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# Quantification of bovine mammary stem/progenitor cells by laser scanning and flow cytometry*

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**Due to the presence and activity of mammary stem cells (MaSC), growth and remodelling of mammary gland during puberty and lactation cycles is possible. In this study the number of putative mammary stem/progenitor cells was examined in 20 months old non-pregnant Holstein-Friesian heifers. Cells were double-stained with fluorescent dye-conjugated antibodies against stem cell antigen-1 (Sca-1) and fibronectin type III domain containing 3B (FNDC3B), and were analysed using scanning cytometry and flow cytometry. Nuclei were counterstained with Hoechst 33342. Scanning and flow cytometry revealed 2.43±0.32% and 1±0.37% of MaSC in total cell number, respectively. Sca-1$^{pos}$FNDC3B$^{pos}$ cells did not express estrogen receptor (ER*α*), confirming their undifferentiated phenotype. In conclusion, scanning cytometry is a preferable method for evaluation of the number and localization of MaSC *in situ*, whereas, flow cytometry with cell sorting enables further genomic and biochemical analyses of isolated cells.**

**KEY WORDS: flow cytometry / mammary gland / progenitor cells / scanning cytometry / stem**

To confirm the presence of mammary stem cells (MaSC), a number *in vitro* and *in vivo* studies on rodents were conducted with the use of transplantation experiments, electron microscopy, functional techniques, flow cytometry, scanning cytometry, microarrays and mammosphere cultures. Unfortunately, until now a universal molecular stem cell marker for identification of these cells, has not been found. There are similarities as well as inter-species differences in the expression of cell-surface markers on the epithelial subsets [Lim *et al*. 2010]. There are only few reports on

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quantification of stem/progenitor cells in bovine mammary gland. Capuco [2007] described a method of MaSC identification based on stain (BrdU) retention by stem cells. Stem cells appear to retain labelled DNA for extended periods because of their selective segregation of template DNA strands during mitosis. These cells are described as label-retaining epithelial cells (LREC). BrdU-label-retaining epithelial cells were detected immunohistochemically and quantified. The size of bovine LREC population on average amounted to 0.4%. Research done on mouse and human mammary gland models revealed that Sca-1 can serve as a good marker of MaSC population [Deugnier *et al.* 2006]. In our previous study the number of Sca-1 positive cells located in mammary tissue of 2-year-old heifers was estimated by scanning cytometry [Motyl *et al.* 2011]. The analysis revealed that cells expressing Sca-1 protein comprised about 2% of total cell number in tissue sections. Sca-1 positive cells were Erα-negative indicating a possibility that Sca-$1^{pos}$ cells form a population, which localizes in the mammary stem/progenitor niches, and is important for the renewal of bovine mammary gland during development and tissue regeneration. Transcriptomic analysis of Sca-$1^{pos}$ cell population in comparison to Sca-$1^{neg}$ cells showed that the differentially expressed genes were involved in biological processes, such as signal transduction, development, protein metabolism and protein modifications, cell structure, motility, immunity and defence.

The present study compared two cytometric methods – scanning cytometry and flow cytometry – used to quantify bovine mammary stem/progenitor cells. To identify these cells a new combination of two molecular markers: stem cell antigen-1 (Sca-1) and fibronectin type III domain containing 3B (FNDC3B) were applied.

## Material and methods

### Tissue sampling

Mammary tissue was obtained at slaughterhouse from 20 months-old non-pregnant Holstein–Friesian heifers (n = 10), free of clinical signs of mastitis. Udders were removed and mammary tissue was collected and fixed for immunohistochemistry, scanning cytometry and flow cytometry.

### Immunohistochemistry (IHC)

Samples were fixed in 4% phosphate-buffered formalin and after 48 h stored in 70% ethanol (POCH S.A., Gliwice, Poland) until further processing. Tissues were dehydrated and paraffin embedded, according to standard histological technique. The paraffin blocks were cut into 5 μm slices, which were then mounted on silanized microscope slides. Next slides were de-paraffined in xylene and hydrated in a graded series of ethanol to PBS. For antigen retrieval slices were heated in a microwave (650 W) in 400 ml of 10 mM citrate buffer (ph 6.0) according to Capuco [2007]. Next, they were rinsed with PBS. The slices were then labelled with rabbit anti-FNDC3B polyclonal antibody (Santa Cruz Biotechnology), in a 1:200 dilution overnight in

darkness at 4°C. DAKO Envision system was used according to manufacturer's protocol. Additionally a standard protocol for HE staining was performed. The results were analyzed on Bx-60 light microscope (OLYMPUS), using Microimage software (OLYMPUS).

**Immunofluorescent staining of tissue sections for scanning cytometry**

Paraffin tissue samples were prepared as in the IHC staining. For scanning cytometry tissue slices were labelled with rabbit anti-FNDC3B polyclonal antibodies (SANTA CRUZ Biotechnology) and Alexa Fluor® 647-conjugated anti-rabbit secondary antibody, and mouse anti-Sca-1-FITC-conjugated antibody (BD PHARMINGEN, USA). Nuclei were counterstained with Hoechst 33342. Scanning cytometry analysis was performed using Olympus Scan^R screening station (OLYMPUS Poland, Sp. z o. o., Warsaw, Poland), and combined analysis software (SCAN^R Analysis v. 1.3.03). The results were statistically evaluated using Microsoft Excel 2003 software (MICROSOFT Corporation, Redmond, WA, USA).

**Immunofluorescent staining, flow cytometry and isolation of mammary stem/progenitor cells**

A cumulative sample of approximately 200 g of mammary tissue was obtained within 20 min after slaughter, and transported to the laboratory in 500 ml Hank's balanced salt solution with $Ca^{2+}$ and $Mg^{2+}$ (HBSS, Sigma-Aldrich, USA) containing penicillin G (100 μg/ml), streptomycin (100 μg/ml), gentamicin (100 μg/ml), and fungizone (5 μg/ml), at 20°C. All antibiotics were purchased from Life Technologies, INVITROGEN, USA. Further procedures were performed under sterile conditions in laminar flow hood according to the protocol of Wellnitz and Kerr [2004] with own modifications. Tissue samples were minced with opposing scalpels to 1-5 $mm^3$ pieces, rinsed several times with HBSS to remove blood, and placed into 1L baffled Erlenmeyer flask containing 250 ml of digest mixture (HBSS with 0.5 mg/ml collagenase IV, 0.4 mg/ml DNAse I, 0.5 mg/ml hyaluronidase I–S, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone). All enzymes were purchased from Sigma-Aldrich. The digestion proceeded for 3 h in an orbital shaker (37°C; 100 rpm). Cells were then filtered through metal strainers (1 mm pore size, purchased in SIGMA-ALDRICH, USA) and centrifuged for 5 min at 150×G. Pellets were resuspended in 1/2 volume HBSS, filtered through metal strainers (0.5 mm pore size, purchased in SIGMA-ALDRICH, USA), and centrifuged for 5 min at 150×g. Pellets were resuspended in 1/2 volume HBSS, then filtered through cell strainers (100 μm; BD FALCON, San Jose, CA, USA) and centrifuged for 5 min at 150×G. The same procedure was applied two more times, with cell strainers of 70 and 40 μm diameter (BD FALCON, USA) in order to obtain single cells. After the last centrifugation the cells were resuspended in PBS at $10^6$ cells per ml. Cells were stained with anti-Sca-1-FITC antibody (BD PHARMINGEN, USA), rabbit anti-FNDC3B polyclonal antibody (SANTA CRUZ BIOTECHNOLOGY) and ALEXA FLUOR® 647-conjugated anti-rabbit secondary antibody. Cell viability was

evaluated by co-staining the cells with propidium iodide (2 μg/ml). The cytometric analysis was performed using FACSAria™ cell sorter equipped with 375-nm near UV laser, 488 nm (blue) laser, and 633 nm (red) laser (BD BIOSCIENCES, San Jose, CA, USA), and next Sca-1$^{pos}$FNDC3B$^{pos}$ and Sca-1$^{neg}$FNDC3B$^{neg}$ cells were sorted. Cell samples and collection tubes were maintained at 4°C.

**Analysis of ER*α* expression**

After cell sorting RNA from Sca-1$^{pos}$FNDC3B$^{pos}$ and Sca-1$^{neg}$FNDC3B$^{neg}$ cells was isolated using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. Isolated RNA samples were dissolved in RNase-free water, and RNA quantity was measured using NanoDrop (NANODROP TECHNOLOGIES Inc., Wilmington, DE, USA). A two-step RT-PCR was performed. Total RNA was reversely transcribed to cDNA by using Enhanced Avian HS RT-PCR Kit (SIGMA-ALDRICH, USA) according to the reaction conditions recommended by manufacturer. Next, real time-PCR was performed with a STRATAGENE Mx3005P QPCR System (AGILENT TECHNOLOGIES, USA) using SYBR® Select Master Mix kit (APPLIED BIOSYSTEMS, Life Technology, Canada). ERα transcripts were amplified using primers: forward 5'- GAAGTGGGCATGATGAAAGG-3' and reverse 5'-AAGGTTGGCAGCTCTCATGT-3'[Kenngott *et al.* 2011], and GAPDH (housekeeping gene) was amplified using primers: forward 5'- CTTCAACAGCGACACTCA -3' and reverse 5'-CCAGGGACCTTACTCCTT-3'. The PCR reaction involved two-step preincubation: 2 min at 50°C, and 2 min at 95°C, followed by 40 cycles composed of denaturation (95°C for 15 s), annealing (58°C for 15 s), and extension phase (72°C for 1 min), followed by: 1 min at 95°C, 30 s at 55°C, and 30s at 95°C. Additionally, the obtained products of amplification were electrophoresed on 2% agarose gel with ethidium bromide, and then visualized by a UV transilluminator. Images were taken using Kodak 1D 3.5 visualization system (NEW HAVEN, CT, USA).

**Results and discussion**

To identify putative mammary stem/progenitor cells we applied double labelling with anti-Sca1-FITC-cojugated antibody [Motyl *et al.* 2011] and antibody against fibronectin type III domain containing 3B (FNDC3B) co-stained with ALEXA FLUOR® 647-conjugated secondary antibody [Choudhary and Capuco 2012]. These two markers are considered to be putative markers of mammary stem/progenitor cells. Since Sca-1 is expressed not only by epithelial stem/progenitor cells but also by non-differentiated cells of bone marrow origin it cannot be used as a single marker, defining a stem cell population. Therefore, in the present study we decided to use an additional potential stem/progenitor cell marker – FNDC3B to determine the epithelial stem/progenitor cell population. According to Choudhary and Capuco [2012] FNDC3B is a novel MaSC marker. The authors demonstrated that FNDC3B positive cells showed increased telomerase activity, which is a feature characteristic of stem and progenitor

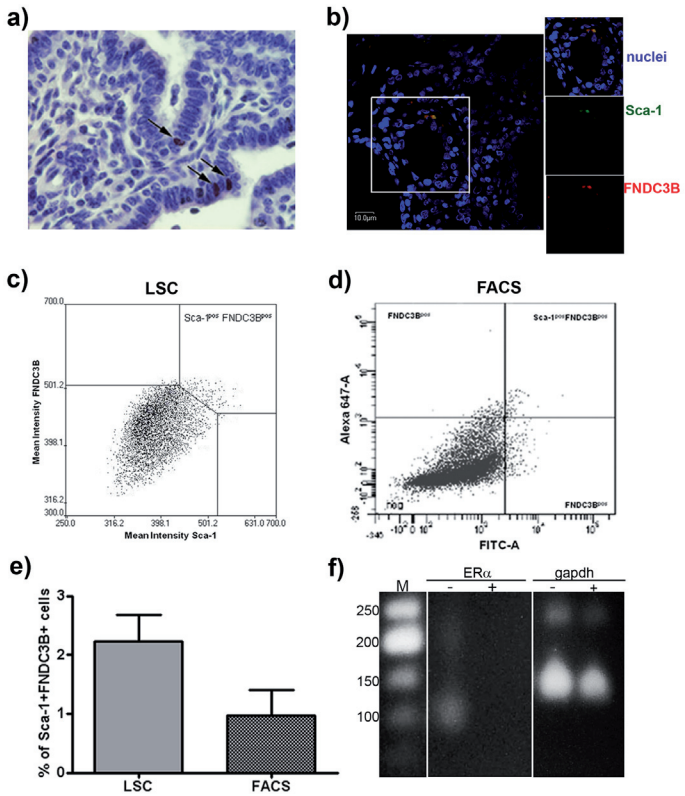


Fig. 1. Characterization of bovine mammary stem cells (MaSC) population; **a)** representative immunohistochemical staining of mammary tissue sections, showing localization of FNDC3B$^{pos}$ cells in HF heifer's mammary tissue (black arrows), image taken under 20× lens of bright field microscope, nuclei were stained with H&E; **b**) representative confocal images of mammary tissue sections stained with anti-Sca-1 and anti-FNDC3B antibodies (green and red fluorescence, respectively), showing localization of Sca-1$^{pos}$FNDC3B$^{pos}$ cells in HF heifer's, nuclei were stained with Hoechst33342 (blue fluorescence), **c)** representative cytogram acquired by scanning cytometer (LSC), showing the population of mammary cells expressing Sca-1 and FNDC3B antigens in HF heifers; **d**) representative cytogram acquired by flow cytometer (FACS), showing the population of Sca-1$^{pos}$FNDC3B$^{pos}$ cells in HF heifers; **e)** comparison of the number of putative MaSC obtained by LSC and FACS analyses, expressed as the percentage of Sca-1$^{pos}$FNDC3B$^{pos}$ cells in total population of mammary epithelial cells, the results are presented as means ± SD; **f)** estrogen receptor alpha (ERα) and GAPDH (reference gene) expression in Sca-1$^{pos}$ FNDC3B$^{pos}$ (+) and Sca-1$^{neg}$ FNDC3B$^{neg}$ (-) cells, expression was examined by RT-PCR in cells sorted by FACSAriaII.

cells. Immunohistochemical analysis of FNDC3B expression in mammary tissue sections of heifers revealed its localization mainly in the basal layers of epithelium (Fig. 1a). The co-localization of Sca-1 and FNDC3B allowed more accurate identification of putative MaSC located preferentially in the ductal epithelium (Fig. 1b). We assessed their number using both scanning cytometry (Olympus Scan^R screening station and

combined analysis software) and flow cytometry with cell sorting (FACSAria™, BD Biosciences) (Fig. 1c, d). The scanning cytometry of Sca-1$^{pos}$FNDC3B$^{pos}$ in tissue sections revealed 2.43±0.32% of total cell number. Application of flow cytometry provided results showing significantly lower number of double labelled cells, which only accounted to 1±0.37% of total cells analysed (Fig. 1e). The undifferentiated state of Sca-1$^{pos}$ FNDC3B$^{pos}$ cells was additionally confirmed by comparison of the expression status of estrogen receptor alpha (ERα) gene in Sca-1$^{pos}$FNDC3B$^{pos}$ *vs*. Sca-1$^{neg}$FNDC3B$^{neg}$ cells. Real-time RT-PCR results revealed that only Sca-1$^{neg}$FNDC3B$^{neg}$ cells expressed ER*α* transcripts (Fig. 1f).

Comparing these two methods, the question arises which of them gives more accurate results and a wider range of possibilities. The scanning cytometry gives the possibility to use collected, preserved and stored over time tissue samples. It allows using the same material in a number of stainings. It also enables to compare mammary glands of many animals in the same staining procedure without the risk of errors in subsequent repetitions. This method enables to locate cells with a specific phenotype in heterogenous tissue structure [Godlewski *et al*. 2009], estimating stem/progenitor cell number without interfering in tissue structure and gives the possibility to visualize counted cells. It allows for assessment of the quality and specificity of the staining and helps to refine the methodology. On the other hand, only flow cytometry and cell sorting gives the possibility of isolation of the putative stem/progenitor cells, which allows conducting further research on these cells. For example, we were able to further confirm the undifferentiated state of Sca-1$^{pos}$ FNDC3B$^{pos}$ cells on the basis of their ER*α* gene expression status (Fig.1f), taking into account previous reports demonstrating that ER*α*$^{neg}$ cells isolated from the mammary tissue have a high stem/progenitor activity [Sleeman *et al*. 2007, Capuco *et al.* 2012]. The lower number of estimated putative progenitor/stem cells obtained using fluorescence-activated cell sorting (FACS) could be caused by their loss during isolation and limited vitality of these cells. During the procedure of cell sorting we observed that the number of sorted Sca-1$^{pos}$FNDC3B$^{pos}$ cells decreased along with the extending sorting time.

In conclusion, scanning cytometry is a preferable method for evaluation of mammary stem/progenitor cell number with their simultaneous visualization and localization within mammary tissue, whereas, flow cytometry with cell sorting allows for their more detailed genomic and biochemical analyses.

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