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*Original article*

# Role of extracellular matrix and prolactin in functional differentiation of bovine BME-UV1 mammary epithelial cells

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## Abstract

Interactions between extracellular matrix (ECM) and epithelial cells are necessary for proper organisation and function of the epithelium. In the present study we show that bovine mammary epithelial cell line BME-UV1 cultured on ECM components, commercially available as Matrigel™, constitutes a good model for studying mechanisms controlling functional differentiation of the bovine mammary gland. In contact with Matrigel BME-UV1 cells induce apicobasal polarity, and within 16 days form three dimensional (3D) acinar structures with a centrally localized hollow lumen, which structurally resemble mammary alveoli present in the functionally active mammary gland. We have shown that the 3D culture system enables a high expression and proper localisation of integrin receptors and tight junction proteins in BME-UV1 cells to be induced. This effect was not obtained in cells grown in the classical 2D culture system on plastic. Moreover, ECM highly stimulated the synthesis of one of the major milk proteins, β-casein, even in the absence of prolactin. Our results show that contact with ECM plays an important role in the lactogenic activity of bovine MECs, however, prolactin is necessary for the efficient secretion of milk proteins.

**Key words**: bovine mammary epithelial cells, ECM, prolactin, β-casein

## Introduction

The general features of mammary gland functional development is universal for animals and humans, however some differences in growth rate and hormonal control of the process can be distinguished between species. Before puberty the growth of the mammary gland is mainly characterised by an increase in connective tissue and deposition of fat, while the epithelium elongates only moderately, forming first a ductal network. In ruminants mammary ducts develop as compact, highly branched structures within loose connective tissue called the terminal ductal units (TDUs) (Capuco and Ellis 2005). An accelerated ductal extension and branching begins during puberty, and is coordinated by the hypothalamic-pituitary--ovarian axis. Finally, at gestation mammary growth becomes exponential, and is driven by pregnancy hormones, giving rise not only to more extensive ductal branching, but also to the development of alveoli, built by functionally differentiated secretory epithelial cells, showing the ability to synthesise and secrete milk components during lactation. Alveolar morphogenesis occurs mainly in response to increased

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levels of estrogen, progesterone, and prolactin (PRL), however the role of growth hormone (GH), corticosteroids, thyroid hormones, and some growth factors such as: IGF-I and EGF are nowadays well established (Briksen and Rajaram 2006). Nevertheless, the main hormone responsible for the final differentiation of the alveolar epithelium is PRL, which is involved in proliferation and differentiation of mammary epithelial cells (MEC) during pregnancy, stimulation of milk protein synthesis, and secretion of milk components into the lumen of the alveoli (recently reviewed by Bachelot and Binart 2007).

Beside hormonal regulation the development of a functionally active mammary gland depends also on stromal-epithelial interactions. The stromal compartment is comprised of mesenchymal cells and extracellular matrix (ECM) (Kass et al. 2007). The ECM, playing a crucial role in the differentiation of mammary epithelium, mainly consists of collagen IV, and laminins, which are cross-linked by entactins. The mammary stroma secretes additionally fibronectin and tenascins, forming the full composition of the ECM (Katz and Streuli 2007). Signals from ECM are translocated into the cells via specific integrin receptors localized in the basal surface of the polarized mammary epithelium. It has been shown that β1-integrin plays a crucial role in MEC differentiation (Naylor et al. 2005).

The three-dimensional (3D) culture system serves as a good *in vitro* model for studying the interactions between ECM and mammary epithelial cells during their functional differentiation. Unlike monolayer cultures on plastic, MEC grown in 3D on ECM components recapitulate numerous features of mammary epithelium *in vivo,* including the formation of acini-like spheroids, with a hollow lumen, apicobasal polarization of cells, basal deposition of basement membrane components, and the ability to produce milk proteins (Weaver et al. 2002, Debnath et al. 2003). The most commonly used ECM derivative in this culture system is the reconstituted basement membrane (rBM) derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, commercially available as Matrigel™. Although many interesting studies with the use of 3D MEC culture have been published, very few describe the regulation of bovine MEC differentiation in the context of their interaction with ECM components (Delabarre et al. 1997, Kozłowski et al. 2009, Riley et al. 2010). The present study shows the rate and mechanisms of the proper development of acinar structures (mammospheres) formed by the bovine mammary epithelial cell line BME-UV1 cultured on Matrigel. Our special interest was focused on the influence of the rBM on the synthesis of milk protein, β-casein and the relation between the effects of ECM and prolactin on functional differentiation of bovine MEC.

## Materials and Methods

### Cell culture

The bovine mammary epithelial BME-UV1 cell line was cultured in routine culture medium (a mixture of DME/F-12, RPMI-1640 and NCTC 135 in proportions of 5:3:2 by vol.) enriched with α-lactose (0.1%), glutathione (1.2 mM), bovine insulin (1.0 μg/ml), bovine holo-transferrin (5.0 μg/ml), hydrocortisone (1.0 μg/ml), L-ascorbic acid 10 μg/ml), 10% (v/v) heat-inactivated FBS, penicillin-streptomycin (50 IU/ml), fungizone (2.5 μg/ml), gentamycin (50 μg/ml) and incubated in an atmosphere of 5% $CO_2$ / 95% humidified air at 37°C. All cell culture media and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). In order to obtain a monolayer culture cells were plated on plastic plates and maintained until confluence. For 3D culture BME-UV1 cells were treated with trypsin and resuspended in culture medium. 100 mm culture plates (Corning Inc., Lowell, MA, USA) or 8-well Lab-Tek Chamber Slides (Nunc, Inc, Naperville, IL, USA) were coated with 200 μl or 25 μl (per well) of Matrigel (BD Biosciences, USA) respectively, and left to solidify for 30 min. at 37°C. The cells were plated at a concentration of 25,000 cells/ml on culture plates, or 5000 cell/ml on Lab-Tek chamber slides. In the case of both culture systems the medium was replaced every second day. In the studies on the role of prolactin in the functional differentiation of BME-UV1 cells the culture medium was additionally supplemented with prolactin (3 μg/ml) (Sigma-Aldrich). The analysis of cell polarization, and functional development was performed after 3, 6, 9, 12, and 16 days of 3D culture.

### Confocal microscopy

BME-UV1 cells cultured on Lab-Tek chamber slides coated with Matrigel were fixed in 3.7% paraformaldehyde for 20 min, and permeabilized with 0.5% Triton X/PBS for 10 min. The cells were then incubated overnight with primary antibodies against: E-cadherin, ZO1 (Santa Cruz Biotechnology Inc., USA), or cleaved caspase-3 (Cell Signaling Technology Inc., USA). After primary incubation, and washing with PBS, cells were incubated with Alexa Fluor488 secondary antibodies (Life Technologies, Invitrogen, USA) for 1 h at room temperature. In the case of Ki-67 staining cells were only incubated with Ki-67-FITC-conjugated antibody (Life Technologies, Invitrogen) for 1 h at room temp. Additionally the cellular nuclei were stained with 7-aminoactinomycin D (7-AAD, 5 μg/ml) (Sigma-Aldrich). Cells were visualized using a confocal laser scanning microscope FV-500 system (Olympus Optical Co, Hamburg,

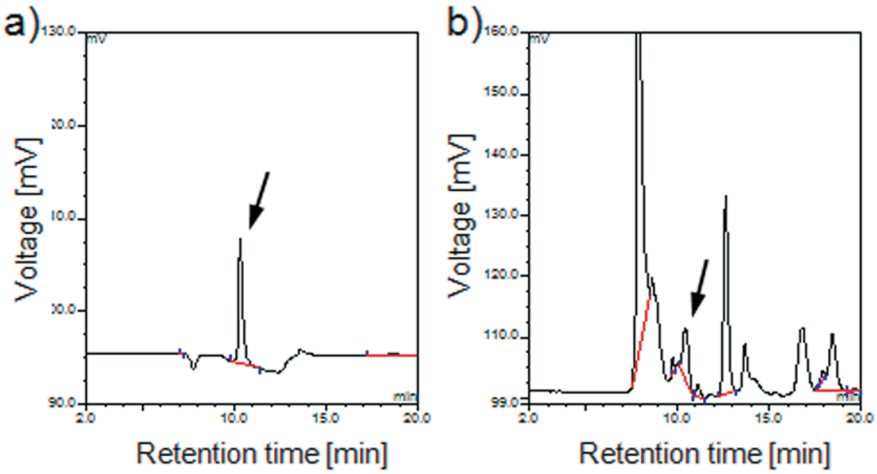


Fig. 1. Representative chromatograms of β-casein detected using RP-HPLC: a) chromatogram of the β-casein standard diluted in a non-used culture medium; b) β-casein detected in the experimental culture medium.

Germany). The combination of excitation/emission were: Argon 488 nm laser with 505-525 nm filter for Alexa Fluor 488 and HeNe 543 nm laser with 610 nm filter for 7-AAD nucleus staining.

### Western-blot analysis

Cells were grown on tissue culture plates until 90% confluence, in the case of 2D culture system, or for 3, 6, 9, 12, or 16 days on plates coated with Matrigel, in the case of 3D culture system. Next cells were pelleted by centrifugation at 14000 rpm at 4°C for 5 min, and frozen at – 80°C until protein extraction. Protein extracts were isolated from cell pellets using RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate and 1 mM PMSF) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich) for 30 min at 4°C. Protein concentration in the lysates was determined using Bio-Rad Protein Assay Dye Reagent according to the producer's instructions (Bio-Rad Laboratories Inc., USA). Proteins (50 fg) were resolved by SDS-PAGE and transferred onto PVDF membrane (Sigma-Aldrich). For immunostaining membranes were first blocked with 5% nonfat dry milk in TBS (20 mM Tris-HCL, 500 mM NaCl) containing 0.5% Tween 20, and then incubated with chosen primary antibodies at 4°C over night. After primary incubation and washing in TBST buffer appropriate horseradish peroxidase-conjugated secondary antibodies were applied (at 1:5000 dilution) (Santa Cruz Biotechnologies, Inc.) for 1h at room temp. Chemiluminescence was developed with the use of Amersham ECL detection reagent, based on the manufacturer's protocol (GE Healthcare Bio-Sciences Corp., USA). For densitometric analysis X-Ray films were scanned, and integrated optical density (IOD) was measured using Kodak 1D 3.5 software (New Haven, CT, USA).

### RP-HPLC analysis

High-performance liquid chromatography (HPLC) was used to determine β-casein concentration in the culture medium. All reagents were HPLC grade and purchased from J.T. Baker (Mallinckrodt Baker, Inc., USA), except for β-casein standard supplied by Sigma-Aldrich (catalog No. C6905). The media samples were purified through PTFE filters. Identification and quantitative analysis of β-casein was made on a liquid chromatograph (Dionex Corporation, USA) equipped in the UV/VIS detector. Beta-casein standard was diluted in the non-used culture medium for BME-UV1 cells, and used for validation of the method. The separation was performed using the Hypersil BDS C18 column, 5 µm, 150 x 4.6 mm at 1.0 ml/min flow rate, detection at 220 nm. Separation was conducted when isocratic condition was reached. The mobile phase consisted of sodium phosphate buffer (0.1M) : methanol (80:20). The content of β-casein was calculated as a sum of chromatographic peak area of β-casein standard and expressed in µg per 1 ml of medium. Figure 1 shows representative chromatograms of β-casein standard in the non-used culture medium, and β-casein detected in experimental culture medium.

## Statistical evaluation

The results were statistically evaluated using One Way ANOVA and Tukey's multiple range tests with GraphPad Prism™ version 5.00 software (GraphPad Software, Inc., La Jolla, CA, USA); $P \leq 0.05$ was

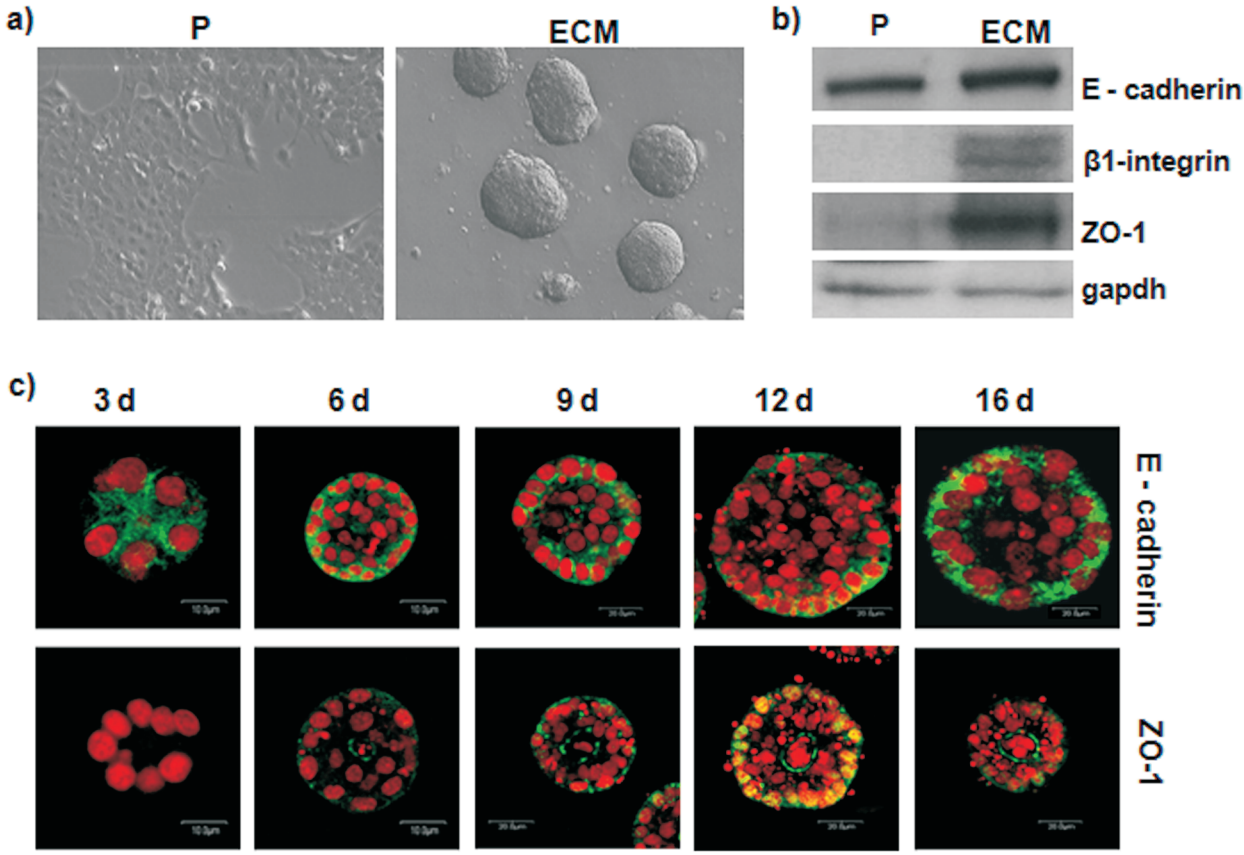


Fig. 2. Comparison of morphology and biochemical characteristics of BME-UV1 bovine mammary epithelial cells cultured on plastic (P), or Matrigel (ECM): a) Phase-contrast microscopy images of cells cultured in 2D system (P) for 24h, or 3D system (ECM) for 9 days; b) Western-blot analysis of the levels of E-cadherin, β1-integrin, and ZO-1 in cells cultured in 2D (P) and 3D (ECM) culture systems; expression of gapdh was used as a loading control; c) confocal images of BME-UV1 cells cultured on Matrigel for 3, 6, 9, 12, 16 days, stained with antibodies against E-cadherin, or ZO-1 (green fluorescence); DNA was counter-stained with 7AAD (red fluorescence).

regarded as significant, and $P \leq 0.01$ as highly significant.

## Results

### Polarization of BME-UV1 bovine mammary epithelial cells cultured on reconstituted basement membrane

BME-UV1 cells plated on Martigel formed spherical cell organoids, while the cell culture conducted on plastic surface resulted in the formation of a two-dimensional monolayer, with a typical cobblestone morphology of epithelium (Fig. 2a). A comparison of the expression of three chosen polarization markers was performed to examine the polarization status of the cells in both culture systems (2D and 3D). BME-UV1 cells were cultured on plastic until they reached 90% confluence in order to obtain optimal cell-cell contact, during which cell adhesions were well established. Simultaneously the cells were cultured for 3 days on plates covered with Matrigel, during which round shaped clusters were established. After the indicated period cells from both culture systems were lysed, and the proteins were isolated to analyze the level of three polarization markers: β1-integrin (basal surface receptor), E-cadherin (adherens junction protein), and ZO-1 (zonula occluden -1, tight junction protein). Western-blot analyses have shown that the expression of β1-integrin, and ZO-1 was highly increased in cells grown in the presence of ECM components, while the level of E-cadherin was comparable in cells cultured on both surfaces: plastic and Matrigel (Fig. 2b).

The localization of both studied junction proteins: E-cadherin, and ZO-1 was then assessed in the acinar structures formed by BME-UV1 cells cultured on Matrigel. Cells were grown in the 3D culture system for 3, 6, 9, 12, or 16 days. Confocal microscopy analysis of the immunofluorescent staining revealed that the proper, lateral localization of E-cadherin was observed from the earliest days of cell culture on Matrigel; however, in the later time-points the expression was detected only in the outer layer of cells, which were in direct contact with rBM. The E-cadherin

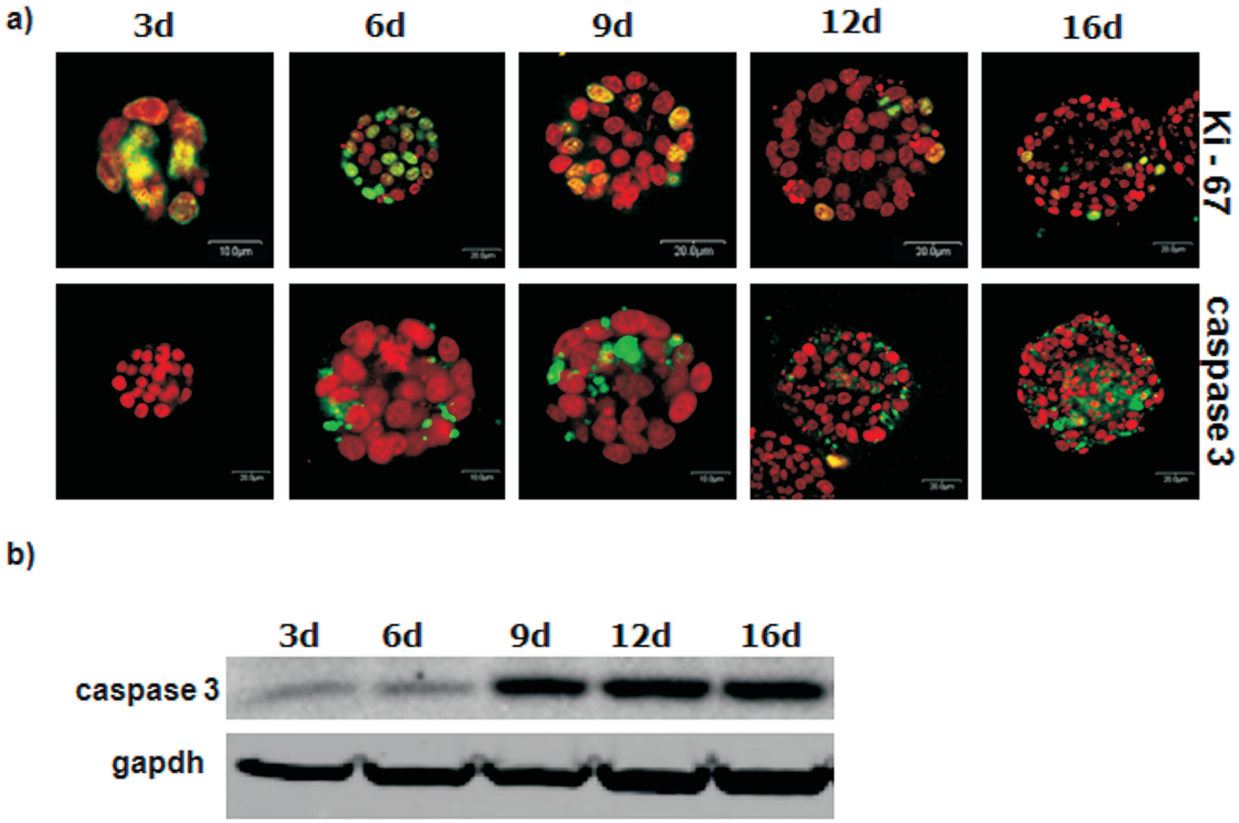


Fig. 3. Growth and differentiation rates of BME-UV1 cells grown in 3D culture system: a) confocal images of BME-UV1 cells cultured on Matrigel for 3, 6, 9, 12, 16 days, stained with antibodies against Ki-67, and cleaved caspase-3 (green fluorescence); DNA was counterstained with 7AAD (red fluorescense); b) changes in the level of cleaved caspase-3 in cells cultured on Matrigel for, 3, 6, 9, 12, and 16 days, analyzed by Western-blot; expression of gapdh was used as a loading control.

staining was not observed in the centrally localized cells, which gradually decayed in the acinar structures grown for a period longer than 9 days (Fig. 2 c). ZO-1 was localized mainly in the apical surface of the outer ring of the cells forming mammospheres, and the expression increased during the time of the experiment (Fig. 2c).

## Rate of growth and differentiation of BME-UV1 cells grown in 3D culture system

The proliferative activity of bovine MEC cells was judged on the basis of expression of proliferation marker: Ki-67 protein. Additionally we examined the process of apoptosis, using antibodies against cleaved (active) caspase-3, as this type of cell death is known to be responsible for the lumen clearance in the final stages of mammospheres development. In both experiments cells were cultured on Matrigel for 3, 6, 9, 12, or 16 days, followed by immunofluorescent staining and confocal microscopy analyses.

Confocal images of the acinar structures formed by BME-UV1 cells cultured on rBM showed that bovine mammary epithelial cells proliferate extensively during the first 6 days of the 3D cell culture (Fig. 3a). On day 9 of the experiment a substantial number of cells did not exhibit Ki-67 expression, which indicates that the process of growth arrest begins in the second week of culture on Matrigel. At this time however, the hollow lumen was not yet observed, as the structures were completely filled with cells. During the next 7 days of cell culture all the cells underwent cell cycle arrest, as indicated by the lack of Ki-67 staining. The activity of caspase-3 was noted in the centre of the mammospheres on the 12$^{th}$ day of culture (Fig. 3a). After 16 days, a hollow lumen could be observed. Additionally, the expression of cleaved caspase-3 was examined using Western-blot technique in cell extracts from the mammospheres formed by bovine MEC grown on Matrigel for 3, 6 , 9, 12, and 16 days (Fig. 3b). A gradual increase in the protein level was detected in the samples from day 9, 12, and 16 of the cell culture.

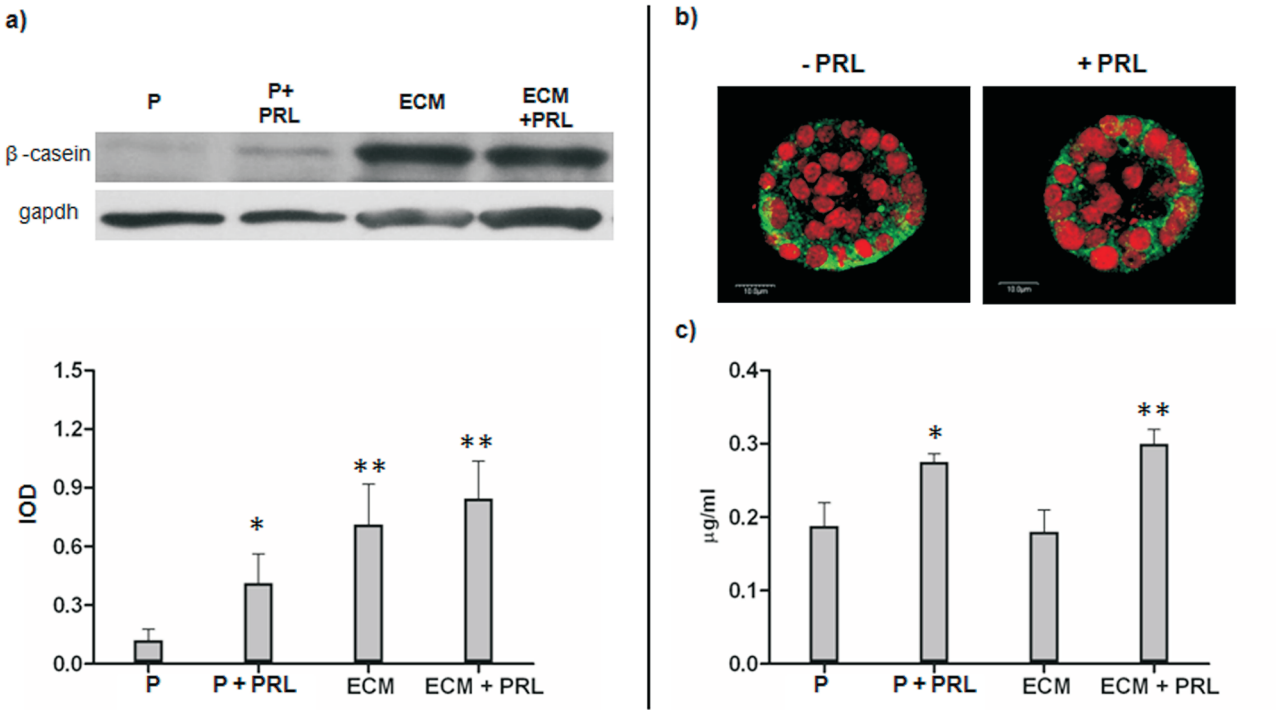


Fig. 4. Effect of ECM and prolactin on synthesis and secretion of β-casein by BME-UV1 cells: a) Western-blot analysis of the level of β-casein in cells cultured on plastic (P) or Matrigel (ECM), with (+PRL) or without (-PRL) prolactin in the medium; expression of gapdh was used as a loading control; the graph below represents the analysis of integrated optical density (IOD) of the corresponding Western-blot bands; b) Confocal images of BME-UV1 cells cultured on Matrigel for 12 days with or without PRL, and stained with antibodies against E-cadherin (green fluorescence); DNA was counterstained with 7-AAD (red fluorescence); c) RP-HPLC analysis of β-casein concentration in culture media collected after 48h incubation of BME-UV1 cells cultured on plastic (P) or Matrigel (ECM), with (+PRL) or without (-PRL) prolactin. Results are presented as means +SEM from four separate experiments; * – statistically significant difference ($p < 0.05$), ** – statistically significant difference ($p < 0.001$) in comparison with culture on plastic (P).

### The effect of ECM and prolactin on synthesis and secretion of β-casein by BME-UV1 cells

The functional differentiation of mammary epithelial cells is reflected by their ability to synthesize and secrete milk components. In the present study we examined the effect of ECM on the functional differentiation of BME-UV1 cells. Beta-casein, one of the major milk proteins produced in the bovine mammary gland, was chosen as a marker of functional differentiation, and its protein level was assessed in the lysates from cells cultured on plastic (when they reached 90% confluence), and cells grown for 3 days on Matrigel. In both culture systems (2D and 3D) two types of media were used: supplemented (+PRL) or not supplemented with prolactin (3 µg/ml). Additionally, the culture media were collected, and concentration of β-casein was measured with the use of RP-HPLC. Our results have shown that ECM significantly increased the synthesis of β-casein in BME-UV1 cells in comparison to the level of this protein obtained from the monolayer cell culture (Fig. 4a). The addition of PRL caused a significant increase in β-casein expression in cells grown in both types of culture system, in comparison with the monolayer not supplemented with this hormone. However, when we compared the β-casein levels between samples isolated from cells grown on Matrigel, which were, or were not, exposed to PRL, the stimulatory effect of this hormone was no longer significant. Moreover, the addition of PRL to the culture medium was also not necessary for the formation of fully developed mammospheres by BME-UV1 cells (Fig. 4b). Nevertheless, the stimulatory effect of PRL could be noted in regard to the secretion of β-casein into the medium. BME-UV1 cells were able to release milk proteins into the environment in both culture systems. In both cases the amount of secreted β-casein detected in the culture media was significantly higher when cells were supplemented with PRL (Fig. 4c).

Since our experiment revealed that the expression of β-casein was already significantly higher in bovine MECs after 3 days of cell culture on Matrigel in comparison to cells grown as a monolayer, we decided to investigate whether the synthesis of this milk protein undergoes further changes during the development of mammospheres formed by BME-UV1 cells. We therefore performed a Western-blot analysis of

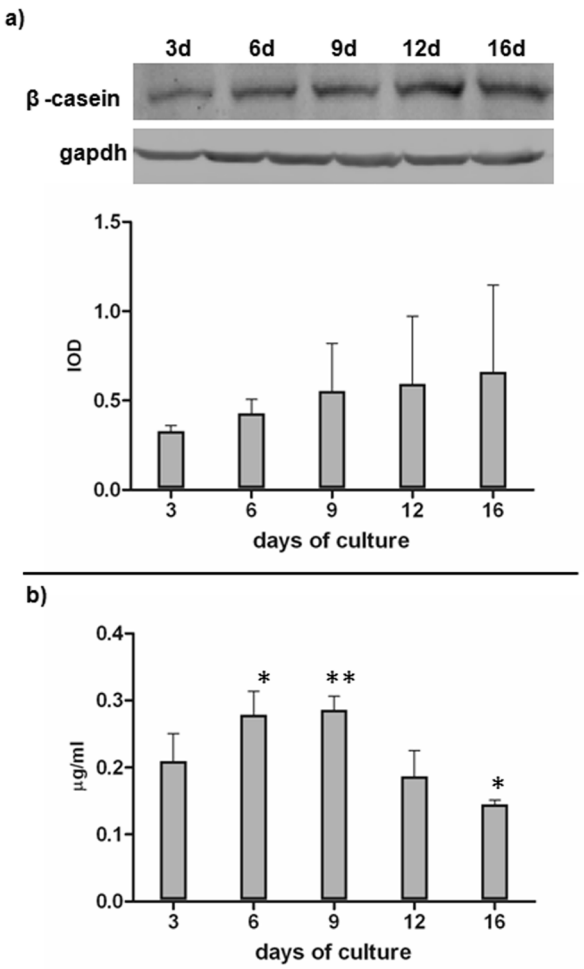


Fig. 5. Level of β-casein synthesized and secreted by BME-UV1 cells cultured on Matrigel for 3, 6, 9, 12, and 16 days: a) Western-blot analysis of β-casein; expression of gapdh was used as a loading control; the graph below represents the analysis of integrated optical density (IOD) of the corresponding Western-blot bands; b) RP-HPLC analysis of β-casein concentration in culture media collected on the 3rd, 6th, 9th, 12th, and 16th day of cell culture. Results are presented as means ± SEM from four separate experiments; * – statistically significant difference ($p < 0.05$), ** – statistically significant difference ($p < 0.001$) in comparison with day 3 of cell culture on Matrigel.

β-casein expression in lysates isolated from acinar structures at different stages of development (3, 6, 9, 12, and 16 days of cell culture). Simultaneously the culture medium was collected on each indicated day of the experiment to quantify the concentration of the secreted β-casein using RP-HPLC. The results indicated that the synthesis of β-casein had a tendency to increase during the time of 3D culture, manifesting the progress of the functional differentiation of MECs during the development of acinar structures (Fig. 5a). Interestingly the amount of secreted β-casein, detected by RP-HPLC, was increasing significantly during the first 9 days of cell culture, but in the later days (day 12 and 16) the level of this milk protein was reduced (Fig. 5b). On the 16$^{th}$ day of the experiment the concentration of β-casein in the medium was significantly lower in comparison to the level found in the medium from day 3 of the 3D culture (Fig. 5b).

## Discussion

The present study describes the stages of functional differentiation of the BME-UV1 bovine mammary epithelial cells cultured on rBM (Matrigel), which form 3D spheroids with a centrally localized hollow lumen, resembling the alveoli of the functionally active mammary gland. Our earlier (Kozlowski et al. 2009) and present observations have established that the development of fully organized and functionally active acinar structures is obtained within 16 days of cell culture on Matrigel. During this time cells undergo cell cycle arrest by the end of the first week of culture, followed by proper polarisation of the cells which are in direct contact with Matrigel (Fig. 2c, Fig. 3a). In the second week of 3D culture the centre of the spherical structures formed by BME-UV1 cells was gradually cleared, as the cells lacking direct contact with Matrigel underwent apoptosis, detected by the increasing activity of caspase-3 (Fig. 3a, b). Programmed cell death induced by cell detachment from the ECM (known as anoikis) was previously described in studies on human mammospheres, indicating that apoptosis is required for lumen formation (Debnath et al. 2002). The rate of development of the acinar structures formed by BME-UV1 cells resembled the rates obtained for mouse and human cell lines grown in the 3D culture system, which formed fully developed mammospheres between day 10 and 15 of cell culture ( Debnath et al. 2002, Xian et al. 2005).

In our study the spacial organization of the BME-UV1 mammary epithelial cells was assessed on the basis of chosen polarization markers: β1-integrin, E-cadherin, and ZO-1. Cells cultured on plastic exhibited only the expression of E-cadherin at levels comparable to the 3D culture (Fig. 2b). The two other markers were detected at very low levels in the monolayer culture, while their expression was significantly increased in BME-UV1 cells cultured on Matrigel (Fig. 2b). The occurrence of E-cadherin in BME-UV1 cells in both cell culture systems used (2D and 3D) was not surprising, as this protein is involved in the formation of the adherens junctions, critical for cell-cell adhesion and contact of the epithelial cells (Streuli, 2003). Since the cells were cultured on plastic until they reached 90% confluence, they were able to develop the necessary cell-cell contacts. However the low expression of the two other polarization markers used in this study indicates that the classical 2D culture approach is not sufficient to retain proper expression of the molecules involved in maintaining bov-

ine MEC contact with the environment. Confocal analysis of the spacial organization of the two junction proteins: E-cadherin, and ZO-1 in BME-UV1 cells cultured on rBM showed that the cells obtain proper apicobasal polarization in the acinar structures, detected from day 6 of the 3D cell culture (Fig. 2c). The expression of the tight junction protein: ZO-1 was very low in the bovine MECs forming a non-polarised monolayer (Fig. 2b). The development of tight-junctions (TJs) directly contributes to the maintenance of cell surface polarity, by formation of a barrier, which prevents diffusion of lipids, and proteins between apical and basolateral domains of the epithelium (Itoh and Bissel 2003). In the 3D system the expression of ZO-1 was detected starting from the early days of bovine MEC culture (Fig. 2b); however, proper apical localization of this marker was detected in the second week of acinar structure development (Fig. 2c). ZO-1 is associated with regulation of cell proliferation, as it forms complexes with the ZO-1-associated nucleic-acid binding protein (ZONAB), which plays the role of a transcription factor regulating the expression of cyclin-dependent kinase 4 (cdk4) (Balda et al. 2003). Since it takes part in formation of TJs it is also involved in regulating the direction of milk secretion, which is properly released into the alveolar lumen. During lactation TJs in the alveolar epithelial cells are highly impermeable to avoid leakage of milk from the lumen, although they are leaky in the MECs of the pregnant animals (Itoh and Bissel 2003). In our study β1-integrin – the receptor of the basal surface of epithelial cell membrane was detected only in BM-UV1 cells cultured on Matrigel (Fig. 2b). It was previously shown that this integrin subunit plays a crucial role in mediating the signals from the basement membrane into the epithelial cells, leading to the proper polarization, and spatial organization of the cells forming alveoli and ducts of the mammary gland (Naylor et al. 2005). Moreover, active integrin signalling was proven to be essential for driving the functional differentiation of MECs. Genetic ablation of β1-integrin in a primary 3D culture model inhibited the ability of prolactin to activate the Jak/Stat5 signalling pathway involved in the synthesis of milk proteins (Naylor et al. 2005).

The present results showed that BME-UV1 cells are able to express β-casein in both culture systems (2D and 3D); however, differences in the amount of synthesized milk protein, and responsiveness to PRL, were noted between the two culture conditions (Fig. 4). The synthesis of β-casein by bovine MECs in monolayer culture was dependent on the presence of PRL in the medium. It is well established that this lactogenic hormone is responsible for the induction of the expression of milk proteins. In the bovine mammary gland it was shown that milk production is regulated coordinately by PRL and GH. Growth hormone is necessary for the maintenance of the mammary cell population during the lactation period, while PRL regulates the expression of milk proteins (Wall et al. 2006). Interestingly, our study revealed that the culture of BME-UV1 cells on ECM components evoked a high level of β-casein synthesis even in the absence of prolactin (Fig. 4b). Moreover, PRL was not necessary for the proper development of mammospheres by BME-UV1 cultured on Matrigel (Fig. 4a). Our observations are consistent with the results of other groups working on the 3D culture system of MECs, which showed that the initiation of alveoli-like morphology formation was primarily driven by the ECM, and the acinar structures were formed even in the absence of PRL (Naylor et al. 2003, Riley et al. 2010). However, Riley and coworkers (2010) have noted in their microarray study of the transcriptional profile of primary bovine MECs cultured on Matrigel, that PRL is necessary for the high expression levels of milk protein genes. In our study the β-casein expression, detected at the posttranslational level, did not differ significantly between cells grown on rBM in the presence or absence of PRL (Fig. 4b). The previous work of Schmidhauser and coworkers (1992) revealed that the expression of milk protein genes depends on cell interactions with the basement membrane, as an ECM-response element exists on the promoter region of the β-casein gene. Moreover, signals from ECM, initiating MEC functional differentiation, are directly mediated by integrin receptors. Although prolactin did not seem to be necessary for the proper polarization of bovine MEC, it exhibited an important role in the secretion of the milk protein into the environment (Fig. 4c). BME-UV1 cells forming a monolayer on plastic surface, or acinar structures on Matrigel, showed a significant increase of β-casein secretion into the culture medium in the presence of PRL. This observation is in agreement with the *in vivo* studies of Wall and co-workers (2006), who noted that administration of an exogenous PRL to cows during early lactation elucidated an increase in milk yield similar to that observed with increased milking frequency. Prolactin is also involved in the regulation of tight junction permeability in the mammary gland during lactation, enabling the proper secretion of the milk proteins into the lumen of the mammary alveoli (Stelwagen et al. 1999). This may explain our results, showing that in the presence of PRL a gradual decrease in concentration of β-casein was observed in the culture medium in the final days of 3D culture (on days 12 and 16), although the synthesis of this milk protein in the cells increased throughout the entire time of the experiment (Fig. 5). The declining levels of β-casein in the medium coincided with the final stages of acini development, during which the lumen clearance, and proper localization of TJs was observed (Fig. 5c). Therefore, the fully organized mammospheres, for-

med by BME-UV1 cells, must have exhibited the secretion of milk components dominantly into the lumen, decreasing the amount of β-casein detected in the culture medium. Our observations are in agreement with the early studies of Barcellos-Hoff et al. (1989), and Blatchford et al. (1998), who characterised the development of acini by mouse mammary epithelial cells cultured in the 3D system, and showed that the alveoli-like structures were capable of vectorial secretion of proteins. High levels of milk proteins were detected in the luminal compartment of spheroids in the later days of cell culture, when the authors observed final formation of fully developed structures.

In conclusion, the present study has shown that the use of rBM in the culture of BME-UV1 cells creates a good model for studying the mechanisms controlling alveolar morphogenesis, and functional differentiation of the bovine mammary gland in *in vitro* conditions. ECM components are necessary for proper polarization and final differentiation of bovine BME-UV1 mammary epithelial cells. ECM exhibits a highly stimulating effect on synthesis of the milk proteins, which seems to be superior to the action of prolactin; however, PRL is necessary for efficient milk secretion by bovine MEC. The use of the 3D culture system in *in vitro* studies of the mammary gland of ruminants allows verifying differences in the development of the functionally active alveoli of humans, rodents and ruminants to be verified.

## Acknowledgments

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