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Bovine mammary epithelial cells retain stem-like phenotype in long-term cultures

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ABSTRACT

The detection and characterization of bovine mammary stem cells may give a better understanding of the cyclic characteristic of mammary gland development. In turn, this could potentially offer techniques to manipulate lactation yield and for regenerative medicine. We previously demonstrated that adult stem cells reside in the bovine mammary gland and possess an intrinsic regenerative potential. In vitro maintenance and expansion of this primitive population is a challenging task that could make easier the study of adult mammary stem cells. The aim of this study is to investigate this possibility. Different subpopulations of mammary epithelial cells emerge when they are cultured in two defined culture conditions. Specific cell differentiation markers as cytokeratin 18 (CK18) and cytokeratin 14 (CK14) were expressed with significant differences according to culture conditions. Vimentin, a well-known fibroblast marker was observed to increase significantly ($P < 0.5$) only after day 20. In both conditions, after prolonged culture (25 days) a subset of cells still retained regenerative capabilities. These cells were able to form organized pseudo-alveoli when transplanted in immunodeficient mice as shown by the expression of cytokeratin 14 (CK14), cytokeratin 18 (CK18), p63 (a mammary basal cell layer marker) and Epithelial Cell Adhesion Molecule (EpCAM). We also were able to observe the presence of milk proteins signal in these regenerated structures, which is a specific marker of functional mammary alveoli. Progenitor content was also analyzed in vitro through Colony-Forming Cell (CFC) assays with no substantial differences among culture conditions and time points. These results demonstrate that long-term culture of a multipotent cell subpopulation with intrinsic regenerative potential is possible.

1. Introduction

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. Mammary stem cells and progenitors provide for net growth, renewal and turnover of mammary epithelial cells, and are therefore potential targets for strategies to increase production efficiency. Appropriate modulation of the homeostasis of these cells can potentially benefit milk yield, persistency, dry period management and repair of mammary tissue when damaged by mastitis (Capuco et al., 2012).

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 (Borena et al., 2013).

To study the functional properties of stem cells, one needs 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 (Blau et al., 2001). 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 level (Stingl et al., 2006). 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 (Martignani et al., 2009) even if suitable in vitro and in vivo assays for testing stem cell properties have been proposed by our laboratory (Martignani et al., 2010).

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In the last years to obtain a large amount of stem cells, some investigators have used an alternative approach in which immortalized mammary cell lines are established from human or rodent tissues. For instance ESA-positive MUC1-negative cell line derived from human mammary cells that was capable of generating ductal-acinar structures in basal membrane gel has been described (Gudjonsson et al., 2002). Complete functional differentiation and synthesis of milk proteins was not shown for these cells. Although cell lines are useful for elucidating molecular pathways, the process of immortalization may introduce artifacts that significantly alter cellular properties and gene expression profiles. An alternative, less biased approach than the use of established cell lines, is the isolation and propagation of normal mammary progenitor cells from primary tissue. This approach, however, has been limited by the lack of suitable systems that allow for the propagation of these cells in an undifferentiated state. In previous studies, in rodents and human, when primary cultures of mammary epithelium were cultured, they underwent limited replication and differentiated in a process regulated by hormonal factors, extracellular matrix, and cell–cell interactions (Muschler et al., 1999; Reynolds and Weiss, 1996; Romanov et al., 2001; Simian et al., 2001). In human it has previously demonstrated that nonadherent mammospheres are enriched in cells with functional characteristics of stem/progenitor cells that may be a feasible methods to isolate and characterize mammary stem cells; 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 (Dontu et al., 2003).

The purpose of this work was to verify the ability of expansion and maintenance of bovine functional mammary stem cells cultured in different media conditions and transplanted in validated in vivo model to analyze their functional and secreting properties. The ability to maintain in long-term culture a stem cell niche should open new perspectives for the study of their physiological regulation that for their manipulation in view of animal husbandry applications.

2. Materials and methods

2.1. Bovine mammary tissue

Bovine mammary tissue was collected from slaughterhouse from 4 to 8 year 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. A piece of tissue was dissected out of the area surrounding the teats and then minced with scalpels. Approximately 10–15 g of tissue were transferred to a 125 ml baffled Erlenmeyer flask 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 (BSA, Fraction V), 300 U/ml collagenase, 100 U/ml hyaluronidase, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Sigma Aldrich, St. Louis, MO, USA). The tissue was then placed in a shaking incubator at 37 °C for 18–20 h. A fraction enriched in epithelial cell aggregates (organoids) was next obtained by centrifugation of the dissociated tissue at 80 g for 30 s and then washed in fresh DMEM/F12 medium at least three times. The organoids were then frozen in 6% dimethyl sulfoxide (DMSO, Fluka, Milan, Italy) containing medium 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 followed by vigorous pipetting for 4 min 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.

2.2. Cell culture

60 mm tissue culture dishes were coated with collagen by incubation for 1 h 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 Epicult®-B Medium (DMEM/F12 mixture 1:1 v/v supplemented with Apo-transferrin, BSA, insulin, EGF, isoproterenol, ethanolamine, 3',3',5-triiodo-L-thyronine, hydrocortisone. Supplements concentration are not provided by the manufacturer (StemCell Technologies) or in SF7 Medium (0.1%BSA, 10 ng/ml EGF, 10 ng/ml Cholera Toxin, 1ug/ml Insulin, 0.5 μg/ml Hydrocortisone, DMEM/F12 v/v). Cells were studied from P0 (from dissociation to the first passage) to P5 splitting them every 5 days (splitting rate ≤ 1:3, a total of 25 days in culture). For immunostaining, 5×10^4 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.

2.3. Xenotransplants

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 5–10 weeks of age as equivalent recipients for the transplants described (Prpar et al., 2012). Concentrated rat tail collagen was prepared as previously described (Richards et al., 1983). Collagen gels were prepared as previously described (Martignani et al., 2009). Each gel contained 1.6×10^5 10T1/2 fibroblasts previously treated with 2 μg/ml mitomycin C and 5×10^4 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 inserted 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 4 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.

2.4. Colony-Forming Cell (CFC) assay

Single cells suspension of bovine epithelial cells were added in number of 500 cells/plate along with 2×10^5 NIH 3T3 mouse fibroblasts previously treated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 2 h. Cells were cultured in human EpiCult B medium 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 h. 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 CCK14 (1:500 dilution, polyclonal AF-64, Covance, Princeton, NJ, USA), CK18 (1:200 dilution, clone KS-B17.2, Sigma-Aldrich) and p63 (1:200 dilution, clone 4A4, Thermo