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Author(s)	Abdelfattah-Hassan, Ahmed; Saadeldin, Islam M.; Ghonimi, Wael
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Instructions for use

Optimization of the isolation process of putative bovine mammary stem cells

Ahmed Abdelfattah-Hassan [1,*], Islam M. Saadeldin [2] and Wael Ghonimi [3]

[1] Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

[2] Department of Physiology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

[3] Department of Histology and Cytology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

*Corresponding Author: Ahmed Abdelfattah Hassan, E-mail: ahmdolla@yahoo.com, a.abdelfatah@zu.edu. eg

Abstract

Mammary gland stem cells are a dormant cell population inside the mammary gland that are cable of differentiation into its all cell types. Increased knowledge on bovine mammary stem cells is of useful importance to the dairy industry, as they are important to mammary gland development and tissue maintenance. Also, bovine mammary stem cells seem a good model for human breast cancer, since mammary cancer is not observed in cows. Proper isolation technique for the putative mammary stem cells is an essential step for the succeeding approaches and experiments using these cells. Therefore, this study was designed to evaluate different steps for the optimum isolation of putative bovine mammary stem cells. This included sites for sampling from the Bovine mammary gland, tissue dissociation steps and different culturing conditions. The optimum conditions for isolating and culturing putative bovine mammary stem cells were sampling as near to the base of the quarter as possible and using 1 hour dissociation step, followed by culturing the cells in tissue culture flasks coated with 100 μg/mL bovine collagen type-1.

Key words: *MaSC, Isolation protocol, Immunofluorescence, Collagen, dissociation time.*

Introduction

Mammary stem cells (MaSC) are an example of adult stem cells that are found in small percentages inside the organ and can differentiate upon request (signals) into more committed cells to re-populate that organ[7]. The mammary gland ability to grow through cycles of gestation, lactation and drying-off throughout cow's lifetime is proposed to be attributed to stem cells that reside inside the mammary gland[10].

A study by Kordon and Smith[5] revealed that a single cell can regenerate an entire mammary gland; following transplantation into cleared mammary fat pads. Also, these cells have been a target for studying cancer stem cells in human breast cancers[7]. Understanding the function of MaSC is essential for discovering treatment strategies for breast cancer and also for the possible use of these cells in regenerative medicine. The isolation and characterization of MaSC is an essential first step toward elucidating their functioning and uses in different species. Unfortunately, in domestic animals, such as cows, scarce information on MaSC isolation is available to date, emphasizing the need for investigating more in this subject.

The identification of mammary stem cells can

be done through the expression of certain cell markers, such as vimentin and cytokeratins (that are known MaSC markers from studies on humans and mice). Vimentin, is used as a mammary stem cells marker in humans[6, 12], and it is negatively expressed on bovine mammary epithelial cells[3]. Cytokeratin 19 (CK-19) have been validated as an in-*vitro* marker of mammary stem cells in humans[11], while cytokeratin-18 (CK-18) is an epithelial cell marker[3].

Scarcity of an optimized and clear protocol for bovine mammary stem cells isolation and of the articles that describe the proper isolation and identification procedure can lead to less clear or even misleading results obtained by different groups. Therefore, the objective of the present work was to evaluate different isolation conditions (site of sampling, dissociation time and using bovine collagen in culture) for the isolation and identification of putative bovine mammary stem cells and outlining the most suitable protocol for obtaining the best results.

Materials and Methods

Sampling:

Samples were taken from apparent healthy mammary gland quarters from 7 Holstein-Frisian cows that were send to a local slaughterhouse in New York (USA). Cow's management and care was as previously reported[1, 9]. Cows suffering any pathologic condition such as systemic diseases, apparent mastitis or with a history of mastitis in one or more quarters were excluded. This study was approved by the Animal Care and Use Committee of College of Veterinary Medicine, Cornell University.

After euthanasia and bleeding of the cows, the whole mammary gland was removed to the specimen worktable (clean and far from air currents and water splashing). The sampling area was cleaned with antiseptic solution and the samples were aseptically obtained from three regions of the same quarter (near the base, at the middle and near the teat) into sterile tubes containing ice–cold sterile 1x PBS (Gibco®, USA) with 2% antibiotic and antimycotic solution [Ab-Am] (Corning, USA), and directly transferred to the laboratory at 4°C.

Isolation procedures:

The isolation procedures were done using sterile materials and under sterile conditions. The samples were washed three times with sterile 1x PBS with 2% Ab-Am, removed into sterile petri dish and cut into small pieces (1 mm^3) using a sterile scalpel blade. The small tissue pieces of the mammary gland were transferred into a sterile bottle, containing 100 mL of 0.1% or 0.5% (w/v) Collagenase (Worthington, USA) in DMEM (Gibco®, USA), then incubated over a magnetic stirrer and the dissociation step was performed at 37°C for three different periods: either 30 minutes, 1 hour or 3 hours.

After that the tissue homogenate was filtered with 100 μm and then 40 μm pore diameter nylon filters (Falcon®, USA), to obtain single cell suspension. The cell suspension was washed twice by centrifugation at 400g for 5 minutes and the pellet was resuspended in 1 mL mammary stem cell medium. The mammary stem cell medium constituted DMEM/F12 (Gibco®, USA), 10% FBS (Atlanta Biologicals, USA), 2% B-27 (Gibco®, USA), 2% Ab-Am (Corning, USA) and 10 ng/mL FGF and EGF (Sigma, USA).

Tissue culture:

The cells were counted using trypan blue exclusion and a hemocytometer and then diluted to 3×10^6 live cells/well in 6-well tissue culture plates (Corning Costar® 3471 and 3516, USA). The plates were incubated in a humidified tissue culture incubator at 37°C and 5% CO_2. The medium was changed every three days.

The plates were either non-coated or collagen-coated using Bovine collagen type-1 (PureCol®, Advanced BioMatrix, USA) according to the manufacturer's instructions. Different collagen concentrations were used (50 μg/mL and 100 μg/

mL). Cells were dissociated with 0.25% Trypsin-EDTA (Corning, Manassas, USA) when reached 80-90% confluency (i.e. filled the tissue culture flask) and resuspended in 3 mL mammary stem cell medium.

The cells were transferred to non-coated or collagen-coated tissue culture flasks (Cellstar®, USA), with medium changed every three days and repeated dissociation process with 0.25% Trypsin-EDTA when reaching 80-90% confluency. Cell growth and shape characteristic were visualized using inverted microscope (Olympus, CKX31).

Immunofluorescence:

Cells from passage five were collected during the dissociation process with 0.25% Trypsin-EDTA and diluted till 5×10^5 cells/mL and cultured in 12 well tissue culture plates. After reaching 90% confluency, the wells were washed twice with sterile PBS 1x, and then were fixed with 4% paraformaldehyde solution for 15 minutes at room temperature and permeabilized using 0.1% Triton-X for 10 minutes. After that the cells were blocked using 10% goat serum (to avoid non-specific reactions). Then the cells were incubated with the primary antibodies: mouse anti-CK18 (dilution 1:50, thermoscientific), mouse anti-CK19 (dilution 1:50, Millipore), mouse anti-Vimentin (dilution 1:50, abcam) and mouse anti-Casein (dilution 1:50, abcam), for 1 hour at room temperature. Then washed twice and incubated with the secondary antibody Alexa-Fluor 488 goat anti-mouse (diluted 1:150, Jackson), for 60 minutes at room temperature in darkness. The cell's nuclei were counterstained with DAPI (diluted 1:10000, Calbiochem, USA) and visualized under fluorescent microscope (Nikon, ECLIPSE TE2000-U).

Statistical analyses:

Resulting data was analyzed using one-way ANOVA with post-hoc group comparisons whenever applicable using PASW v18 package (SPSS Inc., Chicago, IL, USA).

Results

Samples from the same quarter were subjected to different isolation conditions in order to determine which is more suitable for the isolation of bovine mammary stem cells. None of the isolated cells expressed milk Casein or CK-18 antigens, while there was positive expression of CK-19 and Vimentin antigens by immunofluorescence (Figure 1).

Isolation of putative bovine mammary stem cells using different dissociation times:

Mammary gland samples subjected to different dissociation times showed different recovery rates of bovine MaSC (Fig. 2.A). Samples subjected to 1 hour dissociation result in a moderate number of cells (5×10^6 cells; on average), these cells were mostly expressing stem cell markers on passage five (CK-19 and Vimentin). While those subjected to 3 hours resulted in the highest number of obtained cells (1.5×10^7 cells; on average), and on passage five the samples showed variable number of mammary stem cells (variable expression of CK-19 and Vimentin) with other non-desired types of cells (mainly fibroblasts). Finally, samples subjected to 30 minutes resulted in the fewest number of isolated cells (1.8×10^6 cells; on average) and those cells did not survive till passage five for the immunofluorescence test.

Isolation of putative bovine mammary stem cells on collagen-coated or non-coated flasks:

Samples cultured on 100 μg/mL bovine collagen type-1 showed the best morphological characteristics under the inverted microscope, cells were spindle in shape and abundant, and reached confluence faster than samples cultured on either non-coated tissue culture flasks or coated with 50 μg/mL bovine collagen type-1. In the latter two, cells were dispersed and much stretched in shape and grew slower (Fig. 2.B).

Isolation of putative bovine mammary stem cells from the different locations:

Samples collected near the base of the mammary quarter had the best recovery rate of bovine
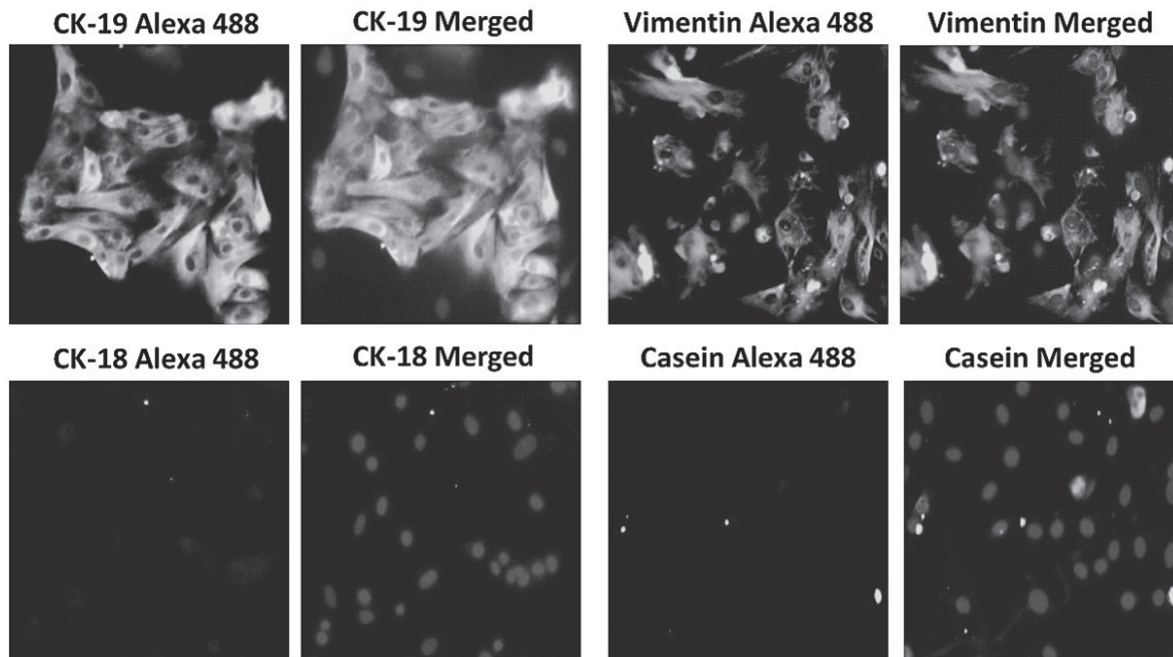
Figure 1. Immunofluorescence of the isolated bovine mammary stem cells at passage five from the results of the optimized isolation protocol, showing the positive expression of CK-19 and Vimentin antigens (green-florescence colored cells in upper row photomicrographs) and negative expression of CK-18 or milk Casein antigens, (lower row photomicrographs). Magnification 400x, visualized with fluorescent microscope (Nikon, ECLIPSE TE2000-U).

mammary stem cells compared to sampling in the middle or near the teat, the latter two had more fibroblasts and less stem cells. These samples were subject to 1 hour dissociation step and were cultured on 100 μg/mL bovine collagen type-1 (Fig. 2.C).

Discussion

The present study evaluated the efficacy of isolation and the quality and number of isolated putative bovine mammary stem cells with changes in dissociation time, collagen concentration and sampling location. Consequently, determining the best protocol to follow when working with stem cells isolated from the bovine mammary gland. In all samples, the positive expression of CK-19 and Vimentin and the negative expression of CK-18 and milk Casein confirms that the isolated cells are mammary stem cells and not milk-producing

epithelial cells, which is in agreement with previous studies in human [3, 5, 11, 12].

From this study the tissue digestion/dissociation time proved to affect the results of the isolation process. Although one would think that more time is better, using 3 hours sample dissociation step resulted in a large number of cells, however, with less number of isolated mammary stem cells compared to the total number of cells. Surprisingly, samples subjected to 1 hour dissociation with collagenase had the best results for the isolation procedure as the cells were mostly stem cells and survived until passage ten. While, 30 minutes were not sufficient to dissociate the stem cells from the samples and the cells were fewer and did not survive in further passaging. From this study sample dissociation time is an important factor in the isolation process and should be considered for future isolations of bovine mammary stem cells or stem cells in general.

The use of substrates that are found in normal

tissue (as Collagen or Fibrinogen) or, very recently, 3D culture techniques have proven to be an essential step for the culture of stem cells in general and mammary stem cells in particular [reviewed in 12]. That is because these cells need appropriate signals from the surrounding medium in order to survive and proliferate. In this study, collagen concentration had an important effect on the quality of the isolated cells and their survival rate. Cells grown on non-coated flasks or coated with 50 μg/mL collagen showed

signs of great surface tension and were stretched. This stretching, certainly, does not resemble the natural shape these cells has in normal tissue where they are surrounded by cells from all sides. And subsequently, the isolated cells did not survive in successive passages and died. Using 3D culture systems (as Matrigel®) is a more realistic approach for culturing MaSC[2]), however it was not performed in this study as it is very expensive and difficult to be used in larger studies.
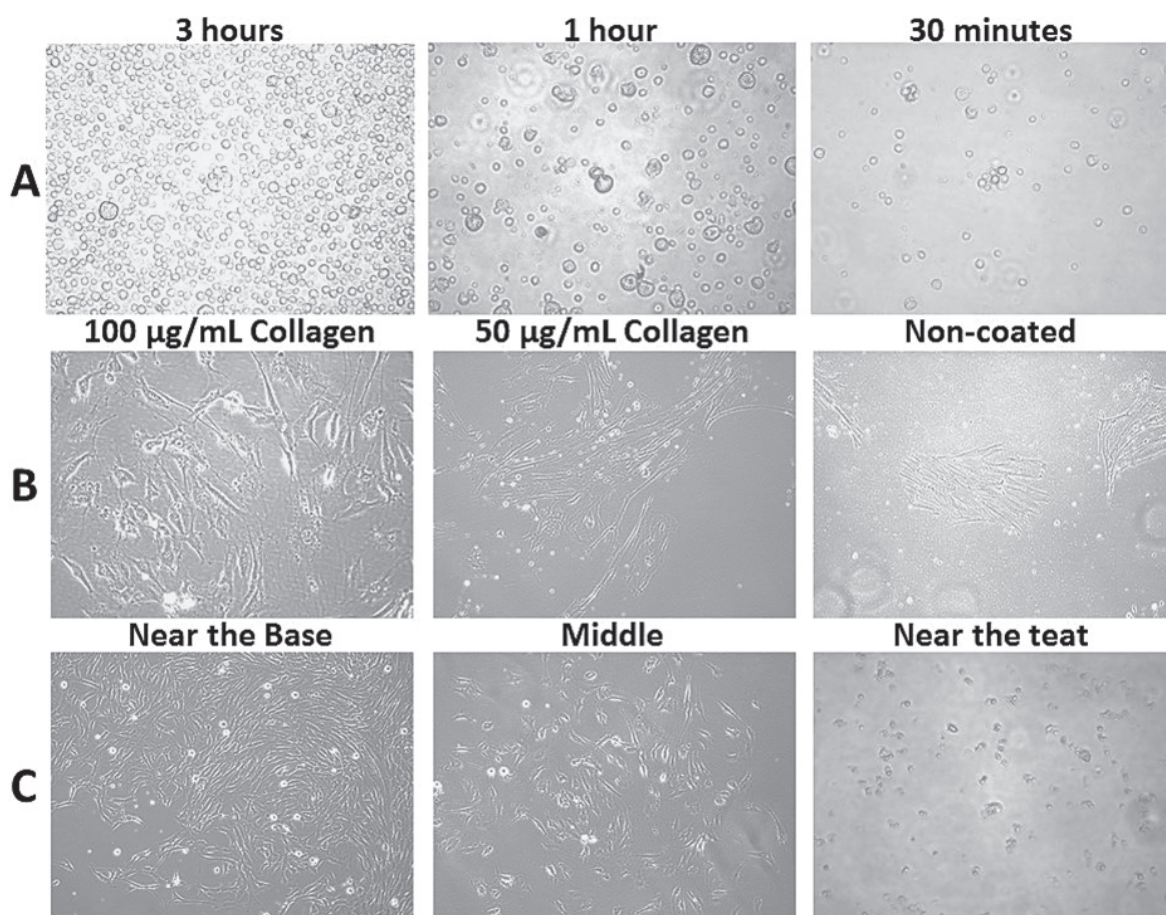

Figure 2. Photomicrographs of the differences in the isolation of putative bovine mammary stem cells with regard to (A) different dissociation times 3 hours, 1 hour and 30 minutes (1.5×10^7, 5×10^6 and 1.8×10^6 cells/mL, respectively), (B) Isolation on 100μg/mL collagen-coated flasks with large number of spindle-shaped cells while on 50μg/mL collagen-coated or non-coated tissue culture flasks with few number of stretched cells and (C) sampling near the base, middle or near the teat showing large, moderate or very few numbers of spindle-shaped cells, respectively. Magnification 200x, viewed under the inverted microscope (Olympus, CKX31).

After optimizing dissociation time and culture method, the sampling site had an effect on the number of isolated stem cells. As, the nearer to the base of the mammary gland the better was the recovery rate of mammary stem cells from the sample. Whereas, samples obtained near the teat had higher number of fibroblasts and very few numbers of stem cells. This is evidenced by the theory that govern the mammary gland development, in which stem cells are present more to the base of the mammary lobule (near the terminal buds) than towards the ducts[4].

In conclusion, this study results recommends the following conditions for successful bovine mammary stem cell isolations: which are sampling as near to the base of the quarter as possible, setting the dissociation time to 1 hour and culturing the cells on 100 μg/mL bovine collagen type-1.

Acknowledgements

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