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**Bovine mammary stem cells: new perspective for dairy science**

E. Martignani, D. Cravero , S. Miretti, P. Accornero, M. Baratta

*Dept. Veterinary Science, University of Turin,Grugliasco (TO), Italy*

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Corresponding author:

Mario Baratta. PhD, MVD, Prof

Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy

Tel 00390116709146

Fax 00390112369146

email: mario.baratta@unito.it

**Abstract**

Mammary stem cells provide for net growth, renewal and turnover of mammary epithelial cells, and are therefore potential targets for strategies to increase production efficiency. Appropriate regulation of mammary stem cells can potentially benefit milk yield, persistency, dry period management and tissue repair. Accordingly, we and others have attempted to characterize and alter the function of bovine mammary stem cells. However, research on mammary stem cells requires tissue biopsies which limit the quantity of samples available. Interestingly, different studies reported recently the identification of putative mammary stem cells in human breast milk and new data are available in ruminants for this issue. In this review we summarized the main achievements in this field for dairy cow science and describe the interesting perspectives open to manipulate milk persistency during lactation and to cope with oxidative stress during the transition period. The exciting possibility that stem cell expansion can influence milk production is currently under investigation. The identification of primitive cell types within cow’s milk may provide a non-invasive source of relevant mammary cells for a wide-range of applications.

1. **Introduction**

The discovery of the presence of stem cells and precursors with high regenerative potential in the mammary gland, hypothetically maintained throughout the course of the productive life of dairy cows sheds an interesting light for basic research which aims to clarify all physiological clues that are involved in milk production. Moreover it opens several scenarios in which increasing or maintaining longer the production of dairy cows by extending the productive life span become possible.

In this review, we try to summarize which scientific aspects have been clarified so far, which ones still need to be studied and to highlight future practical applications of the manipulation of these cells. In particular, recent data are discussed, even in comparison with those already collected for the human species, on the role of stem cells found in milk.

Some of the data reported must necessarily be translated from those found in other species, but the history of mammary stem cells is relatively young and we should take in mind that only in 2006 (see below for references) in two laboratories mammary stem cells were first isolated in the murine species.

1. **Biology of stem cells**

Adult stem cells are multipotent, self-renewing cells that are found in adult tissues. They are long-lived cells that are usually quiescent and have the ability to divide asymmetrically to generate committed progenitors (Regan and Smalley, 2007) These progenitors will ultimately undergo an extensive proliferation and will differentiate along the different lineages that can be found in the tissue where they reside. This behavior allows both for maintaining a primitive population and to provide cell turnover for the tissue, either in case of damage or normal wear of older cells (Hennighausen and Robinson, 1998; Kordon and Smith, 1998).

The mammary gland has a peculiar development, since it takes place at different stages of the life of the animal. Most of the development is after birth of the animal and more specifically right before achieving sexual maturity. However the mammary gland undergoes cyclic remodeling throughout the life of the organism: at each oestral cycle variations in the hormonal milieu cause a limited proliferation of mammary epithelial cells which is followed by a regression phase. These changes are much more prominent during pregnancy: dramatic proliferation and morphogenesis take place and result in appearance of many alveoli (the functional unit of the mammary gland) (Capuco and Ellis, 2005). Prolactin and progesterone drive differentiation of epithelial cells towards a secreting phenotype, that is genes coding for enzymes involved in the biosynthesis of milk components are strongly upregulated. When lactation is over, the mammary gland undergoes involution: cell death occurs to reduce cell numbers and the tissue is remodeled at the same time so that the mammary gland returns to a quiescent state.

The extensive proliferation that the mammary gland undergoes at each cycle (ie at each pregnancy) can be sustained only by an adult stem cell population which resides in the tissue for the whole life of the animal and it’s activated at specific stages by signaling pathways dependant on steroid hormones. Several evidences have been provided for the existence of such a population in the mouse species but only in 2006 two independent research groups isolated this mammary cell subpopulation (Shackleton et al., 2006; Stingl et al., 2006) and described the isolation and purification of stem cells through cell sorting that were able to regenerate in vivo a functional mammary gland when transplanted back in the fat pad of recipient mice. Different committed progenitors were also identified. Two cell lineages can be found in the mammary gland: luminal cells which have the ability to secrete milk in the lumen of the alveoli and myoepithelial cells that are contractile and force the milk through the ductal network to the nipples. Lineage restricted progenitors were detected by these groups, but also bipotent progenitors which could differentiate along both cell lineages but lacked the ability to regenerate in vivo a functional mammary gland.

Other groups further explored the mechanisms that regulate the mammary tissue hierarchy and found that progesterone is one of the main player in causing expansion of the stem cell compartment during oestral cycle and pregnancy (Joshi et al., 2010). However other signaling molecules that were previously studied in other stem population (embryo stem cells) were found to have an important role in self-renewal and cell fate determination: focus was drawn on Notch and Wnt family members (Tanos and Brisken, 2008).

Most of the studies on adult mammary stem cells were done in the mouse and human species. Few data were available on the bovine species: the development of the mammary gland in dairy cows is more similar to the human one than the mouse species, but due to the common management procedures which involve fecundation of the cow soon after giving birth the result is a lactation that is mostly overlapping with another pregnancy and no involution can be observed in dairy animal. At the end of the lactation the signals that would induce cell death and tissue remodeling are overrun by proliferative stimuli generated by continuous progesterone production in the ovary (Capuco et al., 2012).

Recently bovine adult mammary stem cells have been functionally identified by our lab (Martignani et al., 2009) and their identity has been confirmed as they are able to regenerate alveoli that secrete milk when transplanted in immunodeficient mice (Martignani et al., 2010). Lineage restricted progenitors were also identified. They are characterized by a limited regenerative potential and lack the ability to produce cells of both mammary epithelial lineages. These data suggest that a hierarchical cell organization exists within the mammary tissue of ruminants: stem cells are maintained as a small population in a quiescent or slowly replicating state and epithelial cells expansion which is associated with oestral cycles or pregnancy is mainly due to the proliferation of progenitors. Similar data were obtained also in small ruminants (Prpar et al., 2012). Purification strategies aimed at selection and isolation of the different types of progenitors or stem cells however proved difficult due to lack of antibody specific for the bovine and goat species. Recently mammary cell populations based on the expression of specific markers were sorted: basal cells, luminal cells, progenitors and stem cells were found to have a differential expression of these markers, potentially allowing for their isolation and purification (Rauner and Barash, 2012).

However showing the existence of adult mammary stem cells and their putative phenotype in the bovine mammary gland is just the first step on the path of understanding the mechanisms that regulate homeostasis of stem cells and drive the differentiation process.

1. **Mammary stem cell role during mammary gland development, pregnancy and lactation in cow**

We have just mentioned how the complicated and extensive modifications which the mammary gland cyclically undergoes are attributed to adult stem cells, or rather, only to stem cells that have the replication potential to support significant waves of proliferation that determine extensive remodeling during pregnancy (Capuco et al., 2002). These cells may also be involved in the replacement of the epithelial cells that exfoliate into the lumen of the ducts during lactation. There are different populations of progenitors organized with a clear hierarchy: the most primitive cells (stem cells) give rise to all cell types and in all structures, while the progenitors that are derived from them have reduced multipotency, despite their greater number. They are therefore only partially differentiated, and will undergo several divisions, each of which represents a step towards terminal differentiation. In cattle, after an initial quiescent phase, at 2-3 months of age the mammary gland returns to its development: the ducts are developed as extremely compact and greatly branched structures surrounded by loose connective tissue. The elongation of the ducts takes place through a coordinated process of simultaneous growth, branching and extension of the terminal ductal units (TDU) and proliferation of the surrounding connective tissue that invades the mammary fat. When puberty is reached, the mammary growth ends and only small changes are observed during the succession of estrous cycles due to associated hormonal changes. Very different is the situation that occurs when pregnancy happens. In this case the bovine mammary gland undergoes a massive development which starts immediately after fertilization and lasts until parturition: the growth of the epithelium takes place through an increase in secondary branching of the alveolar structures for cell proliferation, which is followed by the cell terminal differentiation(Capuco et al., 2002; Capuco and Ellis, 2005). This important remodeling phase has the purpose either to drastically increase the number of secreting cells and to induce the terminal differentiation, thus the expression of a series of genes that are essential for the biosynthesis of the various components of the milk. The close relation between the progenitor cells with the total mass of epithelial cells that compose the parenchymal portion of the mammary gland acquires a crucial importance when considering the issue of productivity in dairy cattle. Since the amount of milk that a cow is able to produce at any time is closely related to the number of secreting epithelial cells that are present in the tissue, the more the dairy cow will be able to maintain a high number of these cells, the higher will be its production. It has been in fact demonstrated in the lactation curve of dairy cow that after an initial increase in production due to a greater secretory capacity of the epithelial cells, cell number is kept relatively constant and the slow decline of productivity is due to a slow and steady reduction in the number of epithelial cells. This reduction is due to a low apoptotic rate and a slightly lower proliferative activity of mammary epithelium which nonetheless guarantees a constant turnover of secreting cells throughout lactation (Capuco et al., 2003). In this scenario, it is evident that even limited variations in the proliferative activity of stem cells and progenitors may have an important effect on the productivity of the cow. It is also useful to remember that the normal practice of rearing dairy cattle provides that the animal be fertilized about three months after parturition: in this way the time during which the cow is unproductive is minimized, since the subsequent lactation can be initiated shortly after finishing the previous one. The period that elapses between two lactations is defined dry period. If normally in a mammal an involution of the mammary gland has been observed at the end of lactation which reverts back the gland to a quiescent state, this does not occur in dairy cattle. Concomitant pregnancy produces a hormonal pattern that contrasts sharply with the end of lactation and in particular proliferation and mammary morphogenesis is strongly promoted (Choudhary and Capuco, 2012). There is then a phase of regenerative involution concomitant with the dry period, in which the mammary gland does not come to a tissue remodeling, but rather to a consistent cell turnover. This process is crucial to ensure the production of large quantities of milk in later lactation. It is unclear how this massive proliferation should be supported by a population of primitive cells that are "activated" by a specific hormonal framework that ensures the necessary cellular catabolism. It is suggestive that the dry period represents a critical moment for the productivity of dairy cows and the homeostasis of the progenitor cells compartment at this stage is crucial. The significant metabolic stress to which the cows undergo during lactation and the progressive aging of the animal are factors that can affect both these aspects, causing a reduction in the proliferative capacity of progenitors and consequently lower productivity of the animal.

1. **Mammary stem/progenitors cells in milk**

The most common methods for the recovery of mammary cells to analyze their phenotype and their functional properties require the recovery of tissue from the mammary gland, and subsequently its dissociation. This approach may not be possible in case it is necessary to repeat the analyses on the same animal for long periods of time and at regular intervals, as repeated tissue recovery through biopsy may be unacceptable for the welfare of the animal.

Recently the isolation of progenitor cells was described directly from milk in the human species (Cregan et al., 2007; Thomas et al., 2011a). In these works it is shown that in human milk there is a cell population of epithelial identity (shown by the expression of several types of cytokeratins) expressing markers typical of progenitor cells such as nestin, or p63 and that it is able to generate colonies of different mammary subsets, which expand in culture for short periods of time. In human this finding is now paving the way for investigation of the functions of these cells in the breastfed infant and the use of breast milk as a tool to understand the normal biology of the breast and its pathologies (Hassiotou et al., 2013; Twigger et al., 2013).

Milk contains epithelial cells, colostral corpuscles, polymorphonuclear leukocytes, mononuclear phagocytes and lymphocytes(Crago et al., 1979; Smith and Goldman, 1968; Thomas et al., 2011a), with those of epithelial lineage forming the main bulk of cells within two weeks of establishing lactation (Ho et al., 1979). Few years ago it has been hypothesized that these epithelial cells are shed from the ductal and luminal epithelial layers through either a heightened turnover of the secretory tissue, or as a consequence of the mechanical shear forces associated with the continued filling and emptying cycle associated with breast milk synthesis and lactation. The first reports identified putative mammary stem cells from human breast milk through their expression of various cytokeratin markers, CK5, 14 and 19 and nestin (Cregan et al., 2007). Very recently new data available on these cells demonstrated in the cellular component of human breast milkshowing markers associated with haemopoietic, mesenchymal as well as neuro-epithelial lineages. The positive expression of various primitive stem/progenitor cell markers like CD133, Stro-1, nestin and the presence of SP within the total cell population of human breast milk suggests that breast milk may be a novel source of putative stem/progenitor cells (Fan et al., 2010). Furthermore, it has been reported that, at least human milk, contains stem cells with multilineage properties. Breast milk cells from different donors displayed variable expression of pluripotency genes normally found in human embryonic stem cells. These genes included the transcription factors OCT4, SOX2, NANOG, known to constitute the core self-renewal circuitry of hESCs (Hassiotou et al., 2012).

In these years our research group has collected tissue samples from mammary glands from puberty to ten-lactations old cow to measure mammary progenitors frequencies ad we reported an age-dependent distribution of these primitive cells (Martignani E and Baratta M, 2010). These data were gathered by collecting samples of mammary tissue that were subsequently enzymatically digested in order to obtain a single cell suspension that was used to carry on the analysis. However the data points represent only a single moment in the bovine productive life without providing information on variations associated with specific conditions (quiescence, pregnancy, early vs late lactation). Given the technical difficulties and welfare concerns associated with performing repeated biopsies throughout the productive cycle of a dairy cow, as other authors have reported, milk might be an alternative source for progenitor cells. As we previously reported, luminal progenitor cells from the mammary gland possess a high activity of the Aldehyde dehydrogenase 1 (ALDH1) enzyme, which can be detected by administration of a suitable substrate to the cells.

1. **Mammary stem cell role in transition period**

During transition period dairy cows can suffer for an excessive metabolic load and may experience a decreased in the immune system function, thus increasing the risk of disease. Dry period can be consider the time to rest for the mammary gland. During the dry period the mammary epithelial components regress, proliferate and differentiate with the ultimate goal of maximizing milk production during the subsequent lactation. In this period the mammary cells loss is insignificant and therefore the terms involution and regression may be inappropriate. Dry period is necessary to permit reparation or replacement of damaged, lost or senescent epithelial cells and to increase the percentage of cells in the mammary gland prior to the next lactation (Capuco et al., 1997). Cell numbers reflects a balance between cell proliferation and apoptosis. In non pregnant cows, a decline in milk production is expected as lactation progresses, due to changes in cell number rather than to changes in cell activity. In pregnant cows the decline in production may also be related to a secretory capacity reduction. Mammary stem cell manipulation in cow that result in increased cell proliferation or decreased apoptosis will improve the persistency of the lactation curve (Stelwagen et al., 2013). It is evident that variations, no matter how limited in the proliferative activity of stem cells and progenitors may have an important effect on the productivity of the cow. There is then a phase of regenerative involution concomitant with the dry period, in which the mammary gland does not come to a tissue remodeling, but rather to a consistent cell turnover. This process is crucial to ensure the production of large quantities of milk in later lactation (Steeneveld et al., 2013). In this scenario dry period represents a critical moment for the productivity of dairy cows and how homeostasis compartment of progenitor cells at this stage is essential.

Finally, the study of mammary stem cells may shed new light on the ongoing discussion on the most appropriate time of the transition period. In fact, significant metabolic stress to which the cows undergo during lactation and the progressive aging of the animal are factors that can cause a reduction in the proliferative capacity of progenitors and a consequently lower productivity of the animal. The dry period also plays a particularly delicate role in dairy cow in relation to animal well-being, which greatly influences the management when considering the next stage of lactation. Short dry period do not allow for a good regeneration of the mammary tissue, even if they can lower the negative energy balance problems after calving. On the other hand, an extremely long dry period increased body condition of the cows. Multiparous cows can benefit of a short dry period, instead primiparous cows would produce less milk in the second lactation (Annen et al., 2004).

1. **Mammary stem cell and oxidative stress**

An important issue in the management of dry period concerns the oxidative stress (OS) is the result of an imbalance between reactive oxygen species production (ROS), and neutralizing capacity of antioxidant mechanisms (Mantovani et al., 2010). The accumulation of ROS can result in substantial damage to mammalian tissues and aging, and OS is often associated with numerous pathologies (Sordillo and Aitken, 2009). In the dairy cow, at least three interrelated sources of OS can be identified during the transition period. The first OS source is related to the extreme shifts in the cellular metabolism and mobilization of body reserves. The consequence of such an increase of metabolic demand is an augmented rate of ROS production (Bernabucci et al., 2005; Castillo et al., 2005). Secondly, a significant contribution to OS is given by the immune system. After parturition, bacterial contamination can induce a neutrophilic influx into the uterine lumen. Activated neutrophils kills phagocytized organisms by oxygen-dependent mechanisms (respiratory burst), and contribute to the generation of ROS, which can result in tissue damage and inflammation (Bernabucci et al., 2005; Loiselle et al., 2009).

The involuting mammary gland can be a source of OS during the period of intensive mammary epithelial cell replacement (Silanikove et al., 2005), occurring soon after termination of milking and characterized by cell apoptosis and tissue remodelling. Although the complete omission of the dry period has a negative effect on milk production in the ensuing lactation (Collier et al., 2012), many authors consider the reduction/omission of the dry period as an alternative model for milk production (Grummer et al., 2010), in particular because an improvement of energy balance and metabolic status can be envisage (Rastani et al., 2005). An interesting line of research therefore is trying to understand the relationship between oxidative stress and therefore regulation of mammary stem cells. In human it has just proposed that ROS produced by accumulated milk breakdown post-weaning may be the mechanism underlying the selective involution of secretory alveolar luminal cells (Thomas et al., 2011b).

1. **Perspective and future challenge**

The milk-production industry has a significant impact on most national economies. In Europe more than 91 bilions liters of milk are produced annually with costs ranging from 30 to 90 euro per 100 Kg according to the country (Hagemann et al., 2012)(IFNC Dairy report 2011). The sharp rise in the price of grain, as nutrients but now also for fuel production, endangers the milk industry worldwide.. Since global overproduction has been eliminated by increasing milk consumption in the Orient, the ability to meet consumer demand is at risk. The dairy cow is characterized by a typical milk-production curve that rises shortly after parturition, is maintained for a variable period of time and then decreases for most of the milking period. When the lactating cow is pregnant, lactation is terminated at least 40 days before the following parturition, that is the dry period during which the animal is preparing for the successive lactation and does not produce milk. As lactation progresses, a correlation exists between the decline in mammary epithelial cells and the decrease in milk production, with only a very small fraction (0.4%) of the epithelial cells proliferating during that period. Mammary stem cells provide for net growth, renewal and turnover of mammary epithelial cells, and are therefore potential targets for strategies to increase production efficiency. Appropriate regulation of mammary stem cells can potentially benefit milk yield, persistency, dry period management and repair of mammary tissue damaged by mastitis (Capuco et al., 2012). Cows that tend to maintain their peak yield for longer than average are referred to as persistent. Increased persistence is associated with increased milk production and economic profit. Persistent cows are also healthier since they are exposed to less stress caused by consecutive pregnancies and deliveries and lower metabolic, fertility and health risks. Based on murine models and pioneering experiments in cattle, manipulation of the proportion of mammary stem cells appears to be feasible. In theory, persistency in high levels of milk production could double annual milk yield. Thus, an attractive approach to improving the efficiency of milk production involves maintaining the initially high level of milk secretion over a longer period of time. The reduction in the number of "dry periods" also contributes to the efficiency of lactation. Our hypothesis is that the self-renewal potential of the gland (i.e. number of stem cells able to undergo asymmetric division) is the cellular basis for persistency.

Secondarily, the high amount of milk produced over a long time, could allow for the recovery of primitive cells from the mammary gland in a non-invasive way. This could prove a simple and rapid method which can easily provide the necessary amount of cells to monitor the functional status of the bovine mammary gland. It is therefore possible to assume that the complete characterization of these cells in the milk can become a diagnostic tool for the examination of aging of the breast and functionality which can be compromised by infectious processes or poor management, in particular during the transition period.

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