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IN VITRO MAMMARY GLAND MODEL: ESTABLISHMENT AND CHARACTERIZATION OF A CAPRINE MAMMARY EPITHELIAL CELL LINE

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In vitro mammary gland model: establishment and characterization of a caprine mammary epithelial cell line

Demanding transcriptomic studies in combination with challenging experiments in livestock animal species could be replaced by good *in vitro* models mimicking the function of ruminant mammary gland. The objective of our study was to establish epithelial cell line obtained from primary cell culture of lactating goat mammary gland. Mammary tissue from lactating Saanen goat (*Capra hircus*) was digested in collagenase and hyaluronidase solution and plated on plastic flasks. When growing on plastic, typical cobblestone morphology of epithelial cells and larger irregularly shaped cells, corresponding to myoepithelial cells were observed. When growth medium was supplemented with lactogenic hormones (insulin, hydrocortisone, and prolactin) and cells were cultured on plastic for extended period of time at high density, dome-like structures appeared as a result of cell to cell contact induced differentiation. Immunofluorescence staining using antibodies against smooth muscle α-actin, vimentin and various cytokeratins were used to distinguish between different cell types. Cell types of epithelial and myoepithelial cells were confirmed. Complete differentiation of cells was achieved when growing them on a commercial basal membrane matrix preparation which contains laminin, collagen IV, and various growth factors. Cells grown on basal membrane matrix in growth medium supplemented with lactogenic hormones differentiated morphologically and functionally. Spherical structures that resembled the alveoli of lactating mammary gland were observed. Reverse transcription PCR (RT-PCR) was performed on the total RNA extracted from the cultured cells in order to detect the potentially present milk protein mRNAs.

Key words: goats / mammary gland / molecular genetics / cell culture / lactogenesis / caseins / expression / immunofluorescence

In vitro model mlečne žleze: vzpostavitev in določitev značilnosti epitelne celične linije iz kozje mlečne žleze

Zahtevne transkriptomske študije, ki vključujejo poskuse na živih živalih, je mogoče nadomestiti z ustreznimi *in vitro* modeli. Cilj naše raziskave je bil vzpostaviti celično linijo epitelnih celic, pridobljenih iz primarne celične kulture kozje mlečne žleze v laktaciji. Žlezno tkivo koze (*Capra hircus*) sanske pasme smo razgradili v raztopini kolagenaze in hialuronidaze in nacepili v plastične posodice. Pri rasti na plastični podlagi so se pojavile značilne epitelne strukture v obliki tlakovcev in večje celice nepravilnih oblik, ki so po zunanjosti ustrezale mioepitelnim celicam. Ob dodatku laktogenih hormonov (inzulin, hidrokortizon, prolaktin) v medij in po daljšem obdobju rasti ter ob visoki gostoti celic na plastični podlagi so se oblikovale kupolaste strukture, ki so posledica diferenciacije zaradi medceličnih interakcij. Za karakterizacijo različnih celičnih tipov smo uporabili imunofluorescenčno barvanje za α-aktin gladkih mišic, vimentin in različne citokeratine. Z barvanjem smo potrdili prisotnost epitelnih in mioepitelnih celic. Popolno diferenciacijo celic smo dosegli z gojenjem na komercialno pripravljenem matriksu, ki posnema bazalno membrano in vsebuje laminin, kolagen IV in različne rastne faktorje. Celice so se na podlagi iz ekstracelularnega matriksa in ob dodatku laktogenih hormonov morfološko in funkcionalno diferencirale. Nastale so sferične strukture, podobne alveolam mlečne žleze v laktaciji. Z reverzno verižno reakcijo s polimerazo (RT-PCR) smo na izolirani RNA preverili prisotnost mRNA za mlečne proteine.

Ključne besede: koze / mlečna žleza / molekularna genetika / celična kultura / laktogeneza / kazeini / ekspresija / imunofluorescenca

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1 INTRODUCTION

Because of the commercial value of milk there is a great interest in understanding mechanisms involved in milk protein expression and udder resistance to pathogens which cause infectious agalactia or secretion of abnormal milk. Demanding transcriptomic studies investigating mechanisms influencing mammary gland metabolism usually involve *in vivo* experiments. Additionally, treatments *in vivo* can have systemic effects which make controlling the environment of epithelial cells in a predictable way very difficult (Rose & McConoche, 2006). For this reasons adequate *in vitro* model mimicking the function of the mammary gland would be of great importance for the study of physiological, biochemical and immunologic functions of the mammary gland. Furthermore, there are almost no techniques that would allow the maintenance of organs *ex vivo* long enough to permit necessary molecular biological investigations. As such, an enormous potential exists in the use of three-dimensional (3-D) cell culture models as surrogates for tissues. In the recent years, mammary cell culture models were mainly used to study cell differentiation during lactation, innate immune response to infections and response to hormonal induction of lactogenesis in mammary epithelial cells (MECs).

Several ruminant immortalized cell lines such as MAC-T (Huyhn *et al*., 1991) and BME-UV (Zavizion *et al*., 1996) have been established by stable integration of the simian virus large T-antigen (SV40LTA). However, because of their low responsiveness to lactogenic hormones, transformed mammary cell lines were mainly used to study insulin growth factor 1 (IGF-1) metabolism (German & Barash, 2002). It is still not clear how modifications in immortalized cell lines alter physiological pathways of transformed cells, therefore the use of primary cell lines is much more representative of the *in vivo* system maintaining organ-specific functions and signal transduction pathways (Pantschenko *et al.,* 2000).

Growth of primary mammary cell cultures from lactating mammary gland on plastic usually results in loss of tissue specific functions. Cells in this state do not synthesize any of the milk components nor do they have the cellular response of *in vivo* cells (Blum *et al.,* 1989). On the other hand the growth of MECs on pre-formed extracellular matrices results in morphological differentiation as well as in synthesis of milk components (Rose *et al.,* 2002). Kabotyanski *et al.* (2009) studied transcription of β-casein (*CSN2*) and suggested that the expression of *CSN2* is induced synergistically by lactogenic hormones together with local growth factors, cell-cell and cell-substratum interactions.

The objective of our study was to establish goat MEC (GMEC) line, from primary cell culture, that is responsive to lactogenic hormonal induction and capable of expressing milk protein genes. The established GMEC line will be used for further studies of mammary gland differentiation, induction of lactation and infection response.

2 MATERIALS AND METHODS

2.1 ESTABLISHMENT OF CELL CULTURE

Mammary tissue was aseptically removed from the mammary gland of lactating Saanen goat (*Capra hircus*) immediately after slaughter. The gland was wiped with 70% ethanol and chopped up in chunks which were washed in HBSS (Hank's Buffered Salt Solution) medium containing penicillin (200 μg/ml), streptomycin (200 μg/ml), gentamicin (200 μg/ml), ampicilin (200 μg/ml) and amphotericin B (10 μg/ml). Tissue was further sliced in smaller pieces and digested in 100 ml of collagenase (Biochrom AG) and hyaluronidase (Sigma) solution (400 U/ml of each) prepared in HBSS with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) containing all of the above listed antibiotics in the same concentrations. The digestion was carried out at 37 °C with gentle shaking. The digesta were collected at 60, 120 and 180 minutes after the initiation of digestion and were filtered through a steel mesh. The filtrates were put in a 50 ml tube and washed several times with HBSS followed by centrifuging at 1200 rpm for 5 minutes. The cell suspension was filtered through 40 μm mesh, centrifuged and plated on plastic or further resuspended in 90% FBS and 10% DMSO for freezing in liquid nitrogen.

Aliquots of cell suspensions (fractions 1–3) were plated on plastic flasks in growth medium RPMI 1640 (Sigma) supplemented with lactogenic hormones insulin (1 μg/ml), hydrocortisone (1 μg/ml), and prolactin (1 μg/ml). Cells were incubated in a CO_2 incubator at 37 °C, 5% CO_2 and saturated humidity. The medium was changed every 2 to 3 days. For observing dome formation cells were maintained in culture for at least 20 days. When performing passaging the cells were treated with 0.05% trypsin-EDTA (Sigma) and incubated at 37 °C until the cells detached from the plastic dish. The cells were than resuspended in growth medium. Characteristics of the cell line were observed under light microscope.

2.2 IMMUNOFLUORESCENT STAINING

Cells were seeded in 6-well plates on cover glasses and cultured till they nearly reached confluence. They

were washed with cold phosphate buffered saline (PBS) and fixed in a mixture of cooled acetone and methanol (dilution 1:1) at –20 °C. Monoclonal antibodies against smooth muscle α-actin (sc-58669), vimentin (sc-73262) and cytokeratins (K) 14, 18, and 19 (sc-53253; sc-51582; sc-6278; all Santa Cruz Biotechnology) were used to distinguish between different cell types. The cover glasses were covered with solution of primary antibodies (dilution 1:200) in PBS with BSA (3%) and incubated overnight at room temperature. For unspecific binding control cover glass was incubated with PBS – BSA (3%). As a secondary antibody goat anti–mouse–FITC (F4143, Sigma) was used in dilution 1:500 in PBS – BSA (3%). Incubation was carried out in dark for 1 hour, and cells were observed under fluorescent microscope (Nikon Eclipse TE, 2000).

2.3 THIN LAYER METHOD AND 3D CULTURE METHOD

GMECs were grown on Geltrex Reduced Growth Factor Basement Membrane Matrix (Invitrogen) which is a soluble form of basement membrane purified from Engelbreth-Holm-Swarm tumor that gels at 37 °C forming a reconstituted basement membrane. The major components of Geltrex (GT) include laminin, collagen IV, entactin and various growth factors. In both methods GT was thawed at 2 to 8 °C overnight on ice in refrigerator. In thin layer method, used for culturing primary cell line, GT was diluted in cold serum-free RPMI 1640 medium in a concentration of 0.1 mg/ml and sufficient amount was used to cover the entire growth surface. Coated object was placed at 37 °C for 60 minutes or until dry. In 3D culture method 50 µl of GT was used per well of 24-well plate and left at 37 °C to promote gelling of matrix. GMECs were suspended in RPMI 1640 media containing 2% of GT and approximately 10^5 cells were plated per well. The cells were grown at 37 °C in humidified atmosphere of 5% CO2 in air and observed through microscope.

2.4 LACTATION INDUCTION (RT-PCR)

Total RNA was extracted from confluent second passage GMECs, grown on thin layer GT in lactogenic growth medium (as described previously), using TRI Reagent (Ambion) in accordance to manufacturer's instructions. In order to detect potentially present milk

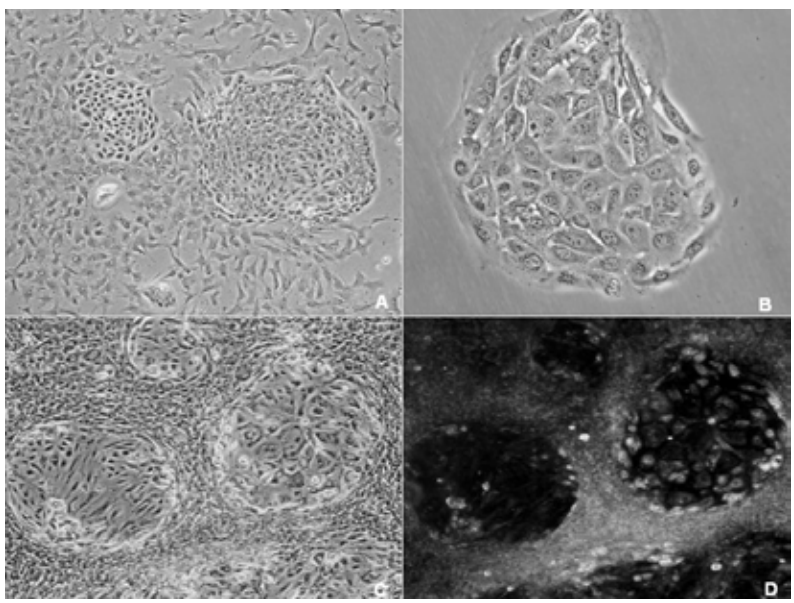

Figure 1: *Goat mammary cell line (second passage) growing as a monolayer on plastic.* ***A.*** *Colony morphology observed after 18 days in culture. Islands of epithelial cells surrounded with myoepithelial cells (magnification × 40).* ***B.*** *Island of densely packed epithelial cells (magnification × 200).* ***C*** *and* ***D.*** *Dome-like structures in 30 day old post-confluent cell line made by light microscopy using different light polarisation (magnification × 40).*

Slika 1: *Kozja celična linija (druga pasaža) v obliki monosloja na plastični podlagi.* ***A.*** *Morfologija kolonij v kulturi po 18. dneh. Otoki epitelnih celic obkroženi z mioepitelnim celicami (40 x povečava).* ***B.*** *Otoček epitelnih celic (200 x povečava).* ***C*** *in* ***D.*** *Kupolaste strukture v 30 dni stari postkonfluenti kulturi – svetlobna mikroskopija z uporabo različnih polarizacijskih filtrov (40 x povečava).*

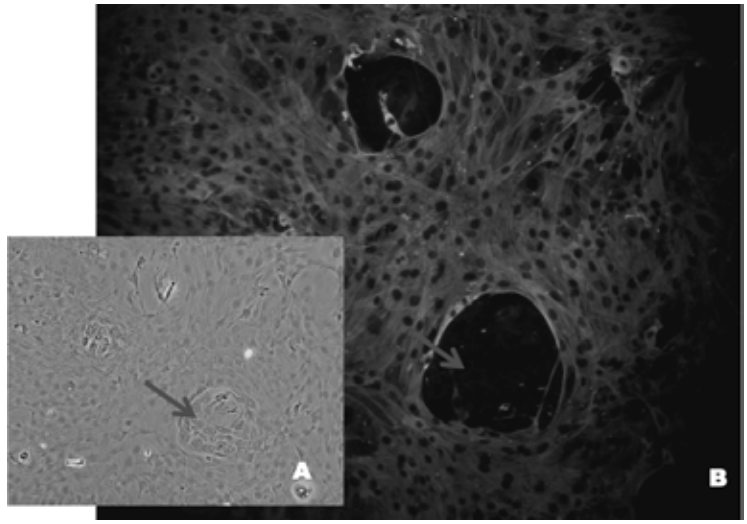

Figure 2: *Cell line labelled with antibodies for smooth muscle α-actin. Arrows indicate island of epithelial cells surrounded with myoepithelial cells.* ***A.*** *light microscopy (magnification ×100).* ***B.*** *fluorescent microscopy (magnification ×100).*
Slika 2: *Celična linija označena s protitelesi proti α-aktinu gladkih mišičnih vlaken. Puščici prikazujeta otok epitelnih celic obkrožen z mioepitelnimi celicami.* ***A.*** *svetlobna mikroskopija (100 x povečava).* ***B.*** *fluorescentna mikroskopija (100 x povečava).*

protein mRNAs reverse transcription-polymerase chain reaction (RT-PCR) was performed using OneStep RT-PCR kit (Qiagen). The PCR primers for amplification of β-casein (*CSN2)* and housekeeping gene β-actin (*ACTB)* were as follows: CSN2a-F: 5'-ACAGCCTC-CCACAAAACATC-3', CSN2a-R: 5'-AGGAAGGT-GCAGCTTTTCAA-3' with product length 206 bp; ACTBa-F: 5'-CCAACCGTGAGAAGATGACC-3', ACT-Ba-R: 5'-CGCTCCGTGAGAATCTTCAT-3' with product length 247 bp. RT-PCR products for β-casein and β-actin (*ACTB)* were isolated from agarose gel using gel extraction kit (Jetquick) and confirmed by sequencing.

3 RESULTS

Digestion of mammary tissue in collagenase and hyaluronidase solution resulted in isolation of heterogenous culture which contained mixed population of epithelial and myoepithelial (smooth muscle α-actin positive) cells. When grown on plastic, typical cobblestone morphology of epithelial cells and larger irregularly shaped cells corresponding to myoepithelial cells were observed (Figures 1 A and B). Dome-like structures appeared as a result of cell to cell contact induced differentiation, when cells were grown for extended period of time at high density (Figures 1 C and D).

The presence of cells representing epithelial and myoepithelial cell type was confirmed by immunostaining. Myoepithelial cells stained positively for smooth muscle α-actin whereas proposed luminal epithelial cells did not (Fig. 2). Cells of caprine cell line stained variously for cytokeratins (K14, K18, and K19) and negatively for mesenchymal intermediate filament protein vimentin.

Complete differentiation of cells was achieved when growing them on basal membrane (GT) matrix. GMECs grown on thin layer of GT matrix in growth medium supplemented with lactogenic hormones were able to express β-casein. Expression of β-casein was confirmed

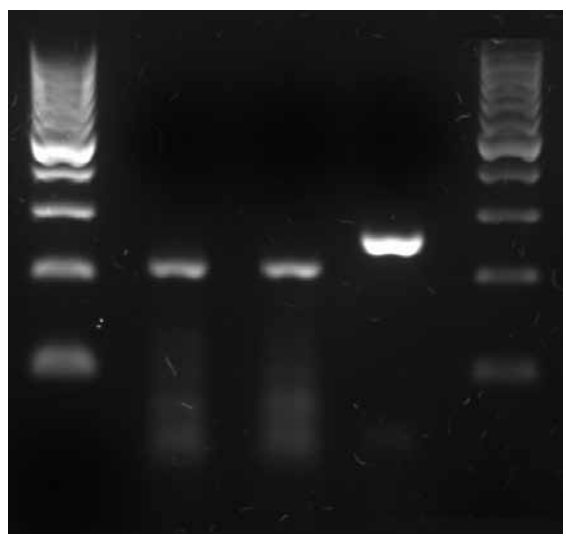

Figure 3: *Agarose gel electrophoresis of RT-PCR products for β-casein (lanes 2,3) and β-actin (lane 4) as control marker.*
Slika 3: *Agarozna gelska elektroforeza RT-PCR produktov za β-casein (stolpec 2,3) in β-aktin (stolpec 4), ki smo ga uporabili za kontrolo.*

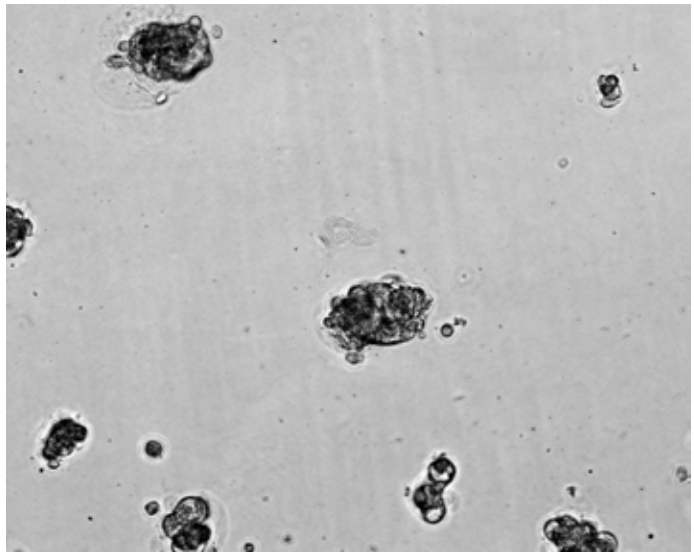

Figure 4: *3D culture of GMECs in GT matrix supplemented with growth medium containing lactogenic hormones (magnification ×100).*
Slika 4: *3D organizacija epitelne celične linije v GT matriksu, gojena v gojišču z dodanimi laktogenimi hormoni (100 x povečava).*

with reverse transcription PCR (Fig. 3). Products for β-casein (*CSN2)* and housekeeping gene β-actin (*ACTB)*, which we used as control, were confirmed by sequencing. This indicates that under lactogenic conditions milk proteins are being produced by GMECs.

When grown on GT matrix using 3-D culture method and supplemented with medium containing lactogenic hormones three-dimensional spheroids resembling acini of lactating mammary gland were formed (Fig. 4).

4 DISCUSSION

We describe the establishment of GMEC line focusing on growth morphology, expression of cytoskeletal proteins and evidence of differentiation. When the dissociated mammary gland cells were grown in vitro some of the cells formed island monolayer aggregates while others existed in free single-cell form. The GMECs were of different types. Epithelial type of cells depended closely on one another, were connected to each other and formed islands of similar densely packed cuboidal cells. Myoepithelial cell growth was observed in individual, random cell pattern at lover density compared to their epithelial counterparts.

Spontaneous domes are formed in post-confluent cell line which in a way is reminiscent of 3D organization of cells. It has been previously shown that formation of dome-like structures was connected with fluid under the epithelial cells that grew on plastic (Pickett *et al.*, 1975). Functional and structural changes that take place in dome-forming cells correspond to cellular changes occurring in vivo when tubules and alveoli are developed in the mammary gland at pregnancy (Zucchi & Dulbecco, 2002).

Specific smooth muscle α-actin monoclonal antibody reactivity was shown in myoepithelial cells. Actin is observed as sheets of filaments in the myoepithelial cell cytoplasm, whereas epithelial cells did not react with this antibody. Since vimentin is a marker of nonepithelial cells (i.e. cells of mesenchymal origin) non-staining of GMECs indicate that there are no fibroblasts in the cell line. Cytoskeletal protein expression is very much dependent on culture conditions and substrate of growth, thus we were not able to determine specific staining for cytokeratins.

The cells isolated from the goat mammary gland undergo three-dimensional organization in Geltrex. We observed formation of mammospheres or acinus-like structures, morphologically similar to those described as deriving from MECs (German & Barash, 2002, Rose *et al.*, 2002). Under this condition casein secretion by GMECs was dependent on the presence of lactogenic hormones. We were able to prove expression of β-casein, which is the major milk protein in goat milk, however we were not able to prove expression of other milk proteins.

When growing in culture, GMECs closely mimic the *in vivo* state of mammary gland, thus providing a suitable cell system model to study complex biological processes and pathways. Compared with monolayer cells in 2D culture, 3D cell culture provides physiologically much more relevant model for studying mammary cell

function. Our GMEC line will be exploited in transcriptomic studies focused on host response during infection, replacing challenging *in vivo* experiments.

5 REFERENCES

Blum J.L., Wicha M.S. 1988. Role of the cytoskeleton in laminin induced mammary gene expression. J. Cell. Physiol., 135: 13–22

German T., Barash I. 2002. Characterization of an epithelial cell line from bovine mammary gland. *In vitro* Cell. Dev. Biol. Anim., 38: 282–292

Huynh H.T., Robitaille G., Turner J. 1991. Establishment of bovine mammary epithelial cells (MAC-T): an *in vitro* model for bovine lactation. Experimental Cell Research, 197: 191–199

Kabotyanski E.B., Rijnkels M., Freeman-Zadrowski C., Buser A.C., Edwards D.P., Rosen J.M. 2009. Lactogenic hormonal induction of long distance interactions between beta-casein gene regulatory elements. J. Biol. Chem., 284: 22815–24

Pantsckenko A.G., Woodcock-Mitchell J., Bushmich S.L., Yang T.J. 2000. Establishment and characterisation of a caprine mammary epithelial cell line (CMEC). *In Vitro* Cell. Dev. Biol. – Animal, 36: 26–37

Pickett P.B., Pitelka D.R., HamamotoT., Misfeldt D.S. 1975. Occluding junctions and cell behavior in primary cell culture of normal and neoplastic mammary gland cells. The Journal of Cell Biology, 66: 316–332

Rose M.T., Aso H., Yonekura S., Komatsu T., Hagino A., Ozutsumi K., Obara Y. 2002. *In vitro* differentiation of a cloned bovine mammary epithelial cell. J. Dairy Res., 69: 345–355

Rose M.T., McConoche H. 2006. The long road to a representative *in vitro* model of bovine lactation. JIFS, 3: 67–72

Zavizion B., van Duffelen M., Schaeffer W., Politis I. 1996. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. *In Vitro* Cell. Dev. Biol. Anim., 32: 138–148

Zucchi I., Dulbecco R. 2002. Proteomic dissection of dome formation in a mammary cell line. Journal of Mammary gland Biology and Neoplasia, 7, 4: 373–384