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Bovine mammary epithelial cells retain stem-like phenotype in longterm cultures

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A B S T R A C T

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.

Keywords: Stem Cell, Cell Differentiation, Bovine, Mammary Gland

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 selfrenewal 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 newstem 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).

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 ductalacinar 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 stemcell 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 5mg/ml dispase and 100 mg/ml DNaseI (Sigma Aldrich) and passed through a 40 μ m 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 μ l of rat tail type I collagen 1.5 mg/ml diluted in 50 ml of PBS).

Dissociated cells were cultured in EpicultR-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 μ g/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 \leq 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 5-10weeks 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 μ g/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 4-5mm 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 contralateral kidney. A slow release pellet containing 2mg 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 $10 \mu\text{g/ml}$ 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 KSB17.2, Sigma-Aldrich) and p63 (1:200 dilution, clone 4A4, ThermoFisher Scientific, Fremont, CA, USA), after validation that all of these cross-reacted with bovine antigens. Colonies containing more than 50 cells (after 7 days of culture) or more 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.

2.5. Immunostaining

Four to $7 \mu\text{m}$ sections were dewaxed and processed either for immunohistochemistry or immunofluorescence as previously described (Martignani et al., 2010). Briefly, after dewaxing, sections were incubated at room temperature for 1 h in TBS-Tween (Tris 0.1M, NaCl 0.07 M, pH 7.6, Tween 20 0.05%, Ultra Pure H₂O v/v) supplemented with 10% goat serum (Sigma-Aldrich) and then for 1 h at room temperature with one or two primary antibodies as required.

Sections were then washed three times with TBS-Tween, secondary antibodies were applied, and the sections were incubated for another hour. Sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). To stain colonies grown in dishes, cells were fixed with methanol/acetone (1:1 v/v) for 30 s to 1 min, blocked with TRIS buffer supplemented with 10% goat serum and then stained as described above. Primary antibodies used were antibodies to human CCK14 (1:500), CK18, p63 (1:200 dilution, clone 4A4, Thermo Fisher Scientific), milk proteins (1:500 dilution, polyclonal Nordic Immunology, Tilburg, Netherlands) EpCAM (1:100 dilution, 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 Invitrogen, 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.

2.6. 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 ($0.5 \mu\text{g/ml}$ in PBS) and photographed with a Leica AF6000 LX (Leica Microsystems, Wetlar, Germany) inverted microscope equipped with a Leica DFC350FX digital camera and motorized stage controlled by the LAS AF software (Leica Microsystems).

For each staining 30 fields at a $40 \times$ 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 -value < 0.05). To compare multiple cell populations ANOVA Test was used.

3. Results

3.1. Pattern of expression of CK14 and CK18 according to media and timing

During the whole time of the culture from day 1 to day 25 it was possible to observe 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. In Fig. 1 we show a representative example at day 5 (P1) and day 25 (P5) for cell types that have been previously related to both luminal and myoepithelial cells in mammary alveoli.

Immunophenotyping revealed that both CK18 positive and CK14 positive cells were detectable throughout the experimental period.

In Fig. 2 we show CK14 or CK18 positive epithelial cells at day 5 (P1) and day 25 (P5) cultured in Epicult B. Similar data are shown for cells cultured in SF7 medium in supplementary data (Appendix: Supplementary Fig. S1). Thus, both media were able to maintain in culture these two different cell populations and the differences for CK14 and CK18 expression for each type of medium along the experimental period are shown in Fig. 3. CK14 positive cells increased significantly from day 5 (P1 - 3.9% ± 0.9% in Epicult B, 5.0% ± 1.2% in SF7) to day 25 (P5 - 26.0% ± 3.9% in Epicult B, 30.0% ± 3.6% in SF7) ($P < 0.05$) and no statistically significant differences were observed due to culture medium used. The highest level of CK18+ cells was observed at day 5 (77.0% ± 5.3% in Epicult B, 76.0% ± 2.9% in SF7) ($P < 0.05$), with a differential pattern of expression: CK18 positive cells showed a significant and rapid variation between day 1 (P0 - 33.0% ± 2.8%), day 5 (P1 - 77.0% ± 5.3%) and day 15 (P3 - 31.0% ± 3.3%) in EpiCult B compared with SF7 medium (P0 - 57.0% ± 4.4%, P1 - 76.0% ± 2.9%, P5 - 55.0% ± 2.8%). A significant reduction ($P < 0.05$) in CK18 positive cells was observed at day 25 (P5 - 30.0% ± 2.6% in Epicult B, 30.0% ± 2.9% in SF7) for both media. Negative population for both CK18 and CK14 increased significantly ($P < 0.05$) after day 15 (P3) in Epicult B (38.0% ± 5.1%) and at day 25 (P5) in SF7 medium (27.0% ± 3.2%). After day 15 we observed a novel double positive (CK14+ and CK18+) cell population - (4.0% ± 1.3% in Epicult B, 3.4% ± 0.6% in SF7) (Fig. 4).

The expression of vimentin, a typical fibroblast marker, was observed to increase significantly ($P < 0.05$) only at day 25 (P5 - 23.0% ± 2.3% in Epicult B, 24.0% ± 3.3% in SF7). Fig. 5 shows the pattern of vimentin expression in fibroblast-like cells. No difference was observed when cells were cultured in the two media.

3.2. CFC assay

At each passage of the culture, CFC assays were performed to evaluate the frequency of cell progenitors during the experimental period in both media. In these assays, 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. Double stained cells for CK14 and CK18 were observed in CFCs performed after day 15. Representative colonies that arose in CFC assays at day 25 (P5) are shown in Fig. 6.

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 Table 1.

3.3. 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 25. After 4 weeks we analyzed the formation of newly generated alveolar-like structures. In Fig. 7 the expression of CK14, CK18, EpCAM, P63 in Epicult Bmedium is shown. Similar results were collected for SF7 medium (data not shown). Interestingly milk proteins expression, a marker of polarized and functional alveolar structures, was detected in the lumen of several outgrowths.

We performed CFC assays with cells dissociated from outgrowths in order to verify the proliferative potential of cell and therefore the progenitor content after xenotransplants. We found the same percentage of basal and luminal colonies in outgrowths derived from cells cultured in Epicult B and SF7 media (Table 2).

4. Discussion

The stem cell niche is the microenvironment in which a stem cell resides. Signals from nearby cells and the extracellular stroma appear instrumental in regulating stem cell activity. There is strong evidence that stem cell activity in the mammary gland is largely dictated by the stem cell niche. Research by Gilbert Smith and colleagues demonstrated that the normal lineages of neural stem cells (Booth et al., 2008) and spermatogonia (Boulanger et al., 2007) are redirected when placed in a mammary environment, permitting them to repopulate a cleared mammary fat pad. This reaffirms the concept of plasticity among somatic stem cells and points to the importance of the stem cell niche (Capuco et al., 2012). Furthermore, a very recent and intriguing perspective is the involvement of microRNA in the regulation of the stem cell niche, as genes responsible for self-renewal and pluri/multi-potency need to be downregulated, while new transcripts are required to specialize in tissue-specific function (Martignani et al., 2011). MicroRNAs are suspected to exert a key role in this complex network of factors that tightly control checkpoints in gene expression with changes in the transcription factors pool.

In this context the maintenance of a stemcell 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*. One of the most used methods is the cultivation of cells in suspension with the formation of mammospheres. A recent work describes a protocol to quantify early common progenitors/stem cells grown as spheres in non-adherent culture conditions. This protocol is based on the combination of two functional tests: the mammosphere assay to maintain and enrich early mammary progenitors/stem cells, and the epithelial colony-forming cells assay to identify and quantify further differentiated epithelial progenitors (Bachelard-Cascales et al., 2012). In this work it has been underlined that mammary stem cell may proliferate and self-renewthroughout several passages and early common progenitor populations are maintained aswell through passages and only partially differentiate.

In our paper we collect the result of two different specific culture media (Epicult B and SF7 media) able to maintain functional properties of stem cell after several passages. To our knowledge it is the first report that shows the possibility to collect mammary stem cell after a long-term culture and open new question about the specific microenvironment that permits to maintain stemness *in vitro* for such a long time.

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. The alternative method is the cultivation in cell suspension and mammosphere formation (Bandyopadhyay et al., 2012; Dong et al., 2013). However, it is intriguing that in a mixed population of differentiated and non differentiated cells, stem cells are able to replicate and to perform after five passages *in vitro* in mammary colony forming cell assay and *in vivo* in multilineage mammary gland reconstitution as freshly isolated adult stem cells. Furthermore, this finding may open new opportunities to expand the stem cell subpopulation for subsequent use in regenerative medicine, 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 (Martignani et al., 2010).

We used two different culture media (EpiCult-B (Eirew et al., 2008) and SF7 (Stingl et al., 1998)) that are validated to culture mammary progenitor cells in order to verify any difference at any time of culture. We observed some medium related differences in cell differentiation in the two main lineages (percentage of luminal cell and basal cells) but in both media we were able to detect double positive CK18 and CK14 cells (EpiCult B at passage 3 and 5, SF-7 at passage 5) a hallmark of the presence of more primitive cells than unipotent progenitors. These differences could be affected by the difference in quality of some of the reagents in the two used medium culture (see Materials and Methods), although it remains difficult to identify the real cause of missing some information. The presence of precursors was also proved by the similar CFC frequency obtained at passage 5 when compared with passage 0. We may argue that during the first passage a significant differentiation occurs, in particular toward the luminal lineage (CK18+ cells), the same compartment where it is thought the adult stem cells are residing (Choudhary et al., 2013). This might indicate that there's a higher proportion of luminal progenitors isolated from the mammary tissue that are lost during the initial phase of the culture, while after several passages, the stem cells that are in the mixed population are able to regenerate progenitors that are located higher in the tissue hierarchy (that is, double positive CK14/CK18 cells). We were not able to determine what factors might promote these phenomena.

However we show a parallel increase of vimentin positive cells, which is a fibroblast marker. This increase can be interpreted as a derive towards an epithelial-to-mesenchymal transition (EMT). An hypothesis is that the increase of fibroblastic-like cells could enrich the culture of factors inducing a condition of stemness. Since the stem cell niche plays a very important role both in self-renewal and differentiation of primitive cells and since in the bovine species epithelial cells are directly in contact with fibroblasts found in the loose connective tissue surrounding the mammary parenchyma (Capuco et al., 2002) rather than adipocytes, it is safe to assume that fibroblasts might be strongly involved in adult mammary stem cells homeostasis. In this case, the widely described EMT process might be important for maintaining undifferentiated mammary cells in long term culture. Studies are being carried out to collect more information. Within the population of primitive cells that we analyzed with immunocytochemistry and CFC assay there are also stem cells as shown by the transplantation in immunodeficient mice. Bilayered alveolar-like structures were observed in mice transplanted with cells cultured either in EpiCult B or SF7. Furthermore, a signal for secreted milk proteins was observed in the lumen of the pseudoalveoli that induced to hypothesize a complete polarization and functional terminal differentiation.

Our conclusion is that it is possible to maintain bovine epithelial cell with stem-like properties in long term culture. Further studies are necessary to understand the mechanism involved in this regulation but along with a better immunophenotyping of bovine mammary stem cell, this may prove fundamental in expansion and enrichment of a stem cell subpopulation *in vitro*.

In dairy science, an ability to regulate the function of mammary stem and progenitor cells may result in an increased lactation efficiency (Choudhary and Capuco, 2012; Rauner and Barash, 2012).

Inducing these cells to generate a higher number of committed progenitors would provide a means to increase production efficiency by increasing the number of secretory mammary cells, 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.

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Contributors

Diego Cravero carried out the biological experiments and analyzed the data. Eugenio Martignani participated in study design and carried out part of the in vivo experiments, Silvia Miretti provided samples and Paolo Accornero contributed to the critical discussion; Mario Baratta conceived the study and elaborated the discussion. All authors read and approved the final manuscript.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rvsc.2014.07.022.

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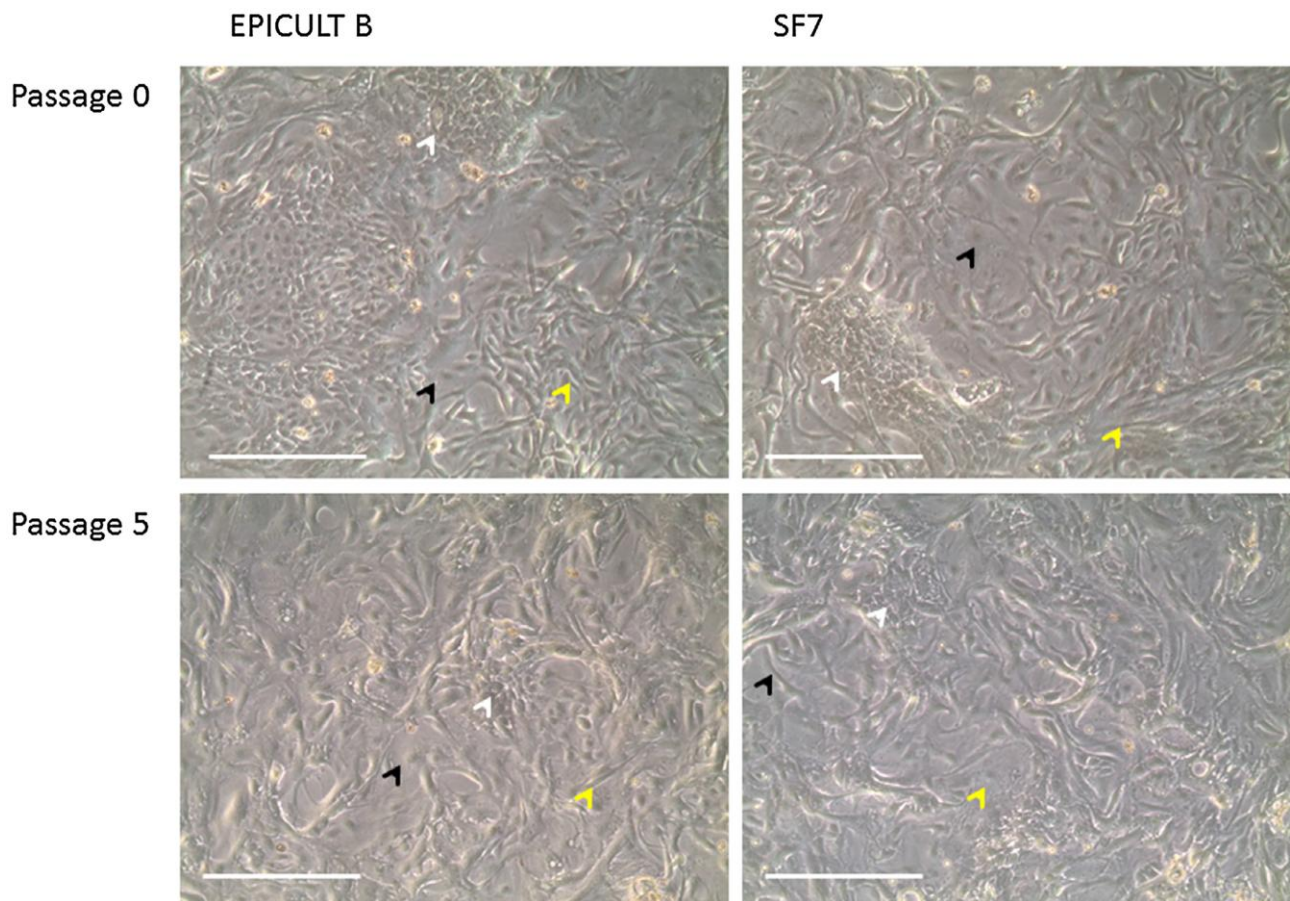


Fig. 1. Bovine mammary cell populations in two different media conditions at the beginning and at the end of cell culture. On the left Epicult B at passage 0 (up) and passage 5 (down). On the right SF7 at passage 0 (up) and passage 5 (down). White arrows indicate cells with 'cobblestone-like' morphology, black arrows indicate cells with large cytoplasm; yellow arrows: fusiform cells. (light micrographs, 10 \times magnification).

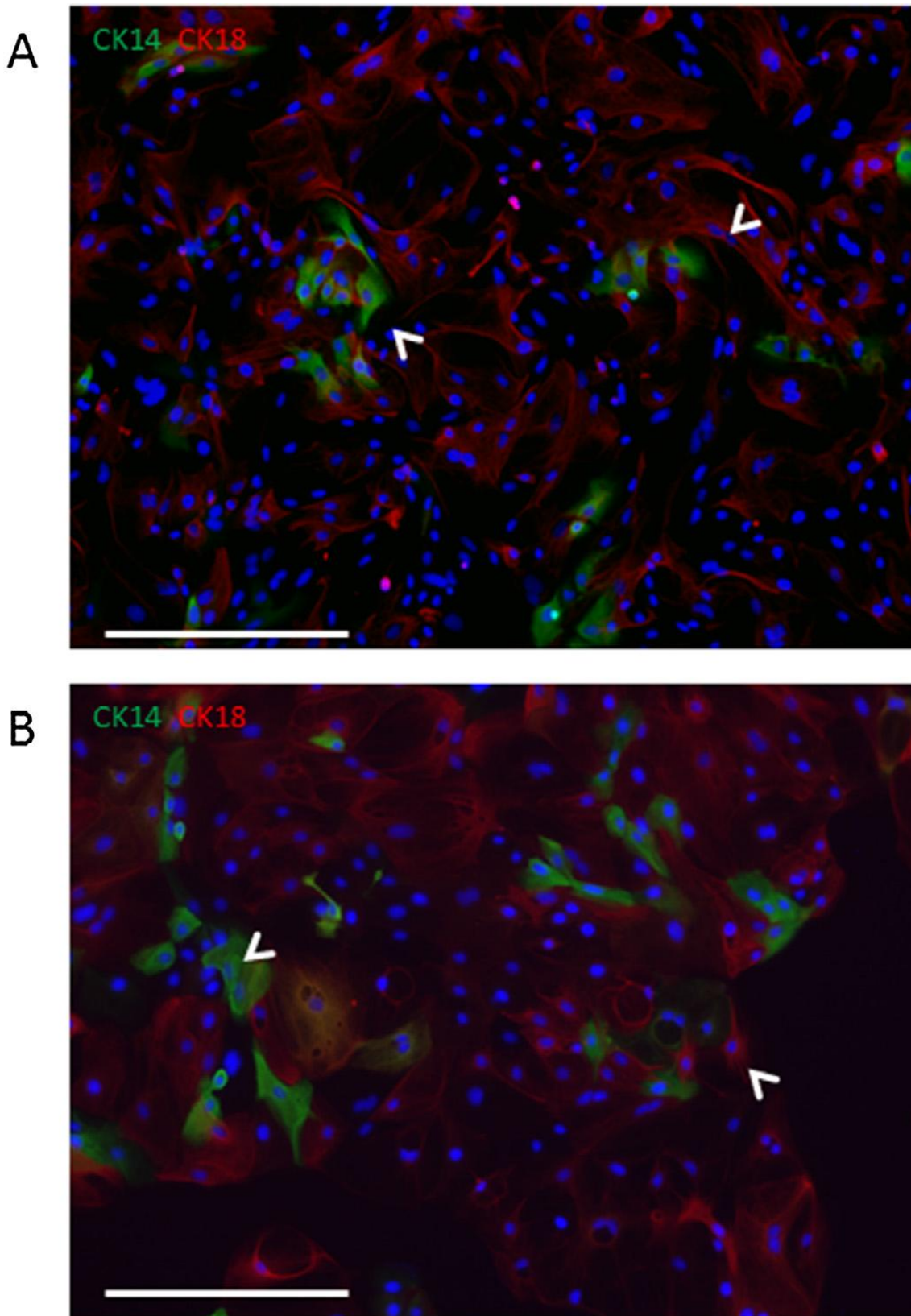


Fig. 2. Immunophenotyping for CK14 and CK18 at passage 1 (a) and 5 (b) in Epicult B (for SF7 see supplementary data Appendix: Supplementary Fig. S1). Arrows indicate CK14+ cells (green) and CK18+cells (red).

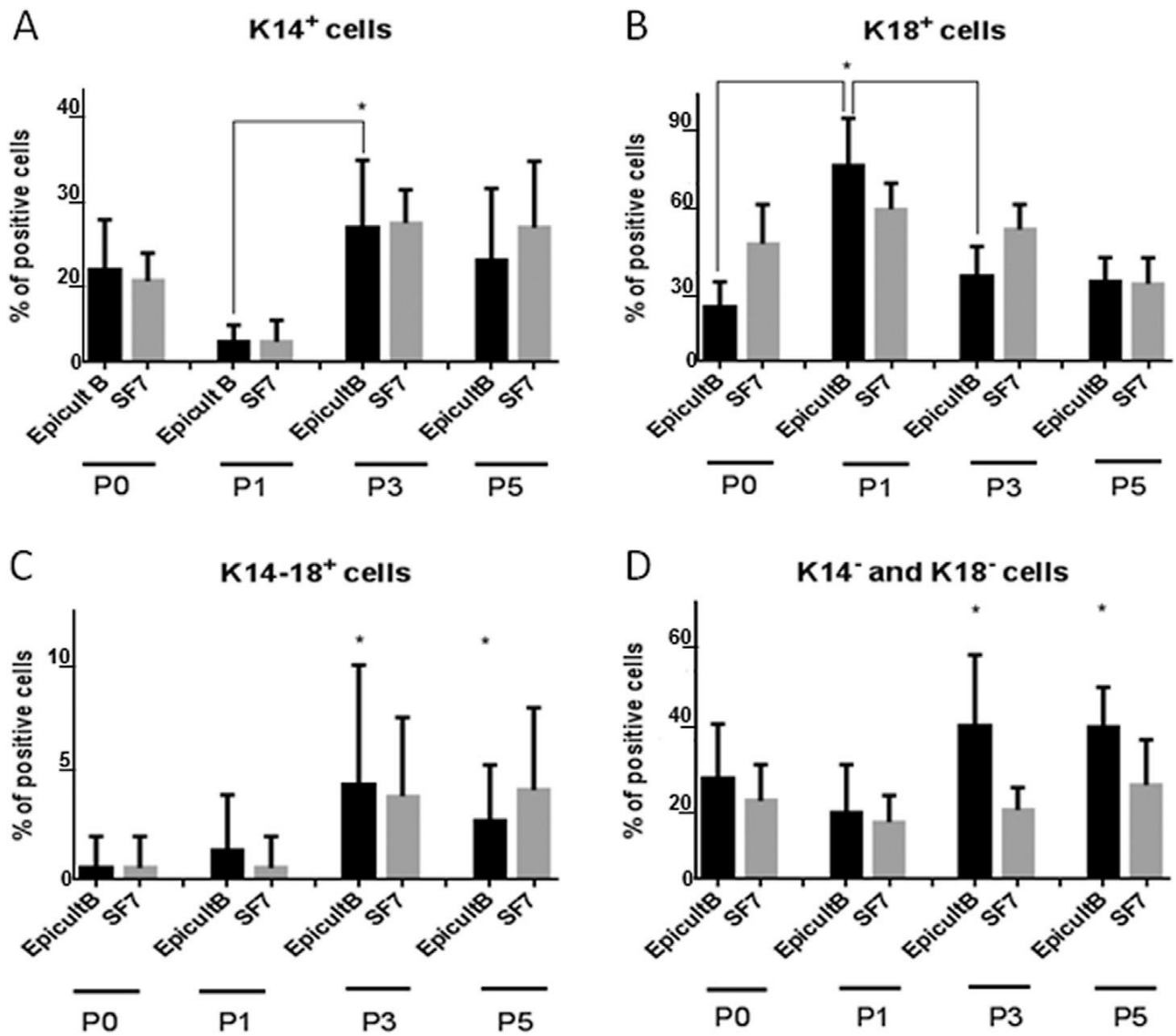
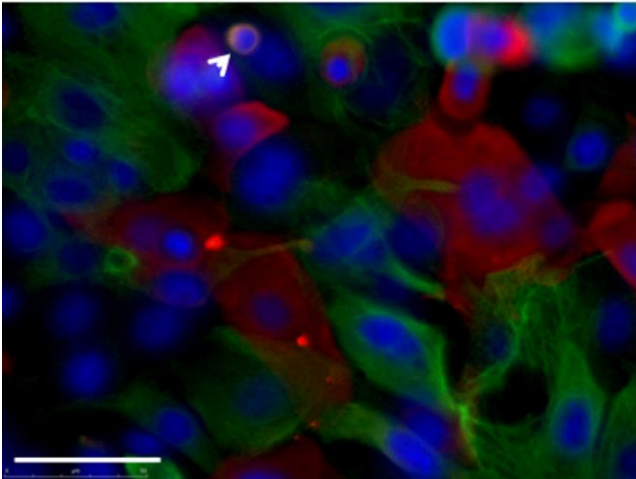


Fig. 3. Variations in CK14 and CK18 positive cells throughout the cell culture in Epicult B and SF7 medium. * means significant difference among passages in the same medium condition (ANOVA analysis, $P < 0.05$).

A



B

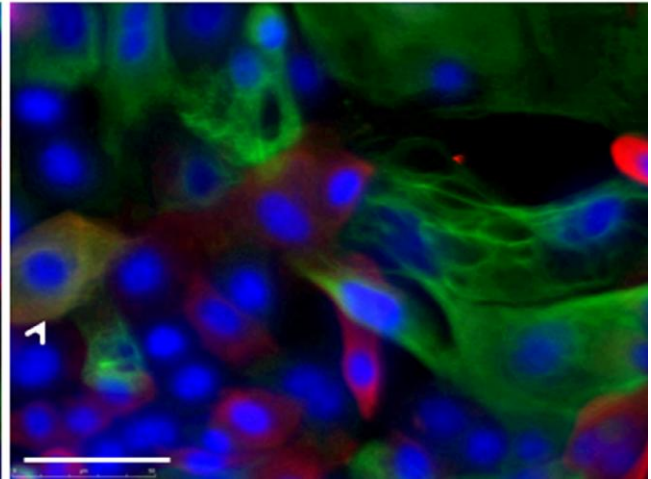


Fig. 4. Expression of CK14 (green), CK18 (red) and CK14-CK18 double positive cells at passage 5 in Epicult B (a) and SF7 (b) medium. Nuclei were detected by Dapi staining. White arrow indicates positive cells for both markers (orange staining).

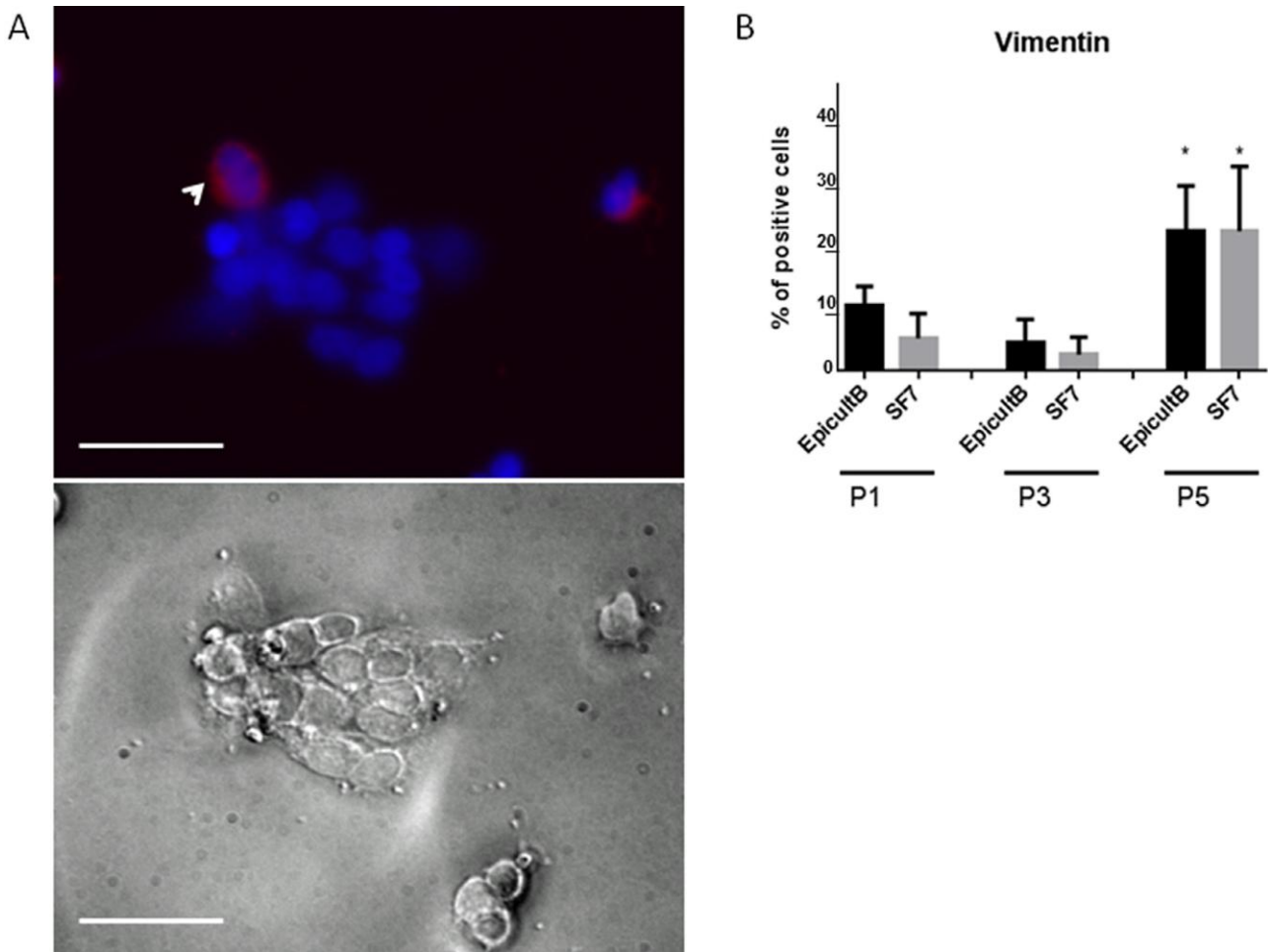


Fig. 5. Expression of vimentin (a, upper panel) and light micrograph (a, lower panel) in epithelial mammary cells culture at P3 in Epicult B (40× magnification). Immunofluorescent labelling staining for vimentin (red) and nuclei (blue, Dapi staining). Panel B shows variations in vimentin expression. * mean significant difference among the different passages for both used medium. ($P < 0.05$, ANOVA analysis). No difference was observed between Epicult B and SF7 medium (Wilcoxon Signed-Rank Test).

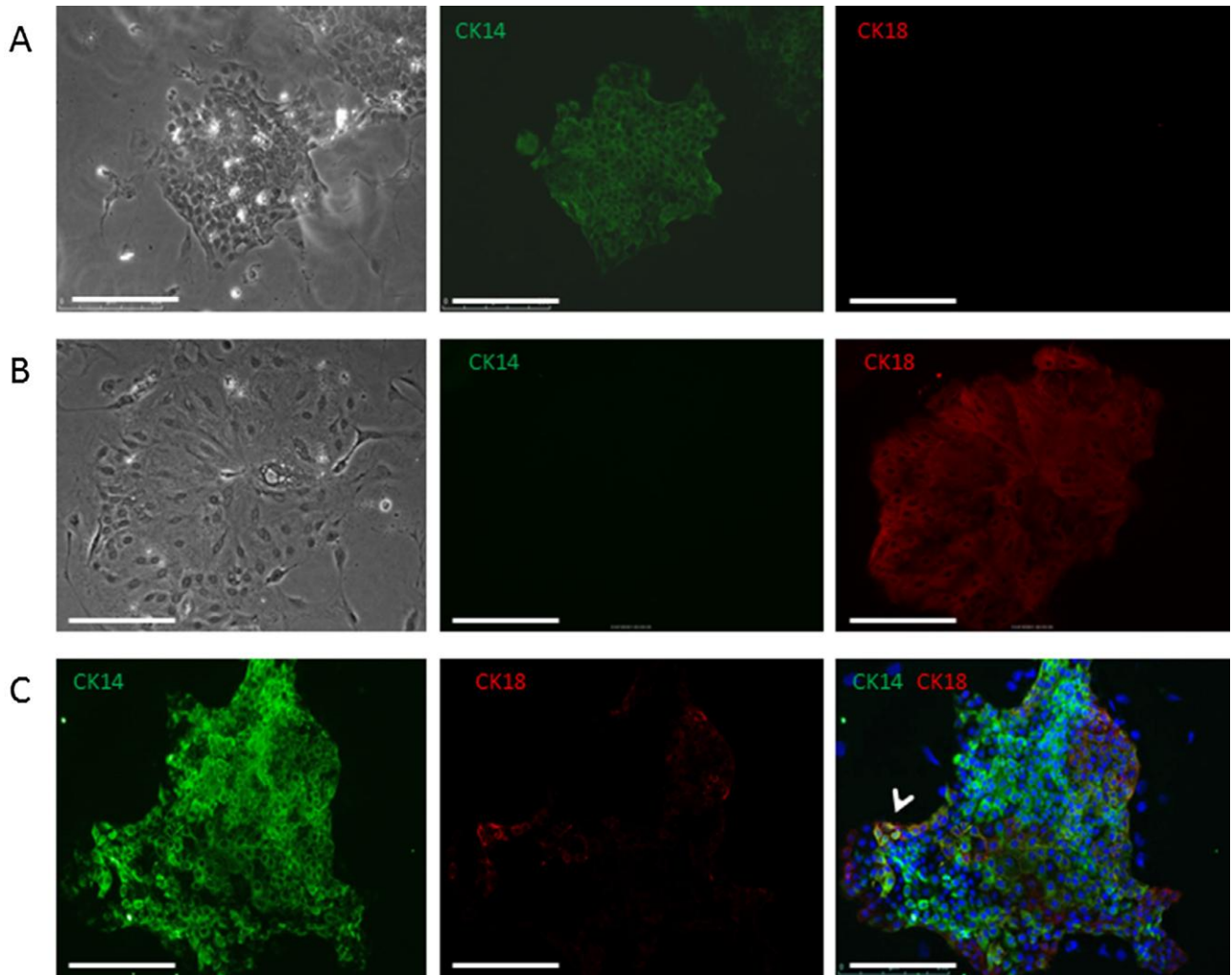


Fig. 6. Representative pictures of a myoepithelial-like colony (panel A), a luminal-like colony (panel B) showing the differential expression of mammary lineage-restricted cytokeratins. Panel C show a novel double positive CK14+ and CK18+ cell population after passage 3 (data not shown) and passage 5. No difference was observed between Epicult B and SF7 medium (Wilcoxon Signed-Rank Test).

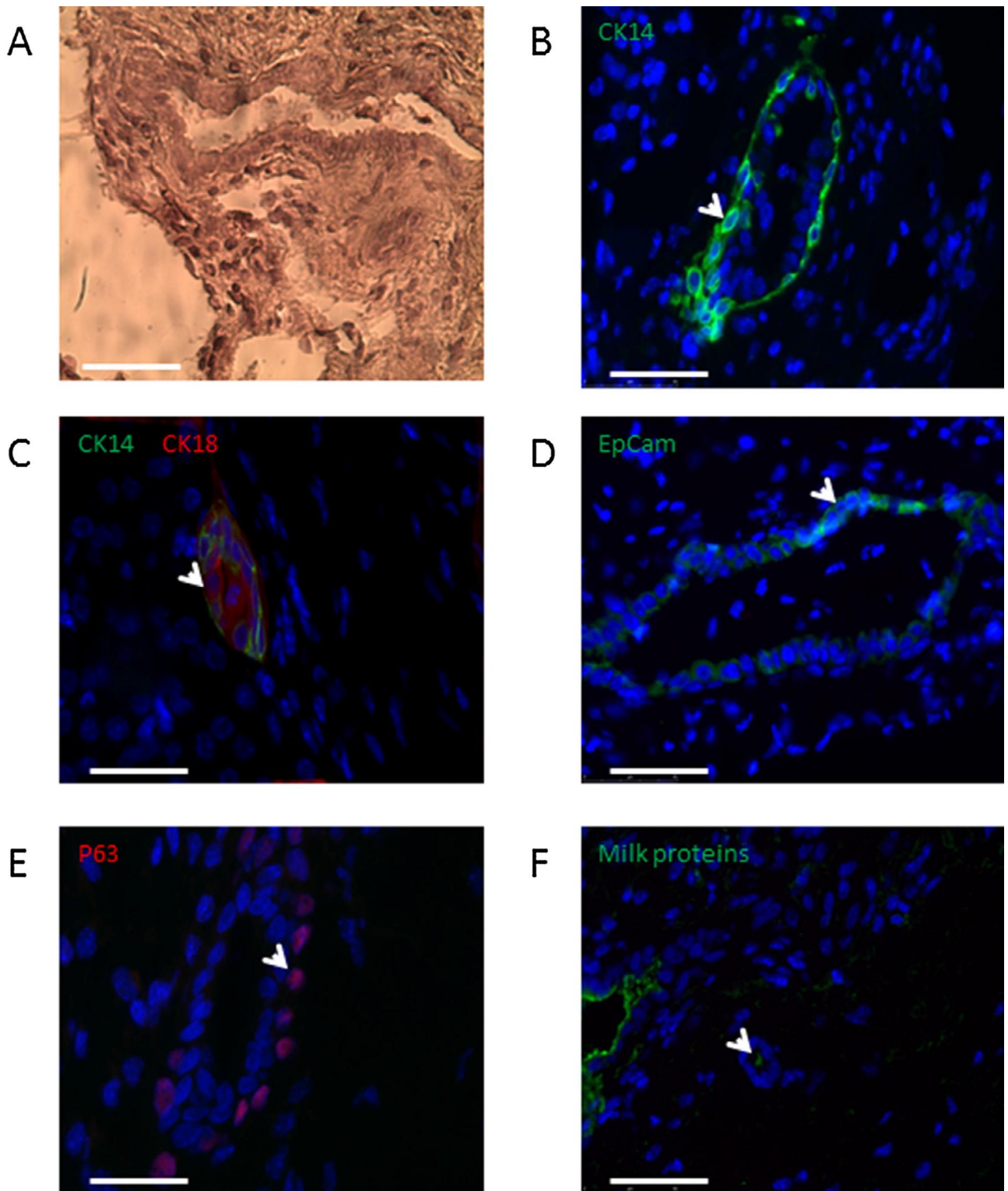


Fig. 7. Expression and spatial localization of detected immunofluorescent markers in regenerated structures in xenograft formed from cells cultured in Epicult B. (A) Hematoxylin and eosin staining; (B) CK14 positive cells in green color; (C) CK 14 (green) and CK18 (red); (D) EpCam staining (green); (E) P63 staining (red); (F) milk protein staining in the lumen. CK14 marks basal cells whereas CK18 marks luminal cells; Epcam marks predominantly luminal compartment and p63 marks basal compartment. Nuclei were counterstained DAPI. Scale bars = 50 μ m (40 \times magnification).

Table 1

The proportional distribution of total clonogenic myoepithelial and luminal progenitors between day 0 (P0) and day 25 (P5) in different medium according to cell morphology.

	P0 Epicult B	P0 SF7	P5 Epicult B	P5 SF7
Myoepithelial-like colony	4.2%	3.0%	4.7%	3.9%
Luminal-like colony	5.1%	3.6%	6.1%	3.2%

Table 2

Percentage of colonies frequency detected from CFC assay after xenotransplant.

	Myoepithelial -like colony	Luminal-like colony	Mixed colony
EpicultB	66%	31%	3%
SF7	62%	30.4%	7.6%