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Bovine mammary stem cells studies – current status – a review*

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Bovine mammary gland is a unique organ with regard to its frequently repeating cycles of growth and involution throughout the life of an animal. In literature there is only scarce information concerning development of its specific epithelium and in particular about the stem and progenitor cells. Knowledge about this subject is essential in terms of further improvement of dairy cows production capacity in the future. It may also provide an answer to the question concerning the lack of carcinogenesic transformation in bovine mammary gland, as frequency of tumours in this tissue equals zero. The morphological features of human mammary gland are more similar to those in cattle than in rodents, commonly used for carcinogenetic studies. The results obtained on the bovine model may also constitute a basis for understanding fundamentals of carcinogenesis in human mammary gland.

To confirm the presence of mammary stem cells, many *in vitro* and *in vivo* studies were conducted with the use of transplantation, electron microscopy, functional techniques, flow cytometry, scanning cytometry and microarrays. Unfortunately, until now no universal molecular marker was found which could make it easier to identify these cells. It has been suggested that the population of cells Sca-1^{pos} CD45^{neg}, for which the lack of steroid receptors is characteristic, may indicate the stem cell population. In our current studies it was found that in the mammary gland tissue, parallel to Sca-1^{pos} CD45^{neg} cells, a population of Sca-1^{pos} CD45^{pos} cells exists which might be of hematopoietic origin. This non-epithelial lineage may enrich the stem/progenitor cell population in the mammary gland facilitating mammary gland renewal.

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Mammary gland is an unusual tubulo-alveolar structure which plays a key role in reproductive strategy of mammals. In spite of various studies carried out on different species, the bovine mammary gland still seems to be an enigmatic structure. It is the result of significant differences between morphology of the mammary gland, as well as its hormonal regulation of growth and lactation in different species [Tucker 1979, Akers 1990, Hovey *et*. 1999, Capuco *et al.* 2002].

The functional part of the mammary gland is composed of an inner layer – luminal epithelial cells surrounded by an outer layer – contractile myoepithelial cells. The fundamental unit is based on actively growing structures called terminal end buds (TEB) in rodents, or grape-like structure – terminal ductule lobular unit (TDLU) in human and ruminant gland [Capuco *et al.* 20026]. Strong histological similarities in ruminant and human mammary development may make cattle a good alternative to using rodents for mammary studies [Ellis and Capuco 2002]. It should also be emphasized, that the expression of the estrogen (ER) and progesterone receptor (PR) within the mammary tissue, and the relationship of ER/PR status to cell proliferation is similar between human and bovine mammary gland [Capuco *et al.* 2002, Capuco 2007]. Moreover, it is interesting that the incidence of mammary cancer in cattle equals zero [Swett *et al.* 1940, Povey and Osborne 1969]. There is a chance that understanding the processes occurring in bovine mammary gland cells will eventually provide clinical benefits for treatment of human breast cancer patients.

Stem cells play a key role in regenerative potential of mammary gland

From an economic point of view gaining control over mammary epithelial cell proliferation may be a key to increasing milk production in dairy cattle [Ellis and Capuco 2002, Martignani *et al.* 2009]. Remarkable regenerative capacity apparent during successive reproductive cycles implicates the existence of stem-like cells that enable the expansion, turnover and replacement of the cell populations in the mammary gland [Capuco and Ellis 2005]. In the year 1959 DeOme [1959] discovered that murine mammary gland has an unexplainable regenerative potential, which he related to the presence of distinct cells that can divide generating both ductal and lobular components of the mammary epithelium. These cells were defined as mammary adult stem cells. Transplantation studies demonstrated that small parts of mouse mammary tissue derived from donor mice could engraft cleared fat pads and these outgrowths could be serially passaged for up to seven transplant generations of donor-derived mammary cells [Daniel 1968]. Kordon and Smith [1998] showed that an entire mammary gland could be regenerated with the progeny of a single cell following transplantation of tissue fragment into cleared mammary fat pads. Multiparameter cell sorting allowed better isolation of the cells and promoted further research on the regenerating potential of a single cell [Shackleton *et al.* 2006, Stingl *et al.* 2006].

During *in vivo* studies of mouse mammary gland Smith [1996] proved the existence of cell population which exhibited proliferative capacity – progenitor cells. Presence of different phenotypes of progenitor cells was shown, one of them leading to secretory lobules outgrowths, other only to ductal outgrowths. Quoted author distinguished also the third phenotype of progenitor cells, which after transplantation led to mixed development of ductal and lobular outgrowths.

Morphological characterization of mammary stem cells

Although numerous recent reports have described existence of stem/progenitor cells in both human and mouse mammary tissue, properties of these cells, their relations and interactions remain poorly understood [Stingl 2009]. Ultrastructural studies have allowed classification of the mammary epithelial cells (MEC) into several subclasses. First and second class contain undifferentiated type I stem/progenitor cells, within which small light cells (SLC) and large light cells (LLC) are distinguished. Third class contains terminally differentiated large dark cells (LDC) – Smith and Medina [1988]. Existence of faintly stained cells was described in MEC of different species: human [Fergusson 1985], mouse [Smith and Medina 1988], rat [Chepcko and Dickson 2003] and prepubertal bovine [Ellis *et al.* 2002]. Spotting difference between stem and progenitor cells is almost impossible because both cell types are morphologically identical [Chepco and Smith 1997]. Both can be characterized by a high nucleus to cytoplasm ratio and a complete lack of cellular organelle, e.g. mitochondria and microfilaments. Lack of microfilaments explains why SLC present such wide diversity of shapes. Another distinct feature of stem cells is the absence of the proteins forming gap junctions [Holland *et al.* 2003]. This also contributes to the shape diversity. Ellis and Capuco [2002] found SLC both single and in couples, which suggest asymmetric and symmetric mitotic divisions. Similarly they have found pairs of both SLC and intermediate cells. This cell type is found in all three tissue zones (cisternal – the parenchyma surrounding the gland cistern, outer – peripheral parenchymal region and medial) in all age groups (2, 5, 8 months). Presence of SLC was confirmed in two year-old, non-pregnant heifers in ultrastructural studies [Motyl *et al.* 2011], which confirms the hypothesis, that these cells are present in the mammary gland of animals of every age. Additionally, Ellis and Capuco [2002] described that SLC frequently contact the ductal lumen and occasionally span the entire distance from basement membrane to ductal lumen. Basally located stem cells are slow-cycling cells which have the ability to respond to the environmental influences and propagate additional stem cells or differentiate along a specific cell line. Some of the basal cells migrate into the suprabasal epithelial layers before undergoing mitosis. Adult mammary gland stem cells appear quiescent and are able to remain in the G_0 state for a prolonged period [Ellis and Capuco 2002]. A method of identification and localization by staining with [^{3}H]-thymidine or bromodeoxyuridine (BrdU) is based on this property. These agents bind a newly synthesized chain of DNA during replication [Cotsarelis *et al.* 1990,

Tumbar *et al.* 2004]. They bind to DNA of all cells present in the tissue, however, in fast proliferating cells they become quickly diluted. Stem cells are slowly proliferating cells and they keep the dye longer in their genome. Mammary epithelial cells which showed the ability of keeping the dye were called the BrdU- label retaining epithelial cells (LREC) – Capuco *et al.* [2009]. It is commonly stated that stem cells are the only cells, which have the possibility of keeping DNA labels included in their genome during mitotic separation [Patt *et al.* 1980, Ellis and Capuco 2002, Zhang *et al.* 2007]. An alternative theory was presented by John Cairns in 1975. His "hypothesis of eternal chain" claimed that stem cells during asymmetric mitotic separation, direct the newly synthesized chain to the cell intended for proliferation, where the matrix DNA is placed in child stem cell [Cairns 1975]. Until now this theory is the source of many controversies [Lansdorp 2007, Rando 2007].

Molecular markers of mammary stem cells

Stem cells, as long-living cells, must be protected against toxic agents. Similarly to tumor cells they are capable of active disposal of toxic agents including stains and dyes. Stem-like cells have the ability to efflux Hoechst 33342 dye [Goodell *et al.* 1996, Alvi *et al.* 2003], which is associated with the expression of transporter proteins from the ABC (ATP Binding Cassette) family. Zhou *et al.* [2001] showed that verapamil, a chemical inhibitor of the ABC protein family of transporters inhibits the efflux of Hoechst in these cells. The ability to eject dye, combined with verapamil-induced blockage of this feature is a most common way for distinguishing the side population (SP), created by stem/progenitor cells. The side population (SP) in bovine mammary gland was estimated as a 0.48% of total cell number [Motyl *et al.* 2011]. Unfortunately, as the determination of the cells is based on their ability to efflux the dye, rather than contain them, it requires constant attention from the observer. Thus, this phenomenon cannot be the basis for automated sorting or quantitative analysis. Studies based on the flow cytometry, showing SP of cells actively ejecting Hoechst dye, suggest the presence of ABC transporters, e.g. Bcrp1 (Breast cancer resistance protein 1) or Mdr-1 (Multi-drug resistance protein 1). However, the evaluation of the presence of these proteins in bovine MECs done with the use of immunofluorescently-labelled antibodies resulted in unspecific binding [Motyl *et al.* 2011]. There are two possible explanations of this observation. Either the primary antibodies had a low affinity to bovine antigens, or several differences in the channel structure between the species exist. Dissimilarities in the type of channel markers were shown in Jonker *et al.* [2005] experiments on mouse and human mammary gland. They observed that SP is not completely ablated on treatment of Mdr1a/1b nulls with a specific and efficient BCRP/Bcrp1 inhibitor. This suggests that another Hoechst 33342 transporter may be upregulated upon the loss of Mdr1a/1b. On the other hand, Merino *et al.* [2009] showed the presence of inter-species allelic variants of ABCG2 transporters in human and murine mammary cell lines.

One of the biggest obstacles in the quest for stem cells is the lack of type-specific differences in the protein expression patterns, which would be helpful in their description. Stem cells have very limited mechanisms of the protein production, which limits the variety of available surface markers. So far the common marker for all stem cells has not been found [Welm *et al.* 2002, Kolek *et al.* 2008]. It seems that some of the following may be helpful in the localization of the stem/progenitor cells: CD24, CD29 (β1 integrin), CD49f (α6 integrin), CD14, CD61 (β3 integrin) and Sca-1 (stem cell antigen-1) – Stingl [2009]. There are some markers described for the human mammary stem/progenitor cells, e.g. EpCAM (epithelial cell adhesion molecule) also known as CD326 or ESA (epithelial specific ant0069 gen), CD49f and MUC1 to a lesser degree [Stingl *et al.* 1998, 2001, Villadsen *et al.* 2007, Eirew *et al.* 2008, Raouf *et al.* 2008, Stingl, 2009]. Functional identification of bovine mammary stem/progenitor cells was done by Martignani *et al.* [2009]. Studies were conducted on a single cell suspension, taken from a 7 month-old Red Angus heifer. It was found, that cells isolated after 4-5 days created three types of colonies. Classification was made on the basis of cell and colony morphology and the expression of specific markers for luminal (CK18, CK19) and myoepithelial (CK14) cells. Colony A had myoepithelial phenotype ($CK18^{neg}$, $CK14^{pos}$), whereas B and C colonies presented luminal phenotype ($CK\ 18^{pos}$, $CK14^{neg}$). Transfer into immunodeficient mice after 3 or 4 weeks enabled to find spherical-shaped structures, organized in a double layer around the lumen. The different colony morphologies suggest the existence of different progenitor populations, each one with a limited differentiation capability.

Bone marrow as a potential source of stem cells in mammary gland

The presence of very small embryonic-like stem cells (VSELs) was described in adult murine organs (pancreas, thymus, spleen, heart, brain), however, the assessment excluded the mammary gland [Ratajczak *et al.* 2008]. These cells were $Oct4^{pos}$, Lin^{neg}, $CD45^{neg}$ and $Sca\text{-}1^{pos}$, were smaller than 5µm, with a characteristic high ratio of the magnitude nucleus to the cytoplasm. Particular attention was focused on the stem cell antigen 1 (Sca-1) which is regarded as a typical molecular marker for multipotential hematopoietic stem cells (HSCs). Its presence was described in mouse mammary stem cells. It has been shown that Sca-1 is an 18 kDa protein from Ly-6 family (Leukocyte antigen). Welms [2002] showed that $Sca\text{-}1^{pos}$ cells retain the ability to contribute progeny to all cell types required to produce normal epithelial outgrowth. There were three stem cell subpopulations defined within the population of mammary epithelial cells: PR^{pos}, $Sca\text{-}1^{pos}$ and one classified as the side population (SP). These results suggest that the localization of the $Sca\text{-}1^{pos}$ is not mutually exclusive with the classification to the SP and a subpopulation $Sca\text{-}1^{pos}$, belonging to SP exists. Research on prepubertal cows showed stem cells expressing ER α and β^{neg} (progenitor cells are ER^{pos}) – Capuco and Ellis [2005]. Unfortunately, there was no reference to the expression of Sca-1. Immunofluorescence studies on mammary tissue of 2 years-

old heifers revealed that Sca-1 did not co-localize with ER [Motyl *et al.* 2011]. The sparsely distributed Sca-1^{pos}, ER^{neg} cells were localized in the basal area of mammary epithelium, without contact with the luminal population of mammary gland cells. These results constitute a basis for hypothesis, that this is a population of very primitive and poorly differentiated cells. Our experiment [Motyl *et al.* 2011] gave opposite results to the study conducted by Sleeman *et al*.. [2007], who showed that in the mammary gland the basal cell population has a CD24$^{+/low}$ Sca-1^{neg} phenotype, while the luminal cells show two types of staining patterns: CD24$^{+/high}$ Sca-1^{neg} ER^{neg}, or CD24$^{+/high}$ Sca-1^{pos} ER^{pos}. This indicates differences in expression of Sca-1 among species.

The fact, that Sca-1 is a marker typical for multipotential HSCs suggests a theory of non- mammary origin of the adult stem cells. Hypothetically, non-mammary cells may be sequestered and reprogrammed to perform mammary epithelial cell functions and to adopt mammary epithelial characteristics during reconstruction of epithelium in regenerating mammary tissue *in vivo* [Sangai *et al.* 2006, Jiang *et al.* 2010]. Our studies on transcriptomic profile of Sca-1^{pos} cells compared to Sca-1^{neg} cells confirmed this theory [Motyl *et al.* 2011]. They revealed a list of 547 genes that were differentially expressed in the populations of Sca-1^{pos} and Sca-1^{neg} cells. Among the statistically significant genes the biggest groups were represented by transcripts that encoded selected regulatory molecules (58), receptors (54), signalling molecules (43), nucleic binding proteins (39) and transcription factors (38). Gene ontology analysis showed that the identified genes take part in biological processes such as: signal transduction (141), development (72), protein metabolism and modifications (54), cell structure and motility (51,) as well as immunity and defence (65). The last group was represented mainly by genes that were up-regulated in the Sca-1^{pos} cells. The more detailed analysis revealed that a large group of these genes is typically expressed in cells of hematopoietic origin [Motyl *et al.* 2011]. This may suggest that the small population of Sca-1^{pos} cells can be formed by cells that do not originate from epithelial lineage, but may enrich the niche of MSCs from bone marrow and participate in the self-renewal of the mammary gland during lactation cycles. Earlier Sangai *et al*. [2006] showed a potential role of bone marrow (BM)-derived, circulating cells in the development of mouse mammary gland. It has been noted that the BM-derived cells are able to rescue postnatal mammary gland development of sublethally irradiated mice [Gouon-Evans *et al.* 2000]. Their further experiments with the use of BM transplantation model in mice showed that the BM could serve as progenitors for myoepithelial cells and periductal fibroblasts of the mammary gland [Sangai *et al.* 2006]. The BM cells obtained from female GFP Tg, and transplanted into mammary gland of sublethally irradiated mice were located in the basal layer of ductal epithelial cells. A similar localization of Sca-1^{pos} cells was observed by Motyl *et al.* [2011]. Many other reports indicate that BM can be the source of progenitor cells for different types of tissue, i.e. skeletal muscle [Shimizu *et al.* 2001, Corbel *et al.* 2003, Zuba-Surma *et al.* 2008], heart [Jackson *et al.* 2001, Orlic *et al.* 2001] and liver [Alison *et al.* 2000, Lagasse *et al.* 2000]. However, our recent study [Motyl *et al.* 2011] on

co-localization of Sca-1 and CD45 (marker of hematopoetic cells) in mammary gland tissue of nonpregnant heifers revealed four cell subpopulations: Sca-1^{neg} $CD45^{neg}$, Sca-1^{pos} $CD45^{pos}$, $Sca1^{pos}$ $CD45^{neg}$ and $Sca1^{neg}$ $CD45^{pos}$. The dominant subpopulation is Sca-1^{neg} $CD45^{neg}$ represented by fully differentiated epithelial, myoepithelial and the stromal cells. $Sca1^{pos}$ $CD45^{neg}$ cells, located in the basal layers of epithelium, are probably stem/progenitor cells of mammary origin. On the other hand, Sca-1^{pos} $CD45^{pos}$ subpopulation localized mainly outside the mammary ducts corresponded to the nondifferentiated cells of BM origin. The last subpopulation of Sca-1^{neg} $CD45^{pos}$ belongs to the differentiated cells of blood origin (probably leucocytes), located in the lumen of blood vessels and infiltrating the connective tissue. This may suggest that all Sca-1^{pos} cells earlier identified by Motyl *et al.* [2011], are stem/progenitor cells of both mammary gland and bone marrow origin that constitute the source of epithelial, myoepithelial and stromal cells during mammogenesis and mammary gland remodeling.

In conclusion we claim, that it is much likely that the number and unique morphological and molecular structure of stem cells predispose the mammary gland to a certain type of growth. If so, it is important to find the solid and substantial characteristics of stem cells responsible for certain features of the gland. The determination of these characteristics should include a comparison of the number and molecular properties of stem cells in the mammary gland of cows of different phenotypes (milk and meat). The fundamental question arises: to which extent high productivity of mammary gland in dairy cows is dependent on the number and molecular features of stem/progenitor cells; and/or also the action of endo- and auto-paracrine factors which stimulate them to proliferate and differentiate? The answer to this question might be the key to identify factors determining the growth of mammary gland and, what follows, of milk production. Maybe, it will be possible to increase the mammary cell number and milk yield potential by regulating the number and activity of stem/progenitor cells.

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