+$ cells was observed at the beginning of the culture P0 (68.2±27.7) and the lowest at P3 (11.1±10.1 ($p<0.05$). After P3 we observed a novel double positive ($CK14^+/CK18^+$) cell population (12.8±10.2%) ($p<0.05$).

Immunophenotyping

In Fig. 2A the flow cytometry analysis shows the presence of mammary epithelial cells positive for CD49f. We observed a remarkable increase over 50% at P3 and P5 compared to P0 (95 % vs 33 % $p <0.05$). In Fig.2B has been depicted the pattern of expression of different cell surface markers. Less evident but present also an increase at P3 of CD24 and CD29. A significant increase ($p<0.05$) of the population of cells $CD49f^+/CD24^+$ was observed at P5 (P0: 0.15% vs P5: 1.13%). Also CD29 expression appears to increase up to 5% at P5 compared to P 0. ALDH1 population is high since the beginning of culture and progressively enhanced along the culture. The overall difference in cell frequency (in percentage) of cell subpopulations detected during the culture are shown in Table 1.

CFC assay

At each passage of culture, CFC assays were performed to evaluate the frequency of cell progenitors during the experimental period in both media. In these assay, distinct large colonies were consistently generated within 6 days at frequencies ranging from 1 colony per 3 to 500 cells plated. Most of the colonies contained cells that expressed markers typical of differentiated luminal ($CK18^+$) or myoepithelial ($CK14^+$) cells. Colonies containing mostly $CK14^+$ cells were comprised of tightly packed small polygonal cells with a reduced cytoplasm. Many of these "myoepithelial" colonies contained larger cells in the center surrounded by smaller cells. Colonies containing $CK18^+$ cells ("luminal" colonies) were comprised primarily of spindle-shaped cells with well-defined nuclei and an extended cytoplasm. Representative colonies that arose in CFC assays at day 35 (P 5) are shown in Fig. B in supplementary data.

The proportional distribution of total clonogenic luminal and myoepithelial progenitors among the different passages and medium conditions according the morphology after crystal violet staining is summarized in Tab.2. An high increase in myoepitelial progenitors was observed at P5.

Xenotransplant assay

We transplanted these cells under the kidney capsule of NOD/SCID mice in order to verify the regenerative potential of mammary epithelial cells at day 35. After 4 weeks we analyzed the formation of newly generated alveolar-like structures. In Fig. 4 the expression of CK14, CK18, EpCAM, P63. Milk proteins expression, a marker of polarized and functional alveolar structures, was detected in the lumen.

DISCUSSION

The maintenance of a stem cell population in vitro has represented a challenge since it is rather difficult to rebuild complex *in vivo* cell-cell and cell-matrix interactions *in vitro*. Our recent work describes how it is possible with two different specific culture media to maintain functional properties of stem cell after several days of culture [10]. Usually to purify and isolate stem cell from mammary gland tissue, cells are analyzed soon after dissociation because the extent of cell differentiation *in vitro* is considered quite high. However, it is interesting that in a mixed population of differentiated and non differentiated cells, it is possible to observe the presence of stem cells able to replicate and to perform a mammary colony-forming cell assay and *in vivo* a multilineage mammary gland reconstitution as freshly isolated adult stem cells. In this study we characterized cell phenotype cultured till to 35 days and we analyzed functionally cell progenitors in vitro and in vivo model.

The characterization of the different subsets of cells in dissociated primary cultures from bovine mammary gland has been performed with flow cytometry analysis based on the expression of different surface markers [13], however not so deeply investigated in this species. We expanded here the characterization by assessing the expression of markers that are related to progenitor populations (CD49f, CD24 and CD29 plus ALDH1). CD49f is part of the laminin-1 receptor and it is expressed in the mammary tissue by basal cells. A high CD49f expression has been associated with myoepithelial progenitors and mammary adult stem cells [3,14], so the data we present here reveal that a significant compartment of CD49f positive cells is maintained and probably increased along the culture instead of the expression of ALDH1 that has been associated with luminal progenitors which are not capable of regenerating a functional mammary epithelium [15]. When analyzing the differences in population frequencies, we found a higher proportions of CD49f positive cells (both in the $ALDH1^-$ and in the $ALDH1^+$ subfractions) in the last period of experimental culture, that means at least two main different epithelial cell subpopulations enriched the culture. We also observed as $CD29^+$ and $CD29^+/CD24^+$ subfractions increased in long-term

culture. It has been reported that in these cell subpopulations resides the stem cell niche as previously reported in human and mouse [3,14]. In our opinion, it may reveal that culture conditions not only retain a primitive cell subpopulation but they permit their selection. It has also to be taken into account, also, the interesting pattern of the terminally epithelial differentiated cells that show a progressive loss of luminal cells and an increase of myoepithelial cells from P0 to P3, that is, after 21 days of culture. At this time-point, we started to observed a small population, not detectable previously, of double-positive cells for both antigens regarded as the most primitive cells in the hierarchy. It is possible to hypothesize that a stimulation of stem cells for an asymmetric division occurs. The reason for this phenomenon is not easily identifiable so far. Further, after 14 days, in P5, we find again a significant presence in the culture of terminally differentiated cells to indicate a new fraction of cells had been made available for the differentiation.

The analysis shows that colony-forming cells are present throughout the culture period considering in the percentage previously reported [5]. This functional test confirmed that cells with significant proliferative properties are maintained in culture and we observed that the number of colonies $K14^+$ appears to increase over time. Within this type of colonies is reported to be present the stem cells subpopulation [5,16]. In addition to this test, we reported that after 35 days of culture a cell population capable of forming alveolar like-structures in vivo with cellular double-layer and luminal antigens such as K18 and EpCAM (if expressed at high levels) [17] or basal such as P63 [18], is present as confirmed by xenografts in NOD-SCID mice. We also observed the presence in the lumen of outgrowth the signal for protein milk indicating a proper polarization and functional activity.

In an attempt to manipulate bovine mammary cell composition toward a higher rate of stem cells, the possibility to study in long-term culture their mechanism of maintenance and/or proliferation can be a useful methodological approach to analyzed putative drugs augmenting symmetric cell division, i.e. in the last years, xanthosine has been investigated for stem cell proliferation in bovine

with contrasting results [19,20]. The approach herein proposed could help with new information on the effect of this molecule to increase mammary stem cell niche.

CONCLUSION

The presence of a significant number of primitive precursors is necessary to generate committed progenitors according to an asymmetric division. Increasing number of secretory mammary cells [21] would provide a support for production efficiency by replacing senescent or damaged cells and by providing a means to decrease nonproductive periods of the mammary gland cycle without negatively impacting milk yield [13,20]. Thus, the possible recovery and expansion of mammary progenitors in long-term culture open new opportunities to expand the stem cell subpopulation. Altering bovine mammary gland production capabilities via cellular manipulation is highly desirable [21] and successful attempts may be applicable for tissue-regeneration studies as it has been recently proposed for human species [22,23] and, finally, for subsequent use in regenerative medicine [24] in mammary gland pathology, particularly in the bovine species where the process of purification of these cells is only partial and a high number of cells is necessary for subsequent clinical applications.

METHODS

Bovine mammary tissue

Bovine mammary tissue was collected from slaughterhouse from 5 to six years old cows. Sample collection was performed with the authorization and under the supervision of representatives of the Veterinary Services of the Italian National Health Service branch of the Ministry of Health as previously described [4]. Briefly, approximately tissue was minced transferred to a 125 ml tube containing 20 ml of a 1:1 v/v mixture of Dulbecco's Modified Eagle Medium/ Nutrient Mixture F12 Ham (DMEM/F12) supplemented with 2% w/v bovine serum albumin, 300 U/ml collagenase, 100 U/ml hyaluronidase, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Sigma Aldrich, St. Louis, MO, USA). After 18–20 hours of incubation, a fraction enriched in epithelial cell aggregates (organoids) was next obtained by centrifugation and stored at -80°C until further processed. To prepare single cell suspensions, organoids were thawed and incubated with a 0.5 mg/ml trypsin solution supplemented with 0.2 mg/ml EDTA and subsequent washing in Hank's balanced salt solution (HBSS, STEMCELL Technologies) supplemented with 2% FBS. Cells were then treated with 5 mg/ml dispase and 100 mg/ml DNAseI (Sigma Aldrich) and passed through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA) to remove remaining cell aggregates.

Cell culture

60 mm tissue culture dishes were coated with collagen by incubation for 1 hour at 37°C with a solution of rat tail type 1 collagen (80 µl of rat tail type I collagen 1.5 mg/ml diluted in 50 ml of PBS). Dissociated cells were cultured in SF7 Medium (0.1%BSA, 10 ng/ml EGF, 10 ng/ml Cholera Toxin, 1ug/ml Insulin, 0.5 ug/ml Hydrocortisone, DMEM/F12 v/v). Cells were studied from P0 (from dissociation to the first passage) to P5 splitting them every 7 days. For immunostaining , $5x10^4$ cells were plated in each well of a collagen coated 96-wells plate and fixed them at passage 0, 1, 3 and 5 for each type of medium.

Colony-Forming Cell (CFC) assay

Single cells suspension of bovine epithelial cells were added in number of 500 cells/plate along with $2x10^5$ NIH 3T3 mouse fibroblasts previously treated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 2 hours. Cells were cultured in human EpiCult B medium (StemCell Technologies) supplemented with 5% FBS, 10^{-6} M hydrocortisone (Sigma-Aldrich), 100 U/ml penicillin and 100 mg/ml streptomycin. The dishes were then incubated at 37°C with 5% CO_2 for 24 hours. Medium was then replaced omitting FBS. Cells were incubated for another 6–9 days and then the cultures were fixed with acetone/methanol (1:1 v/v, Fluka) and either stained with a crystal violet solution (50 mg crystal violet in a 20% methanol solution, Sigma-Aldrich) or immunostained with antibodies to human cytokeratin 14 (CK14), cytokeratin 18 (CK18=, and p63, after validation that all of these cross-reacted with bovine antigens. Colonies containing more than 50 cells (after 7 days of culture) or than 100 cells (after 10 days of culture) were then counted and progenitor frequencies expressed as the total number of colonies obtained per 100 cells.

Immunostaining

Selected culture dishes were processed for immunostaining as described in [4]. Medium was removed from the dishes and cells were fixed with a 1:1 v/v mixture of acetone and methanol for 1 minute. Cells were then washed and blocked with Tris-HCl buffered saline (0.1 M Tris HCl, 0.14 M NaCl, pH 7.6) supplemented with 10% goat serum (all reagents from Sigma-Aldrich Corp). Dishes were then incubated with primary antibodies for 1 hour at room temperature followed by another hour with secondary fluorochrome-conjugated antibodies. Nuclei were then counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at a concentration of 0.5 μg/ml. Primary antibody used were an anti-human cytokeratin 14 (CK14, 1:500 dilution, polyclonal AF-64, Covance, Princeton, NJ, USA) and an anti-bovine CK18 (1:200 dilution, clone KS-B17.2, Sigma-Aldrich); p63 (1:200 dilution, clone 4A4, Thermo Fisher Scientific, Fremont, CA, USA), milk proteins (1:500 dilution, polyclonal Nordic Immunology, Tilburg, Netherlands) EpCAM (1:100

diluition, clone E144, AbCAM, Cambridge, UK). Secondary antibodies used were AlexaFluor® 488-labelled goat anti-rabbit IgG and AlexaFluor® 594-labelled goat anti-mouse IgG (both from Life Technologies, Carlsbad, CA, USA). Negatively stained controls were performed for each antigen by replacing the primary antibody with a suitable isotype (normal mouse IgG or normal rabbit IgG from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at the same concentration.

Flow Cytometry

After picking the colonies from the CFC assays, cells were digested with warm (37°C) trypsin (Sigma-Aldrich Corp) for 2 minutes while pipetting. Trypsin was subsequently neutralized with cold HBSS supplemented with 2% FBS. The resulting single cell suspension was then stained for flow cytometry. Staining for aldehyde dehydrogenase I (ALDH1) was done with ALDEFLUOR™ kit (STEMCELL Technologies) as per manufacturer instruction. Cells were incubated for 30 minutes at 37°C with the ALDEFLUOR substrate and then stained with an R-PE conjugated anti-human CD49f (1: 25 dilution in 50 µl volume, clone GoH3, Santa Cruz Biotechnology Inc., Dallas, TX, USA), FITC conjugated anti-human CD24-FITC or FITC conjugated antihuman CD29- (ImmunoTools GmbH, Germany). DAPI was then added at a concentration of 0.25 µg/ml in order to discriminate live from dead cells. Cells were then run on an Attune® Cytometer (Life Technologies) equipped with a 405 nm and a 488 nm lasers.

Xenotransplants

Ten female NOD/SCID mice were bred and housed at the animal facility of the Department of Veterinary Science of the University of Turin according to the procedures and guidelines approved by the Italian Ministry of Health. Animal work described in this study has been reviewed and approved by the Italian Ministry of Health. Mice were used at 5 to 10 weeks of age as equivalent recipients for the transplants described [25]. Concentrated rat tail collagen was prepared as previously described [26]. Collagen gels were prepared as previously described [4]. Each gel

contained $1.6x10^5$ 10T1/2 fibroblasts previously treated with 2 μg/ml mitomycin C and $5x10^4$ bovine primary mammary cells. At first a 2 cm anterior-to-posterior cut was made through the skin along a median line followed by a smaller incision of approximately 4–5 mm in the abdominal wall directly above the kidney position. The collagen gels were insert under the kidney capsule using fire polished glass Pasteur pipettes. The abdominal wall was then sutured and the procedure was repeated on the controlateral kidney. A slow release pellet containing 2 mg β-estradiol (Sigma-Aldrich) and 4 mg progesterone (Sigma-Aldrich) in silicone (MED-4011, NuSil Technology, Carpinteria, CA, USA) was placed subcutaneously. After four weeks the gels were extracted from the kidneys. For each experiment some gels were fixed in 4% of formalin and then processed for immunochemistry or immunofluorescence, the remaining gels were dissociated with collagenase. The single cell suspension was then used for CFC assays as previously described [5].

Statistic analysis

A quantitative evaluation of phenotype was performed at passages 0, 1, 3, 5 of the cell culture. After immunostaining cells were counter-stained with DAPI (0.5 μg/ml in PBS) and photographed with a Leica AF6000 LX (Leica Microsystem, Wetlar, Germany) inverted microscope. For each staining 30 fields at a 40X magnification were considered to evaluate cell phenotype. Each field covered approximately 1% of the total area of a single well in a 96-well plate. Each different phenotype was expressed as percentage on total cell number. Statistical difference among the treatment was evaluated by Wilcoxon Signed-Rank Test ($P\text{-value}<0.05$). To compare multiple cell populations ANOVA Test was used.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CV carried out the biological experiments and analyzed the data; ME participated in study design and carried out part of the in vivo experiments, MS carried out a section of the immunoassays, AP participated in the design of the study and performed the statistical analysis; BM conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Table 1: Variation (%) of positive cells for CD49f, CD24, CD29, CD49f/CD24, CD29/CD24, ALDH1. * indicates $p<0.05$ vs P0.

Passage	CD49f	CD24	CD29	CD49f/CD24	CD29/24	ALDH1
P0	33,11	0,10	5,16	0,15	0,05	69,44
P1	30,21	0,04	3,01	0,14	0,04	72,10
P3	80,01	0,03	4,52	0,18	0	90,23
P5	95,16	0	10,12	1,13	0,03	93,01

Tab.2 Variation (%)of myoepithelial and luminal colonies. * indicates $p<0.05$.

PASSAGE	% of myoepithelial colonies	% of luminal colonies
P0	2,4	0,6
P1	1,9	0,8
P3	1,5	0,8
P5	7,8	1,3

FIGURE LEGENDS

Fig.1: Variations in bovine mammary cell populations from P0 to P5 positive for CK14, CK18 or both antigens throughout the cell culture in SF7 medium. * means significant difference among passages (ANOVA analysis, $P < 0.05$).

Fig.2 Flow cytometry immunophenotyping panel. (A) CD49f and CD24 expression from P0 to P5 showing antigens-gated populations. Gates: R9 for $CD49f^-/CD24^-$, R10 for $CD24^+$, R7 for $CD49f^+$, R8 for $CD49f^+/CD24^+$; (B) Pattern of expression of positive-staining cells for CD49f, CD24, CD29 and ALDH1 along the culture.

Fig.3 Expression and spatial localization of detected immunofluorescent markers in regenerated structures in xenograft formed from coltured mammary bovine cells. (A) Haematoxylin-Eosin staining scale bar= 250 um (10X magnification) ; (B) CK14 positive cells (green, white arrow) and CK18 (red, yellow arrow); (C) EpCAM staining (green, white arrow); (D) P63 staining (red, white arrow); (E) milk protein staining in the lumen (green, white arrow). CK14 marks basal cells whereas CK18 marks luminal cells; EpCAM marks predominantly luminal compart and p63 marks the nuclei of basal compart. Nuclei were counterstained with DAPI. Scale bars = 50 μm (40× magnification).