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Phenotypic and functional characterization of two bovine mammary epithelial cell lines in 2D and 3D models

Magdalena Arévalo Turrubiarte,1,2 Marie-Hélène Perruchot,1,2 Laurence Finot,1,2 Frédérique Mayeur,1,2 and Frédéric Dessauge1,2

1Institut National de la Recherche Agronomique, UMR1348 Pegase, Saint-Gilles, France; and 2Agrocampus Ouest, UMR1348 Pegase, Rennes, France

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Arévalo Turrubiarte M, Perruchot M, Finot L, Mayeur F, Dessauge F. Phenotypic and functional characterization of two bovine mammary epithelial cell lines in 2D and 3D models. Am J Physiol Cell Physiol 310: C348–C356, 2016. First published December 9, 2015; doi:10.1152/ajpcell.00261.2015.—Immortalized bovine mammary epithelial cells (BME-UV1) and immortalized bovine mammary alveolar cells (MAC-T) have been extensively used as in vitro cell models to understand milk production in dairy cows. Precise knowledge about their phenotype and performance remains, however, unknown. This study aims to characterize MAC-T and BME-UV1 profiles when cultured in two-dimensional adherence, three-dimensional adherence (Matrigel), and three-dimensional no adherence [ultralow attachment (ULA)] supports. MAC-T and BME-UV1 were compared according to their proliferation capacities and to specific cell surface markers CD24, CD326 [epithelial cell adhesion molecule (EpCAM)], CD10, and integrin CD49f (α-6). Cytokeratin (CK14 and CK19), signal transducer and activator of transcription 5, and other proteins (occludin and cadherin-1) were analyzed. BME-UV1 in ULA support expressed higher CD49f marker. A different intensity of CD49 staining allowed the discrimination between the two cell lines in adherent condition. CD10, EpCAM, and CK19 expressions show that BME-UV1 cells have luminal capacity, while MAC-T has a myoepithelial profile with a high expression of CK14. BME-UV1 cells possess a closer committed progenitor profile to their higher expression in aldehyde dehydrogenase and EpCAM. We observed that BME-UV1 cells have a better capacity to form spherical structures, mammospheres, in Matrigel than MAC-T, which was confirmed by the higher mammosphere area. In the ULA condition, BME-UV1 proliferated over the 6 days of culture. Taken together, our results clearly confirm the BME-UV1 luminal profile and MAC-T ductal/myoepithelial-like phenotype.

mammary epithelial cell; bovine mammary alveolar cell; bovine mammary epithelial; CD49f; ALDH

THE DIFFERENTIATED BOVINE mammary gland is composed of alveoli, which conform the secretory tissue embedded by a layer of polarized mammary epithelial cells (MEC) considered as the main functional units for milk production (1). At their apical pole, these cells lead to the alveolar lumen, which contains secreted milk. At their basal pole, they directly interact with contractile myoepithelial cells and stromal tissue (6, 20). The existence of stem cells among other different cell types (27) within the alveoli allows mammary tissue to be regenerated after lactation and involution. Cellular fate through differentiation into a luminal or myoepithelial phenotype depends on autocrine and paracrine regulation, predominantly by the lactogenic hormones (31). All of these cell types are immersed in the stroma or conjunctive tissue, where the extracellular matrix (ECM) makes it possible to link cells to structure and organize mammary tissue. The ECM is a key regulator of MEC function. MECs adhere to the basement membrane via ECM receptors, such as the integrins, a family of glycoproteins that play a central role in the regulation of the matrix microenvironment. Cell surface markers have allowed us to distinguish between cell populations in the mammary gland by flow cytometry (5). To date, only certain cell surface markers have been identified within the mammary gland. The integrins CD29 (B1) and CD49f (α-6) are expressed in luminal and myoepithelial cells (16, 23). However, their rate of expression varies between mammalian species (5). MEC populations retain specific cell surface markers, depending on the mammary gland development stage. Morphological changes in the mammary gland through lactation, known as plasticity of the mammary gland, imply cellular renewal and the presence of stem cell populations with a repopulating capacity. These repopulating cellular populations have been identified by expression of the CD24 cell surface marker (26). Within murine alveolar compartments, the repopulating capacity and the presence of MEC with stem cell properties have been identified by a higher aldehyde dehydrogenase (ALDH) expression (25). During the lactation period, alveoli luminal populations express cell surface marker CD326 [epithelial cell adhesion molecule (EpCAM)], which serves as an important marker for lactating cells (21). The cell surface marker, neutral endopeptidase (CD10), also known as common acute lymphoblastic leukemia antigen (CALLA), has been implicated in cell growth and mammary gland development. It has also served as a marker for cells in the basal layer of ducts and used to evaluate in vitro MEC performance, such as sphere-forming ability, in relationship with their basement membrane (18). The expression of CD24 and CD29 (Lin−CD29hiCD24+) populations represents stem cell populations with a repopulating capacity, as was observed in murine fat pads (28). To identify cell populations, diverse rate expressions in EpCAM, CD24, and CD49f populations have made it possible to discriminate between mature and progenitor luminal/basal cells in human mammary cells (8, 14). In contrast to these results, identification of CD24 and CD49f populations, depending on their rate expression, could determine...
the cellular potent capacity of basal and luminal cells in the bovine mammary gland (22). However, to date, mammary gland cell populations have been studied mostly in humans and mice; therefore, further studies in respect to bovine mammary gland cell populations are necessary.

Currently, the use of immortalized cells as in vitro models is necessary to study mammary glands due to their resemblance to original tissue. In the case of bovine mammary gland studies, a variety of immortal bovine MEC have been established and characterized to understand physiology, growth, and lactation features (9, 11, 12, 24, 33). However, among these immortal cell lines, the bovine mammary epithelial (BME-U1) and the bovine mammary alveolar cell (MAC-T) have been widely used as in vitro models due to their similarity with bovine lactating MECs. The BME-U1 cells are responsive to epidermal growth factor and the insulin-like growth factor I, which are related to mammary growth and development (3). BME-U1 have been used as a bovine MEC model to study involution and apoptotic events because of its significant response to growth factors and apoptotic peptides (32). The MAC-T cells have the ability to synthesize caseins-α and -β during cellular differentiation pathways implicated in lactation (33). The MAC-T cells have been used to study hormone signaling because of its receptivity to hormonal stimuli (13, 20). In general, these studies provide a wide perspective about the differences between the BME-U1 and MAC-T cell performance. In fact, it should be taken into account that BME-U1 and MAC-T cells may behave differently as they were obtained from different animals and established from different research groups. Nonetheless, they were established from MECs. The BME-U1 cells were obtained from pregnant lactating cows and were also transfected with the SV40 large T-antigen; the BME-U1 cells show a domelike morphology typical of epithelial cells (34). The MAC-T cells were obtained from lactating cows and transfected with SV40 large T-antigen, as MAC-T cells have the particularity of possessing cobblestone morphology when cultured in plastic supports (12).

To understand bovine mammary cellular heterogeneity through in vitro models, the aim of the present study was to identify the proliferation activity and phenotype of the BME-U1 and MAC-T cells when cultured in adherent (ADH), Corning ultralow attachment (ULA) surface and Matrigel supports. With the objective of providing accurate knowledge about the BME-U1 and MAC-T cells’ origin, phenotype analysis by cell surface markers, CD24, CD326, (EpCAM), CD10, and integrin CD49f (α-6), served to identify the BME-U1 and MAC-T cells profile and performance in two-dimensional (2D) (ADH) and three-dimensional (3D) (ULA and Matrigel) supports. We also analyzed cytokeratins (CK14 and CK19). The signal transducer and activator of transcription 5 (STAT5), related to lactogenesis and occludin transmembrane tight junction protein (7), were analyzed by the effect of the three supports. In addition, expression of cadherin (CDH)-1 was also evaluated, due to its importance and presence in epithelial cells’ architecture (2). This study should greatly help to define specific biomarkers of MEC populations and to phenotype primary mammary cells within the dairy mammary gland.

MATERIALS AND METHODS

Chemicals and cell culture reagents. Dulbecco’s modified Eagle’s medium (DMEM)/F-12, RPMI-1640, NCTC 135, DMEM x 1, fetal bovine serum, and antibiotic-antimycotic solution were obtained from Gibco (Illkirch, France). Matrigel was obtained from BD Biosciences. Hydrocortisone, a-lactose, glutathione, bovine insulin, ovine prolactin, bovine holo-transferrin, hydrocortisone, l-ascorbic acid, penicillin streptomycin, fungizone, and 0.25% trypsin/EDTA reagents were obtained from Sigma Aldrich Chimie (St. Quentin Fallavier, France).

Cell culture. The original bovine MEC line (MAC-T) was purchased at Nexia Biotechnologies (Quebec, Canada). MAC-T cells were grown in high-glucose DMEM (Dominique Dutscher, Brumath), supplemented with 10% inactivated fetal calf serum (A15-101, PAA, Les Mureaux, France), 100 U/ml of penicillin-streptomycin-fungizone (Dominique Dutscher), and 5 μg/ml of bovine insulin (Dominique Dutscher). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2 and passed twice a week (cells reached confluence within 2–3 days). The immortal mammary epithelial BME-U1 cell line was established by Professor Boris Zavijon (Department of Animal and Food Sciences, University of Vermont, USA). BME-U1 cells used in this study came in the original strain purchased at the Istituto Zooprofilattico Sperimentale Della Lombardia E Dell’Emilia Romagna (Brescia, Italy) and were kindly provided by Professor Antonella Baldi (Animal Nutrition Institute, Faculty of Veterinary Medicine, University of Milan, Milan, Italy). Cells were cultured in routine culture medium (mixture of DMEM/F-12, RPMI-1640, and NCTC 135 in proportions of 5:3:2 by volume) enriched with a-lactose (0.1%), glutathione (1.2 mM), bovine insulin (5 μg/ml), bovine holo-transferrin (5 μg/ml), hydrocortisone (1 μg/ml), l-ascorbic acid (10 μg/ml), 10% (vol/vol) heat-inactivated fetal calf serum, and penicillin-streptomycin (50 IU/ml) in atmosphere of 5% CO2/95% humidified air at 37°C.

MAC-T and BME-U1 cell lines (passage 2 from the original strain) were defrosted and cultivated until confluence in their specific medium during 2 wk before starting the experiment (after four to five passages). Then cells were observed on the microscope to verify their confluence and typical shape, and then harvested with 0.25% trypsin/EDTA at day 0, 1, 3, and 6 for cell viability and proliferation. All of the cells used throughout the experiments had the same treatment before the experiment was started. Cells were harvested with 0.25% trypsin/EDTA at day 6 for flow cytometry and Western blot analysis. For the DNA extraction, cells were plated in a 24-well ADH support at density of 50,000 cells/well. To measure cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cells were added at a density of 200,000 cells/well and were used for qPCR, Western blotting, and flow cytometry analysis. Media were replaced every second day.

MAC-T or BME-U1 cells in plates were trypsinized using 0.25% trypsin/EDTA. Both cell lines are mycoplasma free.

3D cultures. For the 3D study, cells were cultured both on six-well plates coated with Matrigel and on ULA six-well plates (Corning). BME-U1 and MAC-T cells were seeded at a density of 100,000 cells/well. Matrigel plates were coated with 400 μl/well of Matrigel and were left to solidify for 30 min at 37°C. Cells grown in monolayer were trypsinized and resuspended in growth medium with 2% of Matrigel. The cells cultured in ULA plates were cultured at density of 300,000 cells/well. After the 6 days in culture, cells were collected with total medium, centrifuged at 400 g for 5 min (4°C), and washed with cold PBS. The medium of cells seeded in Matrigel-coated plates was recovered, and cells were scrapped in 2 ml of cold PBS, centrifuged at 800 g for 5 min (4°C), and washed with cold PBS.

Protein extraction and Western blot analysis. Proteins were extracted from pelleted cells using the RIPA extraction reagent (Fisher Scientific, Illkirch, France). The soluble protein fraction was collected from pelleted cells using the RIPA extraction reagent (Fisher Scientific, Illkirch, France). The soluble protein fraction was collected after centrifugation at 13,000 g for 10 min (4°C), and protein con-
Flow cytometry analysis. The BME-UV1 and MAC-T cells were obtained at day 6 of culture and dissociated for further flow cytometry analysis. BME-UV1 and MAC-T ADH cells were trypsinized, washed with PBS, and centrifuged at 400 g for 5 min. Cells were counted with TC20 automated cell counter (Bio-Rad) and suspended to further flow cytometry analysis. Matrigel BME-UV1 and MAC-T cells were recovered at 4°C for 1 h with cell recovery solution (BD Biosciences), according to the manufacturer’s protocol. Then cells were dissociated with 1 ml of ReLeSR (Stem Cell Technologies) and incubated at 25°C for 5 min. Cells were sieved twice with PBS and their respective medium through a 30-μm cell strainer (Miltenyi Biotec). Following PBS washing, cells were counted with the TC20 and prepared for cytometry analysis. Cells cultivated in ULA supports were obtained by dissociating them with 1 ml of ReLeSR (Stem Cell Technologies, Grenoble, France) and incubated at 25°C for 5–7 min. Then cells were sieved twice with PBS and medium and prepared for the cell sorting, as described above. Cells obtained from ADH, Matrigel, and ULA supports were prepared for flow cytometry analysis with MACS buffer (autoMACS with 2% BSA) (Miltenyi Biotec). Cells were dispensed at 100,000 cells/ml concentration in each respective tube. Cells were incubated in darkness at 4°C for 30 min with antibodies FITC anti-rat IgG1 CD49f (α-6 integrin), allophycocyanin anti-rat IgG2bcs CD24 (heat stable antigen), phycoerythrin anti-mouse IgG1 CD29 (β1-integrin), Vioblue anti-mouse IgG1 CD326 (EpCam) (Miltenyi Biotec), and anti-mouse IgG1 PE-VIO700 CD10 (CALLA) (Miltenyi Biotec). Antibodies were used against each corresponding isotype control. Then cells were centrifuged at 400 g and resuspended.

Centrations were determined using the BCA Protein Assay kit (Fisher Scientific, Illkirch, France), according to the manufacturer’s instructions. Lysates were combined with sample buffer [50 mM Tris·HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 20% glycerol, and 0.1 M diithiothreitol], boiled for 5 min at 95°C, and resolved using SDS-PAGE. Equal amounts of protein (10 μg) were separated on 4–12% NuPage gradient gels (Fisher Scientific, Illkirch, France) via SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Amersham, Courtaboeuf, France) at 110 V for 80 min. A set of prestained molecular mass standards (protein ladder) was run into each gel. The membranes were incubated with blocking solution (5% blotting grade blocker; Bio-Rad, Marnes-la-Coquette, France) dissolved in Tris-buffered saline-Tween buffer [50 mM Tris·HCl (pH 8.6), 150 mM NaCl, and 0.1% Tween] for 30 min. These membranes were incubated overnight at 4°C with primary antibodies: rabbit STAT5 (C-17 clone) (SC-835, Santa Cruz Biotechnology), mouse CDH-1 (NCH-38 clone) (M-3612, Dako Agilent Technologies), rabbit occludin (71–1500, Zymed Thermoﬁsher), goat CK14 (SC-17104, Santa Cruz Biotechnology), mouse CK19 (NCL-CK19 Novocastra Leica Biosystems), and β-actin (A5441, Sigma). They were also incubated with the appropriate secondary antibody coupled to horse-radish peroxidase used at 1:2,500. The horseradish peroxidase chemiluminescence was visualized using an ECL kit and the ImageQuant LAS4000 Biомolecular Image digital imaging system (GE Healthcare, Velizy-Villacoublay, France). Images generated as gel files were analyzed using the corresponding ImageQuant TL software (GE Healthcare).

DNA concentration by Hoechst 33258 staining. Cellular pellets obtained from the MAC-T and BME-UV1 cells in Matrigel, and ULA culture conditions were dissociated with 1 ml of buffered 0.05 M phosphate, 2 M NaCl, and 2 mM EDTA. Cells were sonicated for 30 s at 4°C and centrifuged at 4,000 g at 20°C for 30 min. Hoechst (Sigma Aldrich) solution was prepared at 1 mg/ml and then diluted to 200 μg/ml with free-nucleic sterile water (Thermo Scientific). Calibration curve of 0, 1, 2, 4, 5, 6, 8, and 10 μg/ml was prepared in amber tubes (Axygen, Corning) with DNA solution (100 μg/ml) (Sigma Aldrich), buffered 0.05 M phosphate, and diluted Hoechst (200 μg/ml). DNA samples were analyzed in 96-well black flat bottom using Mithras LB 940 Multimode Microplate Reader (Berthold Technologies). DNA concentration was calculated based on a regression calibration curve from the DNA dilutions.

BME-UV1 and MAC-T morphology and mammosphere measurement. Cells were observed at ×10 magnification using phase-contrast microscope equipped with an AxioCam MRC (Axio vert. A1, Zeiss). The analysis of mammosphere size and parameter was performed at 0, 3, 6, 8, and 10 days of culture. Image acquisition (0.3 μm/pixel) area was measured at 50-μm scale using the Zen blue software (Zeiss, 2012). Mammosphere area was determined using three representative images of each independent experiment and day.

Table 1. Percentage expression of CD49f, CD24, EpCAM, and CD10 in BME-UV1 and MAC-T cells cultured in ADH, Matrigel, and ULA supports

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<th>ADH</th>
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<th>ADH</th>
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<td>CD49f</td>
<td>99.0 a</td>
<td>97.9 a</td>
<td>98.9 a</td>
<td>99.2 a</td>
<td>52.0 b</td>
<td>98.5 a</td>
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<tr>
<td>CD24</td>
<td>0.6 a</td>
<td>0.0 b</td>
<td>0.0 b</td>
<td>0.2 a</td>
<td>0.0 b</td>
<td>0.6 a</td>
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<tr>
<td>CD10</td>
<td>25.3 a</td>
<td>11.2 a</td>
<td>17.9 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td>EpCAM</td>
<td>3.0 a</td>
<td>2.0 b</td>
<td>1.6 c</td>
<td>1.6 a</td>
<td>1.6 a</td>
<td>1.6 a</td>
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Values are in percent. Cells were cultured during 6 days and then obtained to be analyzed by flow cytometry. BME-UV1, bovine mammary epithelial cells; MAC-T, bovine mammary alveolar cells; ADH, adherent; ULA, ultralow attachment; EpCAM, epithelial cell adhesion molecule. a,b,c Significant difference (P < 0.05).

Fig. 1. Bovine mammary epithelial cells (BME-UV1; A) and mammary alveolar cells (MAC-T; B) CD49f expression by histogram graphic measuring forward scatter (FSC) vs. CD49f-FITC staining intensity (volt). C: dot-plot graphic representation merge of CD49f in BME-UV1 (light gray) and MAC-T (dark gray) populations against unstained (black).
Cell suspension was obtained from ADH cultures and analyzed at different concentrations in 0.3 ml of MACS buffer. Multistaining was performed to determine specific populations. For each particular cell surface marker, the use of propidium iodide made it possible to identify the authentic live cellular population (stained cells). Then acquisition of this stained population was gated to forward-scattered light and side-scattered light, to exclude dead population and delimit cell granularity and size, as well as doublets by adjustments in height and photomultiplier voltage. The isotype of each target antigen served as a control to discriminate nonspecific background during the analysis. Samples were analyzed to collect a data set of 10,000 events. Flow cytometry analysis was performed using a MACSQuant Analyzer 10 cytometer (Miltenyi Biotec). Data were analyzed using MACSQuantify analysis software (Miltenyi Biotec). Obtained results were expressed in percentages by excluding the isotype from the cell surface marker by dot plot analysis.

**ALDH detection.** The BME-UV1 and MAC-T cells line were obtained from ADH cultures and analyzed at different concentrations (100,000, 200,000, 500,000, 1,000,000, and 2,000,000 cells/ml) in ADH supports at 37°C in 5% CO2. Cells were then removed by trypsin digestion and incubated for 45 min at 37°C with Aldefluor (Stem Cell Technologies), according to the manufacturer’s recommendations. Next, cells were centrifuged (250 g) and suspended in Aldefluor assay buffer (Stem Cell Technologies). Cell suspension was then acquired by flow cytometer following the same methodology as described before by MACsQuant Analyzer 10 (Miltenyi Biotec).

**RESULTS**

**BME-UV1 and MAC-T cells possess different phenotypes in their cell surface markers profile.** We decided to use flow cytometry by monostaining and multistaining protocols to characterize BME-UV1 and MAC-T cellular phenotypes. BME-UV1 and MAC-T cells cultured in ADH supports both expressed high levels of CD49f (~99%), considered as an important epithelial cell surface marker. Subsequently, and to study CD49f expression in mammospheres, BME-UV1 and MAC-T cells were cultured in 3D supports (Matrigel and ULA). Our results showed that CD49f expression decreased only in MAC-T cells cultured in ULA (Table 1).

In the mammary gland, cell subpopulations can be discriminated through the intensity of CD49f staining: CD49flow, CD49fmedium, and CD49fhigh. If we look at CD49f intensity staining in ADH support, histograms (Fig. 1) show that BME-UV1 and MAC-T CD49f expression is different with respect to their staining intensity. Merging the BME-UV1 (light gray) and MAC-T (dark gray) CD49f stainings allowed us to confirm this difference of CD49f staining (respectively, CD49fmedium vs. CD49fhigh).

CD10, CD24, and EpCAM cell surface markers are usually analyzed to phenotype mammary cell populations. BME-UV1 cells expressed CD10 in ADH conditions (25.3%), and MAC-T cells showed no expression (Table 1). CD10 expression decreased when BME-UV1 cells formed mammospheres in ULA and Matrigel support. MAC-T cells did not express CD10, whatever cell culture support. Very low CD24 expression was observed in both BME-UV1 and MAC-T cells cultured in all conditions (Table 1). Furthermore, significant differences in EpCAM expression were observed. BME-UV1 cells had a higher expression (3%), whereas MAC-T cells showed a lower expression (0.8%) in ADH supports (Table 1). EpCAM expression decreased when BME-UV1 are cultured in 3D sup-

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**Fig. 2.** Characterization of aldehyde dehydrogenase (ALDH) expression for BME-UV1 (A) and MAC-T (B) cells in ADH culture supports. Data acquisition was made by gating FSC vs. ALDH-positive expression. C: data represent percentage of expression means ± SE. ALDH expression is shown in percentages of three experiments. a,b Superscripted letters are significantly different from each other (P < 0.001).

**Fig. 3.** Western blotting detection of signal transducer and activator of transcription 5 (STAT5; A), cadherin (CDH)-1 (B), occludin (C), cytokeratin (CK) 14 (D), CK19 (E), and actin (control; F). MAC-T and BME-UV1 were cultured in two-dimensional (2D) ADH support and in three-dimensional (3D) nonadherent [ultralow attachment (ULA)] and 3D adherent support (Matrigel) during 6 days. Cells lysates were harvested at day 6, and ~10 μg protein were separated on polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Protein size is expressed in kDa on the left of the gels. The experiment was independently repeated three times.
unstained cells (black). Interestingly, we observed (Fig. 2 and MAC-T (Fig. 2B) MAC-T and BME-UV1 cells. The dot plot shows BME-UV1 the presence of stem and progenitor cell-specific activity in contrast, ALDH high ALDH phenotypes. Expression of ALDH was assessed to determine MAC-T cells were cultured in Matrigel.

ports. Interestingly, EpCAM expression was increased when MAC-T cells were cultured in Matrigel.

**BME-UV1 cells possess progenitor and stem-cell-like phenotypes.** Expression of ALDH was assessed to determine the presence of stem and progenitor cell-specific activity in MAC-T and BME-UV1 cells. The dot plot shows BME-UV1 (Fig. 2A) and MAC-T (Fig. 2B) cell-positive populations and unstained cells (black). Interestingly, we observed (Fig. 2C) a high ALDH+ cell population in BME-UV1 cells (83.01%). In contrast, ALDH+ cell population was slightly represented in MAC-T cells (6.27%).

**BME-UV1 and MAC-T cells express their own adhesion proteins and cellular signaling in response to a specific culture support.** The analyses of different proteins (STAT5, CDH-1, occludin, CK14, CK19) were evaluated at day 6 by Western blot to determine how culture supports can influence their expression. STAT5 expression in MAC-T cells showed a slightly and almost unapparent presence in ADH, Matrigel, and ULA supports (Fig. 3A). BME-UV1 cells presented high and stable expression of STAT5 for ADH, Matrigel, and ULA supports (Fig. 3A). The absence of CDH-1 expression in both MAC-T and BME-UV1 cells was observed when cultured on ADH, but increased in ULA and Matrigel supports (Fig. 3B). As CDH-1, occludin protein was only expressed in Matrigel and ULA supports in both cell lines (Fig. 3C). CK14 was only expressed in MAC-T cells, while CK19 was only expressed in BME-UV1 at 2D and 3D culture conditions (Fig. 3, D and E). Interestingly, we observed a high expression of fibronectin when cells were cultured in ULA, and the expression was substantially higher in MAC-T cells (Fig. 3F).

**MAC-T and BME-UV1 cell proliferation differs between cellular 2D and 3D supports.** To evaluate the influence of 2D and 3D supports on proliferation, DNA concentration was measured in different cell culture conditions. Our results clearly showed higher BME-UV1 cell proliferation activity in Matrigel by its increase in DNA concentration rate from day 1 to day 6 (Fig. 4A) compared with MAC-T cells proliferation. In the same way, BME-UV1 showed higher proliferation ability in ULA; while MAC-T cells DNA concentration was decreased throughout the days (Fig. 4B). These results suggested that ULA had a negative effect on MAC-T cells’ proliferation activity. Concerning proliferation in ADH, we classically observed a higher proliferation of MAC-T cells compared with BME-UV1 cells (Fig. 5).

**Performance of MAC-T and BME-UV1 cells in 2D and 3D supports regarding mammosphere formation.** In Matrigel coating supports, the profile of 3D spherical shapes was more clearly defined for both cell lines. In ULA supports, MAC-T cells formed little round spheres with some cell clusters; whereas BME-UV1 cells presented bigger sphere-shaped cells, typical of the mammospheres described by Kozlowski et al. (15) (Fig. 6). In 3D (ULA and Matrigel) supports, cells underwent an apparent morphological change. Here, we observed that MAC-T cells did not evolve as BME-UV1 cells from day 3 to day 10 in ULA. MAC-T cells form aberrant structures in ULA. Conversely, BME-UV1 cells showed a cluster of 3D sphere-like cells (Fig. 6). Mammosphere area was measured by Zen blue software. The analysis showed that, after day 3, mammosphere size constantly increased until day 10 in 3D Matrigel in both cell lines. The mammosphere surface area formed by BME-UV1 cells almost reached 600 μm², whereas MAC-T cells were ~400 μm² in Matrigel support (P < 0.05) (Fig. 7).
DISCUSSION

To study bovine mammary gland plasticity, immortalized cells are powerful tools. Therefore, in vitro models and cell lines need to be fully characterized. For the first time, this study clearly elucidated BME-UV1 and MAC-T phenotypes by the use of antibodies against specific cell surface markers CD10, CD24, CD326 (EpCAM), integrin CD49f (α-6), and ALDH^+ cell population determination using flow cytometry. We confirmed BME-UV1 and MAC-T cell differences with respect to their cellular population CD49f^+, EpCAM^+, and CD10^+ and their ability to form mammospheres in 3D supports. We demonstrated that differences between BME-UV1 and MAC-T cells in their phenotype and performance corresponded to different cytokeratins (CK14 and CK19) and proteins (STAT5, occludin, and CDH-1) expressions.

The combination between specific cell surface markers and ALDH activity allows now characterizing specific cell populations like mammary epithelial stem/progenitors cells. In human normal breast tissue, high expression of ALDH can serve as a biomarker to identify stem/progenitor MECs. Moreover, MECs with high ALDH activity have often high self-renewing capacities and mammosphere-forming abilities (25). Interestingly, in our study, high ALDH activity was observed in BME-UV1 cells compared with MAC-T cells. Furthermore, we observed that MAC-T cells possess a higher capacity to proliferate in ADH supports than BME-UV1 cells. In contrast, MAC-T cell proliferation seems to be diminished in ULA and Matrigel compared with BME-UV1 cells. In 3D culture supports, MAC-T cells exhibited low ability to form mammospheres and showed aberrant and not well-structured mammosphere cells. The higher ability of these cells to proliferate on plastic is not related to their low ability to form mammospheres in Matrigel and ULA. On the other hand, BME-UV1 cells showed higher ability to form spherical colonies in ULA and larger mammospheres. Dontu et al. (6) demonstrated that non-ADH mammospheres were enriched in early progenitor/stem cells and able to differentiate along all mammary epithelial lineages and to clonally generate complex functional structures in reconstituted 3D culture systems. Thus behavior of BME-UV1 cells cultured on 3D using non-ADH support ULA suggested that these cells would possess progenitor/stem cell characteristic.

Cell-to-cell adhesion and cellular communications are crucial in mammary gland development for regulation and
differentiation of alveolar epithelial cells in late pregnancy and at the beginning of lactation. CDH-1 is one of these crucial proteins required for cell attachment to the stroma and cell motility (10). CDH-1 is a marker of polarization essential for the organization of MEC in 3D (29). As expected, MAC-T cells and BME-UV1 cells did not express CDH-1 in ADH supports and were unable to form adherent junctions and thus to polarize. In contrast, BME-UV1 and MAC-T cells expressed CDH-1 when cultured in Matrigel and ULA supports. Like Kozlowski et al. (15), we observed CDH-1 expression after 6 days of culture in Matrigel and ULA support. Moreover, a well-known tight junction protein, occludin, was highly expressed in BME-UV1 cells cultured in Matrigel and ULA supports. In contrast, lower expression of occludin was found in MAC-T cells. The lower presence of occludin in MAC-T cells could also explain the aberrant structures observed in ULA support, as well as their higher and specific expression of fibronectin. Epithelial cell’s ability to transit into mesenchymal cells (EMT) is a particularity of epithelial plasticity. During EMT, epithelial cells lose their junctions and apical-basal polarity, reorganize their cytoskeleton, undergo a change in the signaling programs that define cell shape, and reprogram gene expression (17). They demonstrated that the dissolution of tight junctions during EMT is accompanied by decreased claudin and occludin expression, and the diffusion of zonula occludens 1 from cell-cell contacts. We can hypothesize that MAC-T cells cultured in 3D on non-ADH support like ULA are able to undergo EMT and switch to mesenchymal cells.

BME-UV1 and MAC-T cells were obtained at different time of mammary development. BME-UV1 cells were obtained from pregnant lactating Holstein cows at a slaughter house (34), while MAC-T cells were obtained from biopsies apparently from lactating Holstein cows (12). Furthermore, dairy animals’ mammary glands have a great capacity for cellular population shift (mammary gland plasticity) during mamogenesis and throughout lactations. Luminal progenitor cells are present during puberty and lactation, whereas myoepithelial cells were highly detected at the involution period (21). According to Borena et al. (5), the most common markers to identify luminal epithelial cells are CK8, CK18, CK19, and mucin-1, whereas CK5, CK14, smooth muscle actin, and vimentin are the most frequently used markers to detect basal myoepithelial cells (5). The strong expression of CK19 and the absence of CK14, whatever the cell culture conditions, suggest that MAC-T cells come predominantly from ductal/myoepithelial origin. In contrast, BME-UV1 cells strongly express CK19 but not CK14. Moreover, STAT5 is expressed constitutively in BME-UV1 cells, whereas its expression is undetectable in MAC-T cells. The overexpression of STAT5 in BME-UV1 cells in 3D cultures corresponds to the gene analysis of Kozlowski et al. (15), where they found the activation of genes involved in milk synthesis. The present study revealed that BME-UV1 and MAC-T cells have different phenotypes and different signaling pathways. This confirms results obtained by Zarzynska et al. (32), where they demonstrated that BME-UV1 and MAC-T cells have opposite responses to hormones. They concluded that BME-UV1 cells are more reactive to hormones of the somatotropic axis and epidermal growth factor, whereas MAC-T cells are more reactive to sex steroids. In 2003, Berry et al. (4) used MAC-T cells to measure the mitogenic activity of extracts from heifer mammary tissue. They concluded that the proliferative responses of MAC-T cells mimic the response of primary MEC but are refractory to estrogen. Differences between BME-UV1 and MAC-T cells seem to come from the physiological status of the dairy cow before mammary sampling. Despite this, we cannot exclude that cells have derived through cell culture passages and have acquired a phenotype dependent of each laboratory cell culture conditions (FBS concentration, insulin concentration, mycoplasma contamination, cell culture plastic brand, etc.). Overall, our results allowed the identifying of common markers and specific markers of both cell lines. However, new biomarkers that allow for a clear and consistent identification of MECs can be used in flow cytometry.

Flow cytometry has allowed us to clearly observe high expression of CD49f in both BME-UV1 and MAC-T cells, and these results confirm their epithelial profile, as described in previous studies (5, 22, 25). Villadsen et al. (30) suggested that ductal MECs expressed high levels of CD49f and CK14. Thus we focused our attention on the intensity of CD49f expression, and we observed that MAC-T cells expressed CD49f high compared with BME-UV1 that expressed CD49f medium. This result, combined with the restricted expression of CK14 within MAC-T cells, suggests that MAC-T cells have a ductal origin. Interestingly, MAC-T cells had a decline in CD49f expression in ULA condition. This might be explained by the intimate relationship between ECM and integrins, where cells are able to respond by defining their polarity and morphology, depending on their support (19). For MAC-T cells, the expression of CD49f seems to be required for cell proliferation and mammosphere formation. The subsequent analysis of EpCAM in MAC-T and BME-UV1 cells in ADH supports showed that EpCAM was expressed significantly higher in BME-UV1 than in MAC-T cells. Our findings correspond with EpCAM expression in luminal mammary cellular populations (18). The presence of EpCAM in MAC-T cells was...
also in accordance with previous studies, where EpCAM seems to be expressed in both basal and luminal cellular populations (21). Moreover, our data indicate that BME-UV1 cells highly expressed CD10 in basal cell culture conditions (ADH). CD10⁺ cells decreased when BME-UV1 cells formed mammospheres in 3D conditions: ULA and Matrigel. These results are correlated with previous studies that reporting CD10⁺ cells have the ability to form mammospheres and have progenitor’s phenotype. These data have been confirmed by the high ALDH activity in BME-UV1 cells. In 2012, Rauner and Barash (22) distinguished two major epithelial cell populations in primary MEC: CD24neg-medCD49pos and CD24med-highCD49neg (22). In BME-UV1 cells, nearly 100% of cells were CD49pos, but we never identified CD24pos cells. This result suggests that cells could lose CD24 expression during their immortalization and putative stem cell markers. In the previous study, primary bovine MEC was from undifferentiated heifer mammary tissue. Thus CD24 could be expressed at specific stage during mamrogenesis in dairy cows.

In conclusion, this study has shown BME-UV1 and MAC-T performances in 2D and 3D supports and has provided evidence about their phenotypical characteristics. This study confirms that BME-UV1 cells have a clear luminal-like profile by expression of CD10, CD49f, and EpCAM, along with higher ALDH and CK19 expression. Moreover, BME-UV1 cells have a high capacity to form mammospheres; this could also be related to their luminal progenitor/stem profile and their lactogenic origin. MAC-T cells have a high ability to proliferate, higher CD49f expression, lower ADLH activity, and CK14 expression means that MAC-T cells have a myoepithelial/ductal profile. Together, these results defined precisely the phenotypes of MAC-T and BME-UV1 cells, and we confirmed their distinct responses to culture support. Moreover, regarding myoepithelial profile and fibronectin production of MAC-T cells cultured in ULA support, it could be a nice model for epithelial-mesenchymal transition studies.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.A.T., M.-H.P., L.F., and F.D. conception and design of research; M.A.T., L.F., and F.M. performed experiments; M.A.T., M.-H.P., L.F., and F.D. analyzed data; M.A.T., M.-H.P., L.F., and F.D. interpreted results of experiments; M.A.T., L.F., and F.D. prepared figures; M.A.T., M.-H.P., L.F., and F.D. drafted manuscript; M.A.T., M.-H.P., L.F., and F.D. edited and revised manuscript; M.A.T., M.-H.P., L.F., and F.D. approved final version of manuscript.

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