Differential expression of living mammary epithelial cell subpopulations in milk during lactation in dairy cows

UNIVERSITÀ DEGLI STUDI DI TORINO

Differential expression of living mammary epithelial cell subpopulations in milk during lactation in dairy cow

Running title: Characterization of bovine epithelial cells in milk

Baratta M[1], Volpe MG,[1] Nucera D[2], Gabai G[3], Guzzo N[3], Faustini M[4], Martignani E[1]

Affiliations:

1. Dept. Veterinary Sciences, Largo Braccini 2, University of Torino, 10095 Grugliasco (TO), Italy
2. Dept. Agricultural, Forest and Food Sciences, University of Torino,
3. Dept. Comparative Biomedicine and Food Science - University of Padova, Italy
4. Dept. Veterinary Medical Sciences, University of Bologna, Italy.

Corresponding author: Mario Baratta, Dept. Veterinary Sciences, University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy Email: mario.baratta@unito.it

Keywords: somatic cell, milk, bovine, epithelia cells, cell precursors

Abstract

Epithelial cells are shed into milk during the lactation process and whilst they generally reflect the cellular characteristics of terminally differentiated luminal cells, recently the detection of more primitive cells was described in human milk where a cell population of epithelial lineage was detected expressing markers typical of progenitor cells. As differences among putative and differentiated, mature epithelial cells lack unique lineage markers herein we report a flow cytometry assay of somatic cell populations that allows multiparametric analysis of subsets in epithelial cells found in milk. Mammary epithelial cells (MECs) were identified for the expression of precursor and/or differentiation markers by using a single-platform 6-color assay. Cells collected from milk samples of 10 healthy dairy cows and were directly analyzed for 6 different markers: CD45, CD49f, cytokeratin 14 and cytokeratin 18, presence of nucleus and cell viability. Milk samples were collected in three different periods of lactation, early lactation (EL=day 0-day30), mid-lactation (ML=day 90-120) and late lactation (LL= 210-250). Here we identify differential expression of precursors and/or differentiated cells markers in MECs present in bovine milk. Myoepithelial cells as indicated by cells staining positively for cytokeratin 14^{+}/cytokeratin 18^{-} were observed to increase from EL to LL ($p<0.05$) with a high correlation with nuclear staining thus indicating potential proliferative activity. Furthermore, a significant increase in $CD49f^{+}$ and $K14^{+}/CK18^{+}$ positive cells has been detected in LL. This assay is a sensitive approach for evaluating the variations in the frequency and features of living primitive/mature epithelial cells, whose reciprocal balance may be significant in understanding the regulation of these cells in bovine milk. This finding opens new questions on the possible role of epithelial cells as biomarkers in mammary gland functionality in dairy cow.

Introduction

Present within the mammary secretions of all mammals are heterogeneous cell populations that differs between species. In most animals, immune cell types are predominant, in particular lymphocytes, polymorphonuclear neutrophils and macrophages. Most studies of cellular fraction of milk report the total count of somatic cell without taking into account the cell viability and cell types. In bovine species this has been observed as low percentage of epithelial cells compared to that observed in the human (Boutinaud and Jammes, 2002). However, mammary epithelial cells (MECs) are also found in milk, caused by shedding during the lactation phase, but the range of cell frequency is different if only the live cell fraction is analysed. It has been reported the possibility of selecting milk epithelial cells with the features of viable and mature alveolar epithelial cells. If immune cells compartment is generally accepted to be a defense of the body against the state of inflammation and/or infection disease of the gland, very little it is known on epithelial compartment and there has been appreciable supposition concerning the derivation and regulation of these cells (Boutinaud and Jammes, 2002). Renew interest in this observation has occurred recently following the discovery that in somatic cells are present different type of epithelial cells according to a hierarchy of precursor cells: recently the detection of progenitor cells was reported from milk in the human species (Cregan et al., 2007; Thomas et al., 2011). In those studies, it has been described how human milk presents different cell populations of epithelial features (identified by the detection of different cytokeratins) expressing specific markers of progenitor cells like nestin or p63. Furthermore, these cells are able to generate cell colonies of different mammary cell subsets. New questions arise from these observations with respect to the functional role of these cells, in particular the interest of different researchers has been addressed

for investigation of the function of these cells in the breastfed infants and in breast pathology (Hassiotou et al., 2013; Twigger et al., 2013).

In dairy science the amount of total somatic cells (SCs), usually called somatic cell count (SCC), in milk is affected by different factors, such as species, breeds, lactation phase, milk yield, individual animal differences and management practices (Rupp et al., 2000). Both SC count and composition affect milk quality, but their relationship is not steadily related, apart from the case of high SCC corresponding to a high concentration of neutrophils in milk. Usually, it is difficult to analyze cell composition since SCC is a total count that doesn't consider the concentration of any other cell types present in the secretion (Li et al., 2014).

In recent years some studies of SCs by flow cytometry reported new tools in the understanding of the cell subpopulations organization, in particular for immune cells (Albenzio and Caroprese, 2011; Piepers et al., 2009). To our knowledge, however, there are few investigations carried out on epithelial subpopulations in relation to the stage of lactation.

Herein, we described a 6 color-immunophenotyping assay to investigate different living epithelial subpopulations cells present in three different phases of lactation in dairy cow. This approach would give the opportunity to further investigate the modification of these cells according to physiological state and/or pathological or ageing in this species which, of course, is of particular importance both for the production of milk and its relation with human health. The procedure is the first report that manages to simultaneously analyze changes in the percentage of these primitive cell populations in milk.

Materials and Methods

Farms and Animals

The trial was conducted at the farm of the Department of Veterinary Medical Science, University of Bologna (Ozzano Emilia, BO, Italy) and conducted according to the European animal care guidelines. The experimental procedures were approved by the Ethical Committee of Bologna University. Cows in the transition period (from 21 days before the expected calving to 7 days after calving) were housed in a bedded-pack, then moved to a free-stall pen for the rest of lactation. Cows were fed long grass hay and a concentrate mixture (on average 3 Kg of concentrate for every 7 Kg of hay) before calving and received a total mixed ration after calving, distributed once a day in the morning. After forestripping into a foremilk cup, the milkers used paper tissues for udder cleaning. Water was available ad libitum.

Study Design

The general udder health status of all lactating cows was determined by analyzing the SCC (somatic cells count). Based on this data, 10 Holstein-Frisian cows in good condition were chosen to analyze with apparently healthy mammary glands with low SCC values (with low SCC values, <100,000 cells/mL). Three different periods were selected: (first period of lactation or early lactation (EL) day 0-30 D after parturition; mid-lactation (ML) Day 90-120 after parturition; late lactation (LL) Day 210-250 after parturition.

Milk Sampling and Processing

Quarter foremilk samples were obtained in accordance with the Veterinary Services Standards of the Italian National Health Service, branch of the Ministry of Health. Before morning milking, teats

were scrubbed with 70% ethanol and the first 2 strips of milk were discarded. Aliquots of 200 mL of milk per udder were collected aseptically in sterile 50-mL BD Falcon tube (BD, Heidelberg, Germany). Ten milliliters was used for SCC assessment according to International Dairy Federation standards (Hamann J, 1996). Milk sample were diluted in PBS buffer at a rate 1:1 to minimize the influence of lipids on the pellet formation. Cells were isolated from the milk using 2 centrifugation steps at 200 × *g* for 15 min at 4°C. Pellets were then washed in PBS to a final dilution of 0.5-1 × 10^6 cells/100 µL.

Flow Cytometry Analysis

Sample processing

The determination of epithelial subpopulations in milk was carried out according to a six-color flow citometry assay: nuclear staining was evaluated with Vybrant DyeCycle Ruby stain, (Ruby) (Life Technology, TermoFisher, India) and living cell with Live/Dead® Fixable (Violet) Dead Cell Stain Kit, (Life Technology. TermoFisher, India). Anti–CD45 antibody (VMRD Inc., Pullman, WA) was used to gate immune cells; anti-human-CD49f –FITC antibody (anti-h-α-integrin-6-FITC, Novus Biological, CO, USA); monoclonal anti-cytokeratin peptide 18 antibody (clone KS-B17.2, SIGMA, MO, USA), anti-cytokeratin 14 antibody (Covance, Life Technology, TermoFisher, India). Anti-CD45, anti-cytokeratin 14 and anti-cytokeratin 18 antibodies were labeled with respectively QDotS525, QDot605 and PE with SiteClick antibody labeling kits according to manufacturer's instructions (Life Technology, TermoFisher, India).

Briefly, 100-µL aliquots of the cell suspension was incubate with 1ul of Violet stain for 30 mins at 4° C in the dark, after 2 washings (250 × *g* for 5 min at 4°C), in PBS + 0.1 mg/ml BSA (PBS/BSA), anti-CD45-QDot525 (0.7 ug/500,000 cells) and anti-CD49f-FITC (1 ug/500,000 cell) were incubated

for 20 mins at 4 °C in the dark. After twice washing PBS/BSA, cells were fixed and permeabilized using the Intracellular Staining Kit (Invitrogen, Life Technology, TermoFisher, India) according to the manufacturer's instructions. Anti-cytoketarin-18-PE antibody (0.5 µg) and anti-cytokeratin-14-QDot605 antibody (1µg) were incubated in 50 µl adult bovine plasma for 30 min at room temperature in the dark. Cells were washed twice in PBS/BSA and resuspended in 1 mL of PBS/BSA. One µl of Ruby stain was added to each tube and allowed to stand for 5 min at 4°C, then stored in the dark until analysed.

Stained samples were analyzed using an Attune Acoustic Focusing Cytometer (Life Technologies); equipped with two lasers (405 nm and 488 nm wave length). Instrument calibration was checked weekly by use of Attune Performance Tracking Beads (Applied Biosystems, Termofisher, India). Individual compensation setting for each fluorophore was carried out by antibody-capture beads (AbC™Anti-Mouse Bead kit and AbC™Anti-Rat-Hamster Bead kit, Molecular Probes, Life Technologies).

Attune Acoustic Cytometer software and BD FACSDiva ™ software were used for data collection and analysis. From 80.000 to 120.000 events were evaluated from each sample. Cells without antibody labeling served as a negative control and were regarded to be a measure for background fluorescence. Fluorescence Minus One control (FMO) was used to appropriately identify in the context of data spread due to the multiple fluorescent signals. (Baumgarth and Roederer, 2000; Bayer et al., 2007). A first gate (R1) was drawn to define the nuclear staining in all events; a second gate (R2) was drawn to identify the living cells in the somatic cell population, excluding the positive population at Violet stain that indicates dead cell population. R3 (negative) and R4 (positive) were used for the separation of immune cell population by using anti-CD45 positive cells. Subsequently, gating R6, CD49f positive cells were identified. Finally R7 and R10 identified

CK14 positive and CK18 positive subpopulations in the epithelial cells were identified and counted as frequency in the total living $CD45^-$ cell population, respectively.

Statistical Analysis

Statistical analysis was performed with SPSS, version 15.0 (SPSS Inc. Chicago, IL). Samples were compared by using non parametric tests between group differences: differences in cell frequencies between lactation stages were investigated using Kruskall-Wallis test. To analyze cell viability among each time point (EL, ML, LL) Mann-Whithey test was used to compare the means.

Results

Gating strategy

The gating strategy used in this analysis is shown in Fig.1 and based on this strategy, it is possible to observe in parallel the expression of markers of mature differentiated epithelial cells ($CK14^+$ for myoepithelial and $CK18^+$ cells for luminal epithelial compartments, respectively) but also a weak co-expression of both markers in more primitive cells (CK14/CK18 double positive cells). Further it is possible to identify a specific cell subpopulation expressing CD49f with or without co-expression of mature differentiated markers CK14 and CK18.

The fraction of the sample analyzed in terms of the criteria "presence of cell viability" was low, ranging from about 4% to 6% according to lactation phase. The live $CD45^-$-cell population was divided according to the characteristics of the scatter in two sub-populations, the first (subset p2) with reduced values of FSC and with low frequency (about 25% of the subset p1, total population), the second one is the majority (subset p3) with high values of FSC. None of the two populations was expressing CD45. The smaller population expresses intensely CK14, but not CK18, while the larger one expresses, more weakly, CK18. Interestingly, the “small” subpopulation $K14^+$ showed a markedly high intensity for the nuclear staining, (22% of P1). $CD49f^+/CD45^-$ cells were detected but the frequency was low between 4-7 % (See Fig.2).

The mean count of total, living non immune system cell detected in somatic cell population, identified as $CD45^-$ cells were from 28% to 75 % according to the stage of lactation. Tab. 1 shows three different periods starting from parturition (early lactation (EL) day 0-30 D; mid-lactation (ML) Day 90-120; late lactation (LL) Day 210-250 with the frequency of epithelial vs. immune cells. Fig.3 shows the percentage of epithelial cell subpopulations in FL, ML, LL groups. Interestingly we observe a significant difference in the level of myoepithelial cells according to the stage of

lactation with an enhancement from 23% to 36% ($p<0.05$). Furthermore, a tendency to an increase in $CD49f^{+}$ and $CK14^{+}/CK18^{+}$ positive cells may be observed in LL even if statistical differences were not observed in this study.

Discussion

In this study we investigated the frequency of putative primitive or mature differentiated markers for epithelial cells and reported that these cell subpopulations are differentially expressed according to the lactation phase. We focused the analysis on the living cell populations directly in milk samples. The novelty of this multiple analysis resides in the possibility to count at simultaneously living MEC in the sample, allowing a direct comparison between subsets as well as investigation of their possible co-expression. Key advantages of this type of analysis is that the analysis may be performed by using the same samples used for SCC milk quality tests on dairy farms. To our knowledge no many studies have been published in the characterization of this cell population fraction, in particular on the identification of primitive epithelial subpopulations within it. In fact, most of the studies identify the different types of cells from a histological point of view that sometimes gives troubles to verify which were the living population and separated that from the huge amount of dead cells from mechanical shedding and immune defense. Furthermore, a high non-specific background fluorescence signal is observed when cell surface antigen analysis is carried out using flow cytometry on a cell sample which contains a high proportion of dead and dying cell (Wing et al., 1990). Thus, we analyze cell viability in SCC and to determine which are dead and residual in the milk or living possibly related to the physiological state of the animal. For this purpose, we used a nuclear marker to separate cells from debris (that are massively especially in early lactation) and cellular staining for dead cells to gate only living MEC at the specific phase of lactation. In this context we analyzed bovine somatic cell for the epithelial compartment taking into account primitive epithelial subpopulations. We adopted three different antigens that characterize the hierarchy of precursor cells in MEC including CK14, CK18 and CD49f (Martignani et al., 2009; Rauner and Barash, 2012) in addition to a pan leukocyte antigen, CD45 or leukocyte common antigen (Boutinaud et al., 2002).

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 (Capuco et al., 2002). The two different cell lineages can be identified 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 (Eirew et al., 2008), mouse (Mikaelian et al., 2006), goat (Prpar et al., 2012) and bovine (Capuco et al., 2002; Fridriksdottir et al., 2005). 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 (Eirew et al., 2008; Stingl et al., 2006).

One finding of interest is the increase in the sub-epithelial $CK14^{+}$/$CK18^{-}$ cells towards the end of lactation. The reasons for this increase may be due to the gradual exhaustion of the inner secreting cell layer at the end of lactation. Thus, this subpopulation may be considered the signal of a reduction in mammary efficiency. This reduction could have a physiological aspect but also different causes should be considered such as mammary gland pathologies and management related to the methods of milking. The animals were routinely checked for clinical inspection and no pathology at mammary level were detected. For this reason, it would be very interesting to verify with further studies if the increase of this epithelial cell subpopulation is directly correlated with the loss of milk-producing capacity of the mammary gland during the last part of lactation. This hypothesis would be expected this cell population a biomarker of production efficiency of the mammary gland.

The observation that $CK14^{+}$ cells highly correlated with nuclear staining also lead us to presume that DNA content in these cells is significantly high as cells would be in proliferative phase. It has been reported previously as in basal layer basal cells, $CK14^{+}$ cells, migrate into the inner layer of the alveolus before starting the proliferative phase. This observation supports the concept that

cell residing basal layer are the primary proliferative cell population (Ellis and Capuco, 2002). From histological analyses it has been reported in different species that lightly staining cell population present in mammary parenchyma may function as mammary stem cells and that light cells accounted for 50% of the cell proliferation mostly resident in basal compartment (Capuco et al., 2002; Chepko and Smith, 1997). Thus, a question arises why in this last of lactation we detected an increase of epithelial cells with this putative property of high proliferation phase.

The presence of CD49f positive cells, even if in low number, may be related also to the reduction in myoepithelial compartment that indicated modification of the myoepithelials genetic program (Garbe et al., 2012). This integrin has been shown to be component of a feedback circuit that regulates the myoepithelial phenotype in mammary epithelial cells from humans and mice (Deugnier et al., 1999; Labarge et al., 2009), suggesting that the basal regulatory machinery may be disrupted in myoepithelial cells, and inappropriately engaged in luminal epithelial cells, during the aging process. Further studies are necessary to deeply investigate this intriguing hypothesis.

Conclusions

In summary, we have reported the expression of epithelial precursors and fully differentiated cells highlighting possible variations in the number and features of MEC subsets in milk. We also reported some evidence tha may support the theory that different distributions of MEC subpopulations may provide more detailed information on the physiology of mammary gland during lactation in dairy cow and, potentially, have an application to evaluate mammary gland functionality.

Acknowledgments

This work was supported by PRIN 2010-2011. Special thanks to Dr. Claudio Ortolani for critical review in flow cytometry data. We thank for Mrs. Cristina Cecere for technical help.

Conflict of Interest Disclosure

All the authors of this manuscript have no conflict of interest to declare.

Legends

Fig.1 Gating strategy used to separate and count MEC from the somatic cells in bovine milk by multicolor flow cytometry assay. (**A**) Typical distribution in somatic cells in milk on physical parameters total events population, R1; (**B**) Gated on R2, total cells were identified on the basis of nuclear staining for their specific and positive signal for Vybrant DyeCycle Ruby stain marker. (**C**) Gated on R3 events, total living cells were defined as Live/Dead® Fixable Violet Dead Cell Stain negative signal from total R17 cell population; (**D**) Gated R6 to separate MEC ($CD45^-$ cells) from immune cells (R7); (**E**) Gated on R11 to obtained CD49f positive cells in MEC; (**F**) gated on R6 to analyze the differentiated mature MEC (basal compartment CK14 positive cells (R12); secreting cells CK18 positive cells (R15); CK14/CK18 double-positive MEC precursor cells (R13).

Fig.2 Analysis of CD45 negative cell population: (A) P2 and P3 are CD45 negative subpopulation according physical parameters; (B) P2 gated for CK14 positive small subpopulation; (C) P3 subpopulation gated for CK18 expression; (D) P1 population gated on nuclear staining signal: CK14 positive cells (green), CK18 positive cell (red).

Fig.3 Frequency of epithelial cell subpopulations in bovine milk according to different phases of lactation. Cell subpopulation are identifies according to the positively expression of CD49f, CK14, CK18. EL = day 0-30 day after parturition; ML = 90-120 day after parturition; LL = 210-250 day after parturition. * means $p<0.05$

Tab 1. Change in the percentage of total viable cells and the immune compartment in the different periods of lactation.

References

Albenzio, M., and M. Caroprese. 2011. Differential leucocyte count for ewe milk with low and high somatic cell count. J. Dairy Res. 78(1):43-48.

Baumgarth, N., and M. Roederer. 2000. A practical approach to multicolor flow cytometry for immunophenotyping. J. Immunol. Methods 243(1-2):77-97.

Bayer, J., D. Grunwald, C. Lambert, J. F. Mayol, and M. Maynadie. 2007. Thematic workshop on fluorescence compensation settings in multicolor flow cytometry. Cytometry B Clin. Cytom. 72(1):8-13.

Boutinaud, M., and H. Jammes. 2002. Potential uses of milk epithelial cells: a review. Reprod. Nutr. Dev. 42(2):133-147.

Boutinaud, M., H. Rulquin, D. H. Keisler, J. Djiane, and H. Jammes. 2002. Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. J. Anim Sci. 80(5):1258-1269.

Capuco, A. V., S. Ellis, D. L. Wood, R. M. Akers, and W. Garrett. 2002. Postnatal mammary ductal growth: three-dimensional imaging of cell proliferation, effects of estrogen treatment, and expression of steroid receptors in prepubertal calves. Tissue Cell 34(3):143-154.

Chepko, G., and G. H. Smith. 1997. Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal. Tissue Cell 29(2):239-253.

Cregan, M. D., Y. Fan, A. Appelbee, M. L. Brown, B. Klopcic, J. Koppen, L. R. Mitoulas, K. M. Piper, M. A. Choolani, Y. S. Chong, and P. E. Hartmann. 2007. Identification of nestin-positive putative mammary stem cells in human breastmilk. Cell Tissue Res. 329(1):129-136.

Deugnier, M. A., M. M. Faraldo, P. Rousselle, J. P. Thiery, and M. A. Glukhova. 1999. Cell-extracellular matrix interactions and EGF are important regulators of the basal mammary epithelial cell phenotype. J. Cell Sci. 112 (Pt 7):1035-1044.

Eirew, P., J. Stingl, A. Raouf, G. Turashvili, S. Aparicio, J. T. Emerman, and C. J. Eaves. 2008. A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. Nat. Med. 14(12):1384-1389.

Ellis, S., and A. V. Capuco. 2002. Cell proliferation in bovine mammary epithelium: identification of the primary proliferative cell population. Tissue Cell 34(3):155-163.

Fridriksdottir, A. J., R. Villadsen, T. Gudjonsson, and O. W. Petersen. 2005. Maintenance of cell type diversification in the human breast. J. Mammary. Gland. Biol. Neoplasia. 10(1):61-74.

Garbe, J. C., F. Pepin, F. A. Pelissier, K. Sputova, A. J. Fridriksdottir, D. E. Guo, R. Villadsen, M. Park, O. W. Petersen, A. D. Borowsky, M. R. Stampfer, and M. A. Labarge. 2012. Accumulation of multipotent progenitors with a basal differentiation bias during aging of human mammary epithelia. Cancer Res. 72(14):3687-3701.

Hamann J. 1996. Somatic Cells: Factors of Influence and Practical Measure. Pages 9-11 in .

Hassiotou, F., A. R. Hepworth, T. M. Williams, A. J. Twigger, S. Perrella, C. T. Lai, L. Filgueira, D. T. Geddes, and P. E. Hartmann. 2013. Breastmilk cell and fat contents respond similarly to removal of breastmilk by the infant. PLoS. One. 8(11):e78232.

Labarge, M. A., C. M. Nelson, R. Villadsen, A. Fridriksdottir, J. R. Ruth, M. R. Stampfer, O. W. Petersen, and M. J. Bissell. 2009. Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. Integr. Biol. (Camb.) 1(1):70-79.

Li, N., R. Richoux, M. Boutinaud, P. Martin, and V. Gagnaire. 2014. Role of somatic cells on dairy processes and products: a review. Dairy Sci. Technol. 94(6):517-538.

Martignani, E., P. Eirew, C. Eaves, and M. Baratta. 2009. Functional identification of bovine mammary epithelial stem/progenitor cells. Vet. Res. Commun. 33 Suppl 1:101-103.

Mikaelian, I., M. Hovick, K. A. Silva, L. M. Burzenski, L. D. Shultz, C. L. ckert-Bicknell, G. A. Cox, and J. P. Sundberg. 2006. Expression of terminal differentiation proteins defines stages of mouse mammary gland development. Vet. Pathol. 43(1):36-49.

Piepers, S., V. S. De, K. Demeyere, B. N. Lambrecht, K. A. De, E. Meyer, and G. Opsomer. 2009. Technical note: Flow cytometric identification of bovine milk neutrophils and simultaneous quantification of their viability. J. Dairy Sci. 92(2):626-631.

Prpar, S., E. Martignani, P. Dovc, and M. Baratta. 2012. Identification of goat mammary stem/progenitor cells. Biol. Reprod. 86(4):117.

Rauner, G., and I. Barash. 2012. Cell hierarchy and lineage commitment in the bovine mammary gland. PLoS. One. 7(1):e30113.

Rupp, R., F. Beaudeau, and D. Boichard. 2000. Relationship between milk somatic-cell counts in the first lactation and clinical mastitis occurrence in the second lactation of French Holstein cows. Prev. Vet. Med. 46(2):99-111.

Stingl, J., P. Eirew, I. Ricketson, M. Shackleton, F. Vaillant, D. Choi, H. I. Li, and C. J. Eaves. 2006. Purification and unique properties of mammary epithelial stem cells. Nature 439(7079):993-997.

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(2):278-284.

Twigger, A. J., S. Hodgetts, L. Filgueira, P. E. Hartmann, and F. Hassiotou. 2013. From breast milk to brains: the potential of stem cells in human milk. J. Hum. Lact. 29(2):136-139.

Wing, M. G., A. M. Montgomery, S. Songsivilai, and J. V. Watson. 1990. An improved method for the detection of cell surface antigens in samples of low viability using flow cytometry. J. Immunol. Methods 126(1):21-27.