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Research Article

Clonogenic assay allows for selection of a primitive mammary epithelial cell population in bovine

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ABSTRACT

Adult mammary stem cells have been identified in several species including the bovine. They are responsible for the development of the gland and for cyclic remodeling during estrous cycles and pregnancy. Epithelial cell subpopulations exist within the mammary gland. We and others showed previously that the Colony Forming Cell (CFC) assay can be used to detect lineage-restricted mammary progenitors.

We carried out CFCs with bovine mammary cells and manually separated colonies with specific morphologies associated with either a luminal or a myoepithelial phenotype. Expression of specific markers was assessed by immunocytochemistry or by flow cytometry to confirm that the manual separation resulted in isolation of phenotipically different cells.

When transplanted in recipient immunodeficient mice, we found that only myoepithelial-like colonies gave rise to outgrowths that resembled bovine mammary alveoli, thus proving that adult stem cells were maintained during culture and segregated with myoepithelial cells. After recovery of the cells from the transplanted mice and subsequent progenitor content analysis, we found a tendency to detect a higher progenitor frequency when myoepithelial-like colonies were transplanted.

We here demonstrate that bovine adult mammary stem cells can be sustained in short-term culture and that they can be enriched by manually selecting for basal-like morphology.

1. Introduction

The mammary gland has a peculiar development, since it takes place mostly after birth [1]. Its parenchyma is a highly branched arborescent structure, in which alveoli constitute the functional units. The mammary epithelium is organized as a bilayer, where the inner luminal cells are responsible for milk production, while the outer myoepithelial cells have contractile capabilities [2]. The two different cell lineages can be identified also by the expression of specific markers. Luminal cells are cytokeratin (CK) $18^+/CK14^-$, while myoepithelial cells are $CK14^+/CK18^-$. This tissue organization is well conserved among different mammalian species, including the human [3], mouse [4], goat [5] and bovine [2,6].

The mammary gland undergoes cyclic remodeling throughout the reproductive life of the organism. During each estrous cycle and more dramatically during pregnancy, epithelial cell number shows a marked increase and more alveoli can be detected in the tissue [7]. Afterwards, involution takes place and the mammary gland revert to a quiescent state [8]. An adult stem cell population exists within the mammary tissue to sustain such a unique behavior. These stem cells have been identified and characterized by assessment of the expression of specific markers by flow cytometry or immunohistochemistry or through functional assays, such as transplantation in immunodeficient animals, to rigorously demonstrate their ability to self-renew and to differentiate. Species in which such characterization was carried out include the mouse [9], human [10] and more recently the bovine [11] and goat as well [5].

As we previously published, the bovine mammary gland is hierarchically organized: a small population of quiescent stem cells gives rise to different lineage restricted progenitors. These progenitors have the ability to extensively proliferate and to generate terminally differentiated cells [12]. They can be identified by their ability to form colonies in vitro over a short time. The cell morphology in these colonies can be used to infer the progenitor type that originated them in order to determine the progenitor content of the tissue of origin. An analysis based on surface markers of the different bovine mammary subpopulations has been previously carried out [13] but no in vivo functional assays were conducted on them.

In the present work we assessed whether the short-term in vitro culture system that is used to evaluate for bovine mammary progenitors can sustain the maintenance of more primitive stem

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cells. Moreover, we show that selection and collection of specific colony types can be used as a phenotype-based method to obtain a rough enrichment of bovine adult mammary stem cells.

2. Material and methods

2.1. Collection of bovine mammary tissue

Whole udders were collected from three different animals (11-17-96 months old animals) at a local abattoir within 2 h of the time of slaughter. All samples were from Piedmontese cattle, a local beef breed. 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.

Mammary tissue was then processed as previously described [11] in order to obtain a single cell suspension to be used for subsequent assays. Briefly tissue samples were dissociated overnight in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM/F12) 1:1 v/v mixture supplemented with 2% bovine serum albumin (BSA), 300 U/ml collagenase type IV, 100 U/ml hyaluronidase type IV-S, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma-Aldrich)

Epithelial aggregates were then separated by differential centrifugation and enzymatically digested with a 0.5 mg/ml trypsin solution supplemented with 0.2 mg/ml EDTA followed by a digestion with 5 mg/ml dispase and 100 μg/ml DNAseI (all from Sigma-Aldrich)

2.2. Colony Forming cell (CFC) assay

CFC assays were carried out as previously described [11]. Briefly, single cell suspensions of bovine mammary epithelial cells were seeded at very low density (1×10^2–1×10^3 cells per 60 mm dish) along with 2×10^5 NIH 3T3 fibroblasts that were previously treated with mitomycin C (10 μg/ml for 2 h).

Cells were cultured for 24 h in human EpiCult B medium (STEMCELL Technologies) supplemented with 5% fetal bovine serum (FBS) and 10^{-6} M hydrocortisone, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma-Aldrich). Subsequently medium was replaced with fresh one without FBS and cells were cultured for 7–8 additional days.

Colonies were then examined under a Leica DM IL inverted contrast phase microscope (LEICA Microsystems) in order to assess their morphology. They were then picked from the dishes with a micropipette and transferred to 1.5 ml test tubes containing Hank's balanced salt solution (HBSS) supplemented with 2% FBS.

2.3. Immunostaining

Selected culture dishes were processed for immunostaining as described in Ref. [11]. Medium was removed from the dishes and cells were fixed with a 1:1 v/v mixture of acetone and methanol for 1 min. 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). Dishes were then incubated with primary antibodies for 1 h 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.

The following antibodies were used in the protocol: anti-human cytokeratin 14 (CK14, 1:500 dilution, polyclonal AF-64, Covance), an anti-bovine CK18 (1:200 dilution, clone KS-B17.2, Sigma-Aldrich) and an anti-p63 (1:200 dilution, clone 4A4, Thermo Fisher Scientific) as well as secondary antibodies: AlexaFluor® 488-labeled goat anti-rabbit IgG and AlexaFluor® 594-labeled goat anti-mouse IgG (both from Life Technologies).

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.) at the same concentration.

2.4. Flow cytometry

After picking the colonies from the CFC assays, cells were digested with warm (37 °C) trypsin (Sigma-Aldrich) for 2 min 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 the ALDEFLUOR kit (STEMCELL Technologies) according to the instructions provided by the manufacturer. Cells were incubated for 30 min 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.). 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.

2.5. Xenografts

Colonies were picked from the CFC assay, separated according to their morphology and single cell suspensions were prepared with the same protocol used for the flow cytometry analysis.

After dissociation, cells were mixed with 10T1/2 mouse fetal fibroblasts that were previously treated with mitomycin C at a concentration of 2 μg/ml for 16 h. Cells were then embedded in rat tail collagen as previously described [11] and transferred under the kidney capsule of female NOD/SCID mice that received at the same time a silicone pellet (MED-4011, NuSil Technology) containing 2 mg of 17β-estradiol and 4 mg of progesterone (both from Sigma-Aldrich). The kidney capsule was chosen as the site for transplantation since it was previously described by other groups as able to support the growth of human mammary epithelial cells [10,14]. Moreover this site allowed for easier recovery of mammary cells at the end of the assay.

Each gel contained 2000 to 6000 epithelial cells and 1.6×10^5 10T1/2 fibroblasts. Six mice were transplanted: gels with luminal-like cells were placed in the left kidney, while gels prepared with myoepithelial-like cells were placed in the right kidney.

After 4 weeks the gels were recovered from the mice. Half of the gels were dissociated by incubation with collagenase (300 U/ml, Sigma-Aldrich) for 5 h at 37 °C in an incubator with 5% CO_2. Cell aggregates were then further digested with warm trypsin (Sigma-Aldrich) for 2 min and then used to perform CFC assays. Cells from a single gel were seeded in an individual dish.

The other gels were fixed in 10% formalin (Sigma-Aldrich) and processed for histological analysis.

2.6. Mice

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 for the transplants described. All surgical procedures were performed under anesthesia using an association of Xylazine and Zolazepam/Tiletamine. All