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Development of an *In Vitro* Goat Mammary Gland Model: Establishment, Characterization, and Applications of Primary Goat Mammary Cell Cultures

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Abstract

Alternatives to animal experiments, based on *in vitro* methodologies, have been suggested and adopted in the last decades in order to completely substitute or to reduce animal numbers in *in vivo* assays. In this chapter we describe methods for establishment, maintenance, and characterization of primary goat mammary epithelial cell cultures (pgMECs) and possible applications for which the derived primary cell model can be used instead of *in vivo* experiments. The established cell lines were grown *in vitro* for several passages and remained hormone and immune responsive and capable of milk protein synthesis. Knowledge on goat mammary cells and their manipulation is applicable to different fields of research; for example, it could be used in basic research to study mammary development and lactation biology, in agriculture to enhance lactation yield and persistency or to produce milk with special characteristics, in biopharma to express recombinant proteins in goat milk, or in biomedicine to study lactation, mammary development, and pathology, including neoplasia. The established cells represent an adequate surrogate for mammary gland; were successfully used to study mammary gland immunity, lactation, and mammary stem/progenitor cells; and have a potential to be used for other purposes.

Keywords: goat, mammary gland, cell culture, mammary epithelial cell, lactation, mastitis

1. Introduction

Goats are one of the oldest domesticated species. They are bred for milk and meat and play an important role in human nutrition, especially in developing countries. Their number is constantly increasing through the years and the population has been estimated to over a billion

(FAOSTAT, 2013). However, goats are not useful only for food production. Because of their anatomical and physiological characteristics, relatively short gestation period, early sexual maturation, and inexpensive and simple maintenance, they are valuable for basic research, for biotechnology applications, and as animal models in medical research. For example, goats are used to study heart and joint diseases [1] and are an excellent model species to study mammary development and lactation [2].

Rodent mammary gland is the most widely studied and has provided many biological insights, but its anatomy and physiology are not fully representative of human or ruminant mammary gland. Morphological development of mammary gland is much more alike between humans and ruminants [3]. Considering the size, arrangement of the mammary gland (two main glands), and mechanism of secretion, which is apocrine in goats and humans, whereas merocrine in bovine, goats seem to be a better choice for modeling human mammary gland, compared to cows or rodents. Goat mammary tissue/cell cultures can serve as valuable models to study lactation, mammary development, and pathology, including neoplasia, which is for unknown reasons extremely rare in ruminants, despite the anatomical and physiological similarities to humans [4]. Additionally, genetically modified ruminants (especially goats) have been used as “bioreactors” for production of recombinant proteins. Recombinant proteins can be controlled by inducible, mammary-specific promoters and expressed in mammary gland, from where they can be relatively easily isolated from milk. For example, the first marketed human recombinant protein produced in transgenic animals was produced and extracted from milk of transgenic goats [5]. Furthermore, transgenic dairy goats can be used for production of milk with special nutritional characteristics [6], which can be beneficial especially in developing countries.

Knowledge on goat mammary cells and goat mammary biology is beneficial to different fields of science, for example, agriculture (enhancing lactation yield and persistency, and producing milk with special characteristics), basic research (understanding mammary biology), medicine (model organisms), and biopharma (expression of recombinant proteins in goat’s milk). In this chapter, we describe methods for establishment and characterization of primary goat mammary cell cultures (pgMECs) and possible applications for which the cell model can be used instead of the mammary tissue. The established cells can be grown *in vitro* for several passages and remain hormone responsive and capable of milk protein synthesis. The cells can be used for basic research of lactation biology, mammary gland immunity studies, mammary stem/progenitor cell identification/isolation, and further applications.

2. Materials and methods

In this section, we briefly describe materials and methods used for establishment, growth, characterization, and procedures with the primary cultures, which apply to the results described in the successive sections.

2.1. Establishment and maintenance of the primary goat mammary epithelial cells (pgMECs)

2.1.1. Tissue processing

Primary cultures were established from mammary tissue of slaughter animals. Lactating goats of different age and nonlactating juvenile goats from approximately four to seven months of age were used for tissue collection. The whole mammary gland was removed immediately after slaughter, wiped with 70% ethanol, and processed under aseptic conditions. First, larger pieces of the glandular tissue were removed from the gland. Alternatively, tissue biopsates can be used instead of whole mammary gland. Different quantities of the tissue can be processed, depending on the desired amount of cells in the culture. In our case, approximately 100 g of the dissected tissue pieces were washed in Hank's balanced salt solution (HBSS), containing penicillin (200 µg/mL), streptomycin (200 µg/mL), gentamicin (200 µg/mL), ampicillin (200 µg/mL), and amphotericin B (10 µg/mL), and mechanically minced with scissors and scalpels. Minced tissue was digested in a 100-ml solution of collagenase and hyaluronidase (400 U/mL of each), prepared in HBSS with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), also containing all of the above listed antibiotics in the same concentrations. The digestion was carried out at 37°C with gentle shaking. Fractions of dissociated cells were collected after 60, 120, and 180 min of incubation, by filtering the contents through a steel mesh and adding fresh solution of the enzymes to the leftovers of the minced tissue. The filtrates were put in 50-ml tubes, washed (diluted) with HBSS, and centrifuged at 1200 rpm for 5 min. The pellets can be resuspended in HBSS and centrifuged several times to remove cell debris. Finally, the resuspended cells were filtered through a 40-µm cell strainer, centrifuged at 1200 rpm for 5 min, resuspended in growth medium, and plated on tissue culture vessels or resuspended in freezing medium (90% FBS and 10% DMSO) for freezing in liquid nitrogen. Major steps of tissue processing are depicted in **Figure 1**.

An alternate to enzymatic digestion, explant culture method is possible. In this case, it is important to mechanically mince the extracted tissue to very small pieces and incubate the finely minced tissue in growth vessels (supplied with growth medium) for several days, using conditions as described hereinafter. After several days, cells will start to explant and attach to culture dishes. Afterward, the tissue pieces can be removed from the vessels and the attached cells passaged into a new dish. To our experience, explant culture will produce lower cell yields; however, the obtained culture might be more enriched in a desired (epithelial) cell type(s) as in the case of enzymatic digestion, where other cell types (e.g. fibroblasts) might be present in a significant amount.

2.1.2. Maintaining pgMECs in cell culture

The cells were grown in RPMI 1640 growth medium, supplemented with 0.1 mM L-methionine, 0.4 mM L-lysine, 2 g/l NaHCO₃, 1 mM Na-pyruvate, 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. For simulation of lactogenic

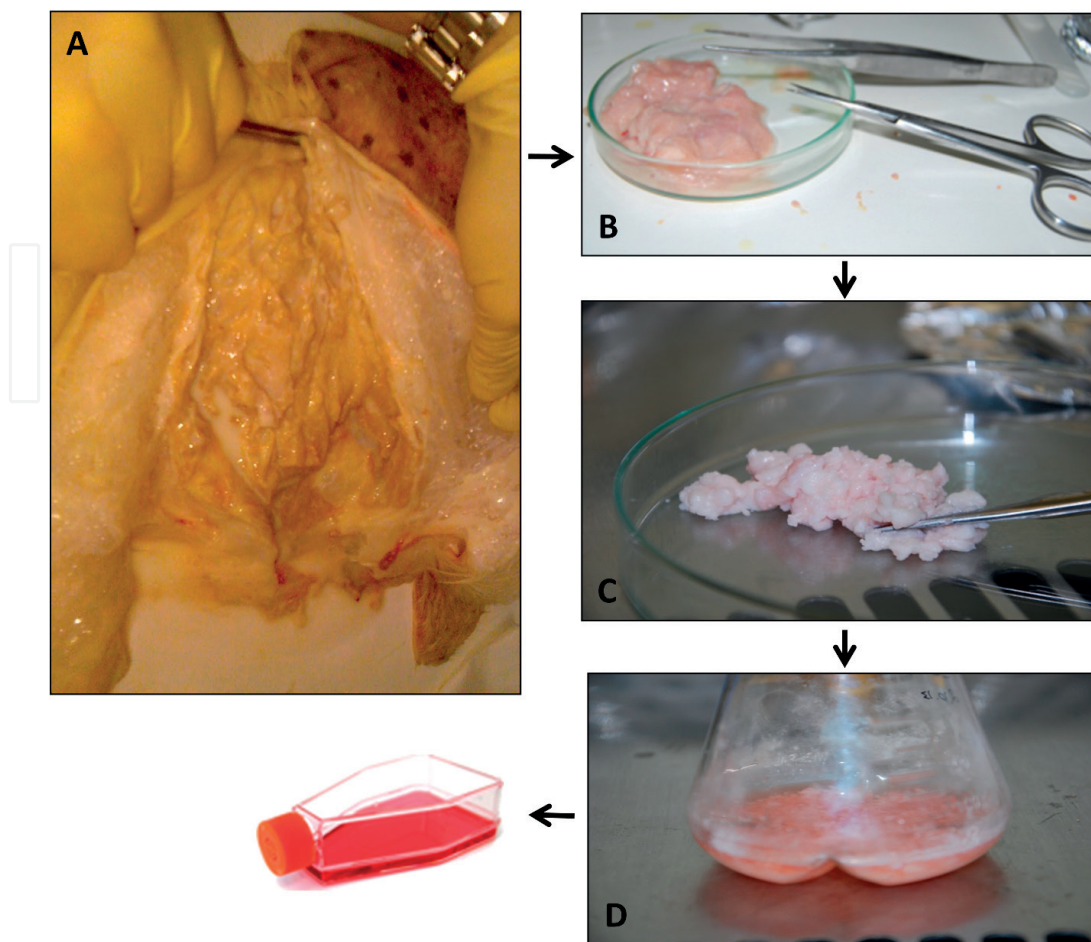


Figure 1. Mechanical and enzymatic processing of the mammary tissue (photo: J. Ogorevc). (A) Removal of the skin covering the mammary tissue. (B) Tissue pieces excised from the gland. (C) Fine mechanical processing of the tissue. (D) Dissociation of the tissue in the cocktail of enzymes. Finally, dissociated cells were collected by centrifugation and seeded in cell culture flasks.

conditions, basic growth medium was supplemented with lactogenic hormones such as insulin ($1 \mu\text{g/mL}$), hydrocortisone ($1 \mu\text{g/mL}$), and prolactin ($1 \mu\text{g/mL}$), and the cells were grown on a commercially prepared basement membrane matrix-covered surface (e.g. Matrigel, Geltrex). The cells were allowed to overgrow the surface, differentiate, and establish cell-cell and cell-surface interactions.

Cells were grown in a 5% CO_2 atmosphere at 37°C , 5% CO_2 , and saturated humidity. Growth medium was changed every two to three days. When performing passaging, the cells were treated with 0.25% trypsin-EDTA and incubated at 37°C until the cells detached from the surface. The cells were centrifuged and resuspended in growth medium in a 1:5 ratio. In case of fibroblast contamination, different detachment times of fibroblasts and epithelial cells can be exploited for refining the culture. Fibroblasts form weaker cell-cell and cell-surface interactions and normally detach faster. This characteristic can be used for enrichment of epithelial cells in the culture, using principle of differential trypsinization. Trypsin-EDTA can be diluted to a lower concentration (e.g. 0.05%) to extend detachment times for better control of the procedure.

The established cells should be routinely screened for possible infections with different mycoplasma species. We suggest PCR-based detection of mycoplasma-specific DNA sequences, using 16S ribosomal RNA universal primers as described previously [7].

2.2. Characterization of the pgMECs

2.2.1. mRNA expression of pgMEC-specific markers

For transcription profiling, total RNA was isolated from the aqueous phase of lysed pgMECs and reverse transcribed into complementary DNA (cDNA), which was subjected to new generation sequencing (NGS) as described in Ref. [8]. Additionally, reverse transcription polymerase chain reactions (RT-PCR) were performed to monitor expression of the selected cell-type specific markers in the culture. Real-time quantitative polymerase chain reaction (RT-qPCR) method was used to determine relative quantities of markers and ratios between them (e.g. expression of caseins in basal and lactogenic medium). Due to poor annotation of the goat genome, PCR primers were designed against *Bos taurus* RefSeq (NCBI) mRNA sequences and cross-matched against the goat reference sequences, if available. We determined expression of markers on mRNA level first and proceeded to protein level (e.g. immunostainings and western blotting) afterward.

In order to detect beta-casein (CSN2) mRNA culprits, we performed reverse transcription polymerase chain reaction (RT-PCR) on pgMEC-derived transcriptome library (cDNA), using the following primer pair CSN2-F: 5'-ACAGCCTCCCACAAAACATC-3', CSN2-R: 5'-AGGAAGGTGCAGCTTTTCAA-3'. The resulting 206 bp product was isolated from agarose gel, using gel extraction kit, and sequenced by Sanger sequencing to validate that the sequences correspond to the portion of the exon seven of the CSN2 gene (GenBank: AJ011019).

Real-time quantitative polymerase chain reactions (RT-qPCRs) consisted of 2× SYBR Green PCR master mix (Life Technologies), water, and 0.5 μM of each primer in a total volume of 20 μl. The cycles were as follows: 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve was determined at 15 s for 95°C, 1 min at 58°C, and 15 s at 95°C. The following primers were used: estrogen receptor 1 (ESR1) forward: 5'-ACAGCATGAAGTGCAAGAACGTGG-3' and reverse: 5'-TGCAAGGAATGCGATGAAGTGCAG-3'; progesterone receptor (PGR) forward: 5'-AAGCCAAGCCCTAAGCCAGAGAAT-3' and reverse: 5'-AGCTGGAGGTATCAGGTTTGCTGT-3'; and CSN2 forward: 5'-ACAGCCTCCCACAAAACATC-3' and reverse: 5'-AGGAAGGTGCAGCTTTTCAA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an endogenous control, using the primer pair: forward: 5'-CATGTTTGTGATGGGCGTGAACCA-3' and reverse: 5'-TAAGTCCCTCCACGATGCCAAAGT-3'.

2.2.2. Immunostainings

The immunostaining protocols often differ for different markers and every marker might require optimization of the protocol. Generally, the protocol consisted of cell fixation for

30 s to 1 min in ice cold acetone/methanol (1:1) or for several minutes in 4% paraformaldehyde. Fixation was followed by permeabilization (not necessary when using acetone-methanol fixation or in case of membrane-bound markers) with 0.3% Triton X-100 for 10 min. After washing with phosphate buffered saline (PBS), cells were blocked with 5–10% fetal serum (it is recommended to use fetal serum from species in which secondary antibodies were produced) and 1–3% bovine serum albumin (BSA) for 60 min. Incubation with primary antibodies was performed overnight at 4°C. Next day, cells were washed with PBS several times and incubated with fluorescently labeled secondary antibodies at room temperature for 1 h. After washing with PBS, cell nuclei can be counterstained with 4',6-diamidino-2-phenylindole (DAPI), washed, and visualized under microscope. In case of paraffinized tissue, sections were deparaffinized using xylene and rehydrated in decreasing concentrations of ethanol. Rehydrated tissue slices were washed in PBS, followed by performing heat-induced antigen retrieval in a microwave oven, using 10 mM sodium-citrate buffer (pH 6). Afterward, the same protocol was used for immunofluorescent staining as described previously for pgMECs. The more detailed protocols and the antibodies used were described in our previous publications [8–10].

2.2.3. Mammosphere formation assay

When performing mammosphere formation assay, a single-cell suspension of the mammary cells was grown in DMEM/F12 medium, supplemented with EGF (20 ng/mL), bFGF (human, 20 ng/mL), heparin (4 µg/mL), cholera toxin (10 ng/mL), hydrocortisone (0.5 µg/mL), insulin (0.5 µg/mL), and B27 supplement (2%), and grown in 6-well ultralow-attachment plates with or without extracellular membrane matrix or in hanging drops, according to the described protocol [11].

2.2.4. Oil Red O staining

Growth medium was aspirated and the pgMECs were fixed in 4% paraformaldehyde for 15 min. Oil Red O (0.5 g) was dissolved in 50 ml of isopropanol and diluted with water (3:2) and then left for 10 min, and the solution filtered through a 20-µm filter. Cells were briefly washed with isopropanol (60%) and incubated with solution of Oil Red O for 15 min at room temperature. The cells were then rinsed with isopropanol and washed under tap water. The formation of lipid droplets (red stain) was observed under bright field microscope.

3. Mammary tissue–derived primary cells as an *in vitro* model

Mammary gland development occurs in stages; proliferation and differentiation of mammary cells are dependent on sexual development and reproduction, which are under control of endocrine system [12]. The gland is primarily composed of mesenchymal and epithelial tissue and the latter is subjected to significant remodeling during lactation cycles. The tissue remodeling involves proliferation and differentiation of the epithelial cells forming functional

glandular tissue, followed by regression of the tissue due to apoptosis and loss of glandular structure and function (involution). The functional part of mammary gland is glandular tissue connected by a branched system of secondary ducts, combining into larger primary ducts, which end in the gland cistern. The milk is synthesized by secretory luminal cells arranged in spherical structures called alveoli, which form larger structures called lobules. Alveoli and ducts of lactating mammary gland are composed of different types of epithelial cells important for milk synthesis and secretion, while connective and fat tissue surround and support the epithelial structures. An alveolus is comprised of a single layer of milk secreting luminal epithelium, surrounded by a single layer of contractile myoepithelial cells, which lie adjacent to the basal membrane, where mammary stem/progenitor cells also reside [10].

In agreement with the “Replacement” of the three Rs principle (3Rs: Replacement, Reduction, and Refinement) adequate *in vitro* model, mimicking the function of the mammary gland allows the study of physiological, biochemical, and immune functions of the mammary gland, substituting *in vivo* experiments. In addition to the ethical issues, cell lines enable use of many technical replicas, better control of the environment, and surmount the problem of variation introduced by animal’s individuality [13] and the problem of systemic effects, which makes elucidation of a contribution of a particular cell type of interest difficult [14]. A main limitation of primary cell models is a finite life span, and a limiting number of available biological replicas, as a derived cell culture, represent a single genotype, while establishment and characterization of a large number of cell cultures/lines are quite a challenging and laborious process. Additionally, cell cultures do not always properly model *in vivo* conditions; therefore, limitations should be considered for each individual purpose.

Several ruminant immortalized mammary epithelial cell lines as MAC-T [15] and BME-UV [16] were generated by genomic integration of Simian virus large T-antigen (SV40LTA). However, transformed mammary cells are genetically and (usually) phenotypically changed. Transient mammary lines show low responsiveness to lactogenic hormones [17] and are not proper approximation of *in vivo* lactation. Genetic modifications and adaptations to growth in cell cultures alter metabolic pathways in continuous cell lines; therefore, the use of primary cells is a much better approximation of the *in vivo* system [18].

4. pgMEC characteristics

4.1. Morphology and growth

The derived primary cell culture consisted of a heterogeneous population of mostly epithelial and mesenchymal (fibroblast-like) cells. Epithelial cells grew in round-shaped densely packed islands of cells with multiple nucleoli and exhibited typical cobblestone morphology. Cells randomly spreading around these islands were larger, spindle-shaped cells, morphologically resembling fibroblasts (**Figure 2**).

Cell proliferation was slow for the first week after seeding dissociated cells in plastic dishes. After the first passage, the cells started to proliferate much faster and overgrew the surface

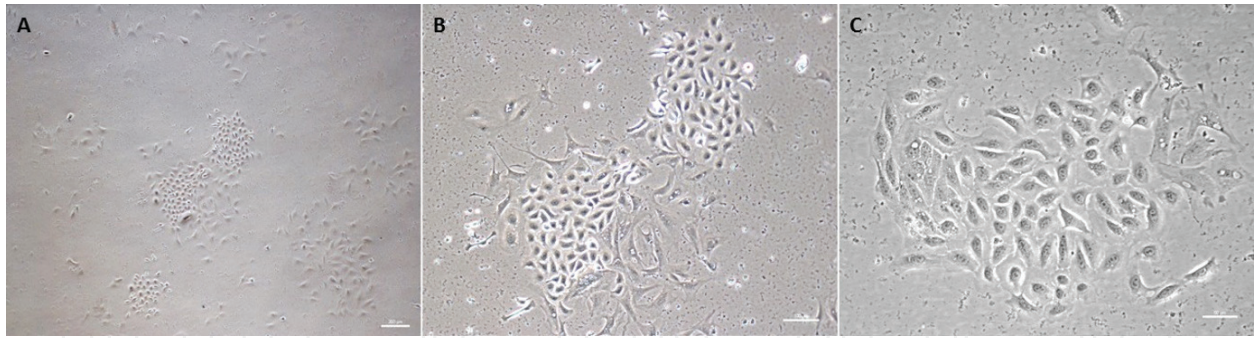


Figure 2. Primary culture 5 days after seeding under bright field microscope (photo: J. Ogorevc). (A) Heterogeneous cell types visible in the primary culture (40× magnification; scale bar = 200 μm). (B) Islands of epithelial cells surrounded by mesenchymal cells (fibroblasts). Cell debris can be observed in primary culture prior to first passaging (100× magnification; scale bar = 100 μm). (C) Densely packed island of epithelial cells (200× magnification; scale bar = 50 μm).

every several days. No changes in proliferation, morphology, or growth patterns were noticed for over five passages (**Figure 3**). When cells were kept at full confluency (without passaging) for extended period of time, they started to show signs of senescence.

4.2. Expression of specific markers

Different cell types express cell type-specific genes, which can be considered characterization markers. A draft of such markers was used to characterize the derived pgMECs and to distinguish different cell types in a heterogeneous primary cell culture.

Transcription profile generated by NGS showed that markers typical of basal/myoepithelial and luminal epithelial cells were highly expressed. The most expressed were different keratins, desmoplakin, and actins. The expression of markers varies based on the number of different cell types/lineages present in the culture and culture conditions, which may favor proliferation of a specific cell type and may promote differentiation (e.g. epithelial-to-mesenchymal transitions).

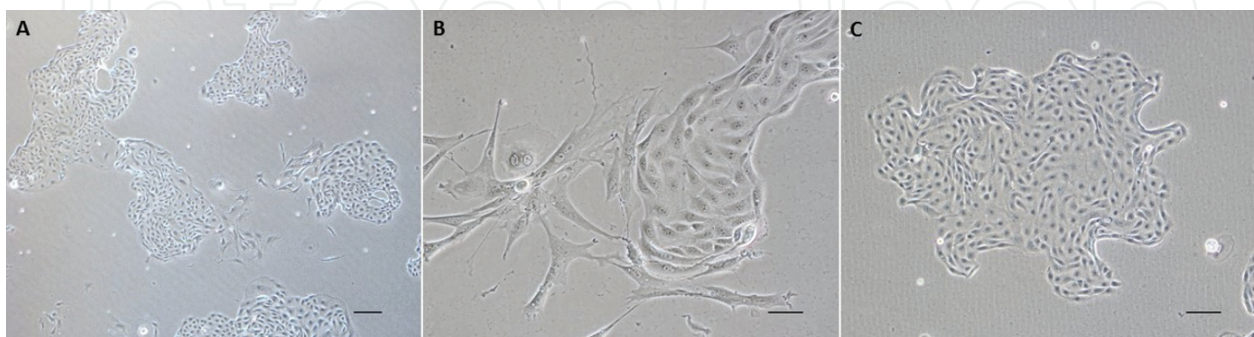


Figure 3. Mammary cell lines after passaging (photo: J. Ogorevc). (A) Epithelial and mesenchymal cells under 40× magnification (scale bar = 200 μm). (B) Island of epithelial cells (right) and mesenchymal cells (left) (200× magnification; scale bar = 50 μm). (C) Enriched culture of epithelial cells after differential trypsinization and removal of fibroblasts (200× magnification; scale bar = 100 μm).

The method of choice for characterization of different cell types in a cell culture is staining cells with tissue/cell type-specific antibodies, which reveal presence and localization of markers in the cells. The analysis of whole-transcriptome mRNA expression and review of previous studies, regarding distinctive mammary-specific markers in different species, represented a rationale for selection of antibodies, potentially useful for characterization of major cell types in goat mammary tissue and the derived cell cultures. Antibody-based characterization is a challenge in ruminants (especially goats) as most of the commercially available antibodies are targeted against human or rodent antigens, while their reactivity in ruminants is generally unknown and has to be determined empirically. To determine the presence of mammary-specific protein markers, immunofluorescent staining with different antibodies was performed.

Based on our results, we suggest cytokeratins (KRT) 14 and 18, as well as vimentin (VIM) as suitable markers for basic characterization of primary mammary cell cultures (**Figure 4**). Namely, cells of mesenchymal origin (e.g. fibroblasts) express VIM (**Figure 4A and B**), KRT 14 is distinctive of myoepithelial (**Figure 4E and F**), whereas KRT18 of luminal epithelial cells (**Figure 4C and D**). Based on these three markers, it is possible to distinguish epithelial cells from mesenchymal cells and distinguish between basal/myoepithelial and luminal cells.

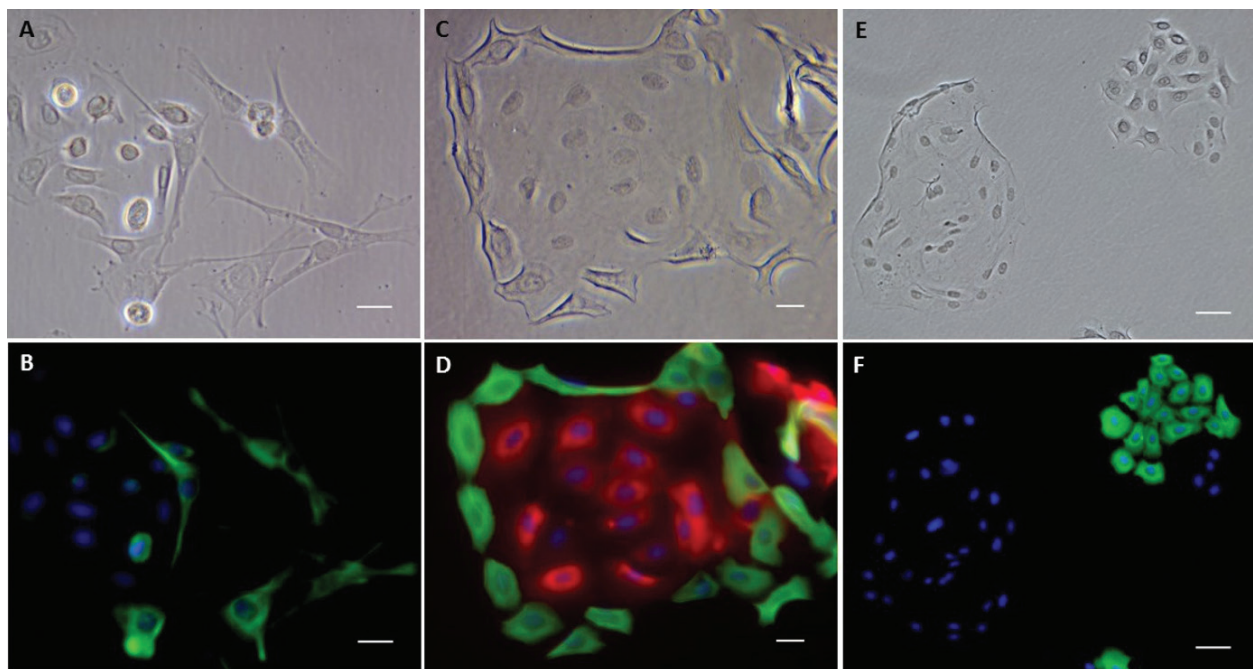


Figure 4. Basic characterization of the pgMECs. Fixed pgMECs under bright field (A, C, and E) and fluorescent illumination (B, D, and F) under 40× magnification (A–D; scale bars = 20 μm) and 20× magnification (E and F; scale bars = 50 μm) (photo: J. Ogorevc). Fluorescently labeled secondary antibodies were used to visualize expression and localization of the markers and a DAPI counterstain was used to visualize the nuclei. (A and B) The cells immunostained with primary antibodies against VIM. Spindle-shaped fibroblasts stained for VIM. (C and D) Double staining for KRT14 and KRT18. Luminal epithelial cells stained for KRT18 (D), whereas myoepithelial for KRT14 (D). Interestingly, when grown at low confluency, cells tended to organize as in alveoli, myoepithelial cells encircling luminal cells. (E and F) Staining for KRT14. Two islands of epithelial cells visible; myoepithelial cells stained for KRT14 (upper right corner), whereas no staining with KRT14 is visible in luminal epithelial cells (left).

Additional markers useful to distinguish epithelial cells from other cell types and to determine epithelial subtypes are different keratins (e.g. 5, 19), epithelial cell adhesion molecule (EPCAM), estrogen receptor 1 (ESR1), tumor protein p63 (TP63), integrin subunit beta 1 (ITGB1/CD29), integrin subunit alpha 6 (ITGA6/CD49f), progesterone receptor (PGR), alpha smooth muscle actin (ACTA2), caseins (e.g. CSN2), and mucin 1 (MUC1).

Additionally, paraffin-embedded sections of goat mammary tissue were stained to compare the expression of the markers between the pgMECs and the mammary tissue (**Figure 5**). Most of the markers showed reactivity in both—the cell cultures and the tissue, whereas some of the markers showed reactivity only in pgMECs (ESR1, CD49F, and KRT5) or only in the tissue (TP63). Tissue sections undergo chemical and physical treatment, which might result in changed conformation and antigen masking. On the other hand, pgMECs adapt to *in vitro* environment, which may alter cell metabolism and expression of markers. Therefore, discrepancies in immunostaining results are possible between the tissue and pgMECs. For example, EpCAM marker was localized in cytoplasmic compartment of pgMECs (**Figure 5A and B**) and was also found in epithelial compartments of goat mammary tissue (**Figure 5C and D**). In case of MUC1, weak signal was observed in pgMECs (**Figure 5E and F**) and strong signal, showing distinctive localization of MUC1 only to apical plasma membranes of secretory (luminal) epithelial cells, was detected in the mammary tissue (**Figure 5G and H**).

4.3. 3D organization—mammosphere formation

Under conditions that do not allow adherence to the surface, differentiated epithelial cells undergo anoikis. Growth under nonadherent, serum-free conditions is a characteristic of mammary stem/progenitor cells, which in such conditions form spherical structures called mammospheres. Spherical structures formed by human mammary epithelial cells contain enriched population of cells capable to differentiate into luminal or myoepithelial cells (bipotent progenitors) [19]. The molecular and cellular processes in mammospheres are similar as those in developing alveoli of the mammary gland [20]. Hierarchically, mammary cells range from terminally differentiated cells to undifferentiated progenitors and stem cells, the latter two being likely targets for malignant transformations in cancer [21]. It was shown that an entire mammary gland can be reconstituted from a single mammary stem cell [22]. Existence of mammary stem/progenitor cells in goat was first demonstrated by [10].

Under nonadherent conditions, irregularly shaped floating masses (organoids) were formed after several days. Aggregates that arose in ultralow-attachment plates (**Figure 6A**) in medium supplemented with basement membrane matrix were rounder and larger in shape as those grown in medium without basement membrane matrix. Immunostaining of fixed mammospheres revealed that luminal (KRT18—positive) and basal/myoepithelial (KRT14—positive) cells were the main constituents of the mammospheres [8]. Additionally, mammospheres were grown using hanging drop method. Hanging drop method is used as one of the *in vitro* tests for determining the pluripotent character of putative stem cells. The spherical structures appeared after several days of growth in hanging drops. They were fewer in number, but larger and more round in shape (**Figure 6B**), compared to mammospheres grown in ultralow-attachment plates. The mammospheres were fixed to glass slides and stained with DAPI and

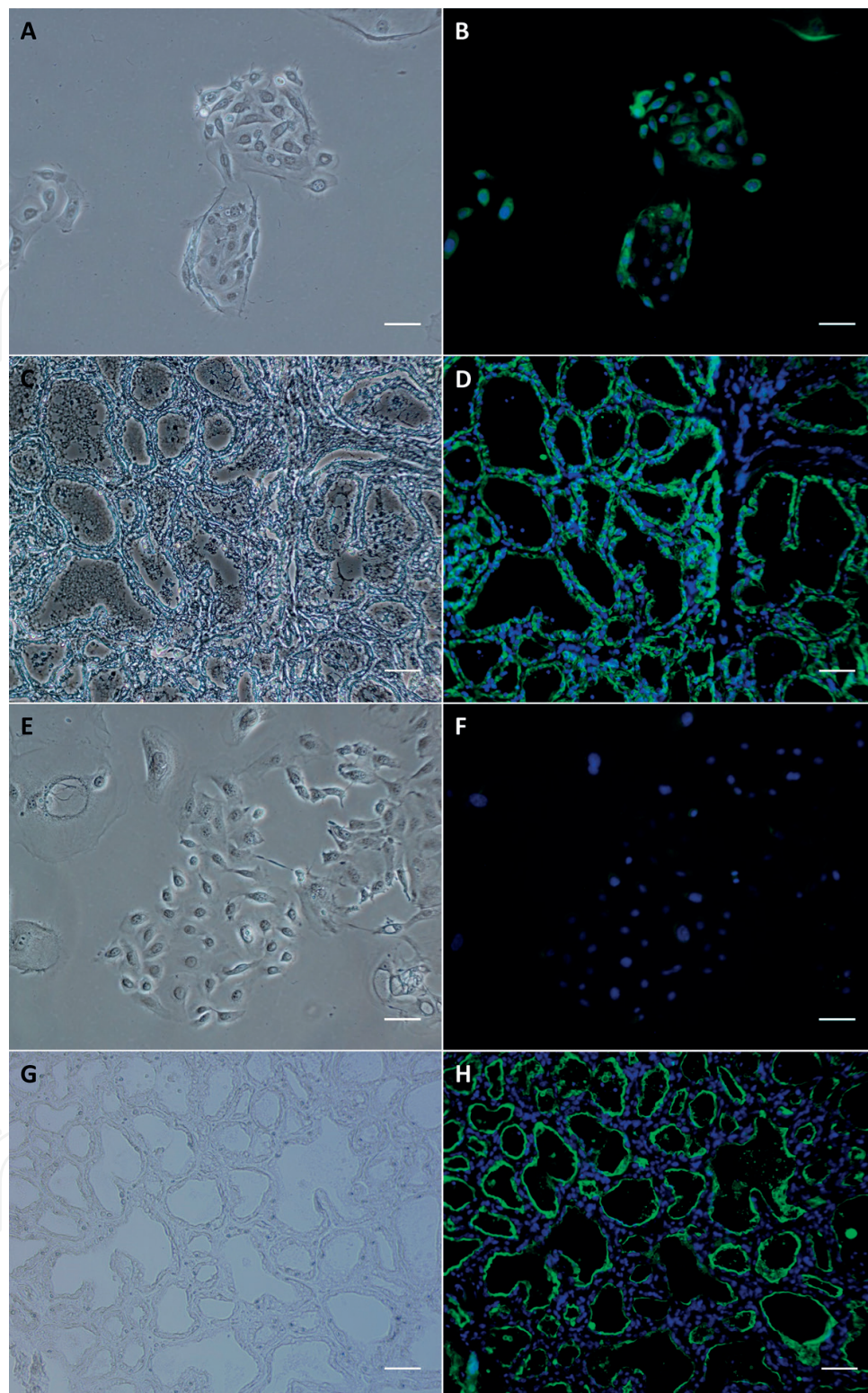


Figure 5. Immunofluorescence of pgMECs and goat mammary tissue under bright field and fluorescent illumination, stained against EpCAM (A–D) and MUC1 (E–H) (20× magnification; scale bars = 50 μm) (photo: J. Ogorevc). Fluorescently labeled secondary antibodies (green) were used and a DAPI counterstain was used to visualize nuclei (blue). The mammary cell culture (A and B) and epithelial cells of the tissue (C and D) stained against epithelial cell-specific marker EpCAM. pgMECs showed weak staining against MUC1 (E and F), whereas strong signal, localized to apical membranes of alveolar structures, was observed in the tissue (G and H).

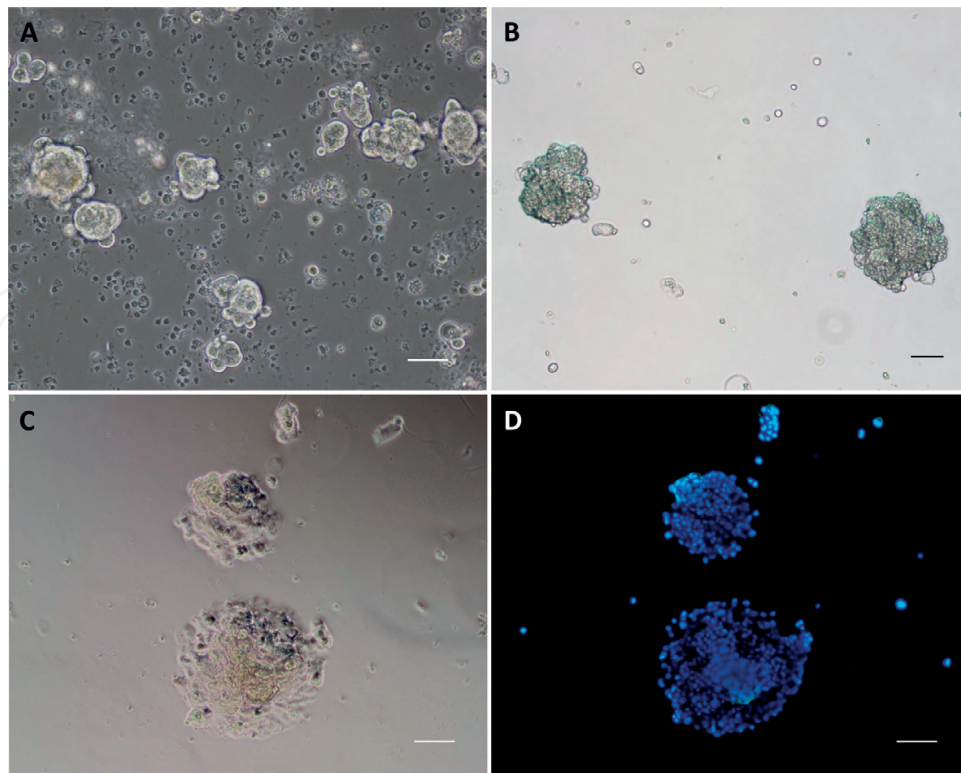


Figure 6. Formation of spherical structures in the pgMECs after 7 days of growth under nonadherent conditions (photo: J. Ogorevc). (A) Spherical structures in low-attachment plates (20× magnification; scale bar = 50 μm). (B) Organoids (mammospheres) grown in hanging drops (4× magnification; scale bar = 200 μm). (C and D) DAPI-stained fixed mammospheres, grown in hanging drops under bright field (C) and fluorescent illumination (D) (100× magnification; scale bar = 100 μm). Number of cells in the organoid structures can be estimated based on the number of stained nuclei (blue).

antibodies raised against KRT14 and 18. They consisted of several hundred cells (**Figure 6D**), which were KRT14- or KRT18-positive. Hanging drop is an efficient method to grow mammospheres from primary mammary cultures. The cells avoiding anoikis and forming organoids probably represent mammary progenitors.

5. Practical applications of the pgMECs

Mammary development, hormone responsiveness of mammary cells, regulation of milk expression, modeling milk composition and coagulation properties, enhancing milk yield, and innate immunity are some of the interests of modern dairy production. The characterized mammary cell lines are useful models to study biology of the mammary gland. For example, we used the cells for infection study with a common mastitis-causing agent in goats—*Mycoplasma agalactiae* (PG2 strain)—and to study differences in expression of the steroid receptors and beta casein in different growth conditions and in different pgMEC lines, derived from tissues of animals in different physiological states.

5.1. Mastitis model

Because of the economic importance for dairy industry and possible health and milk quality risks for consumers, there is a great interest to understand and enhance natural immunity of the mammary gland. Mammary epithelial cells are capable of innate immune response during intramammary infections and represent important barrier against invading pathogens.

In small ruminants, coagulase-negative staphylococci account for most of the mastitis cases, followed by Streptococci, *Staphylococcus aureus*, and other bacteria [23]. Additionally, contagious agalactia caused by *Mycoplasma agalactiae* (*Ma*) is a common cause of intramammary infections (contagious agalactia), especially in Mediterranean regions [24]. Mammary cell lines are often used to study immune response to mastitis, instead of *in vivo* infections. In our study, next-generation sequencing (NGS) was used to assess whole-transcriptomic response of *Ma*-challenged pgMECs 3, 12, and 24 h postinfection [25].

The results show that the infection induced an innate immune response in the infected cells. The pgMECs were capable of recognizing and responding to the pathogen infection (**Figure 7**). The pgMECs responded by induced expression of cytokines (interleukins and chemokines) and other chemotactic agents, activation of complement system, apoptosis pathways, and induction of genes coding for antimicrobial effector molecules (e.g. defensins, lysozyme, and nitric oxide synthase) (**Figure 7A**). The changes in expression were moderate, with no phenotypically visible changes in cell morphology, which corresponds to subclinical course of contagious agalactia *in vivo*. The pathway enrichment analysis showed that the most affected pathways were associated with immune response, steroid and fatty acid metabolism, apoptosis signaling, transcription regulation, and cell cycle regulation. We speculate that physiologically, the *in vivo* immune contribution of the pgMECs is important for recruitment of

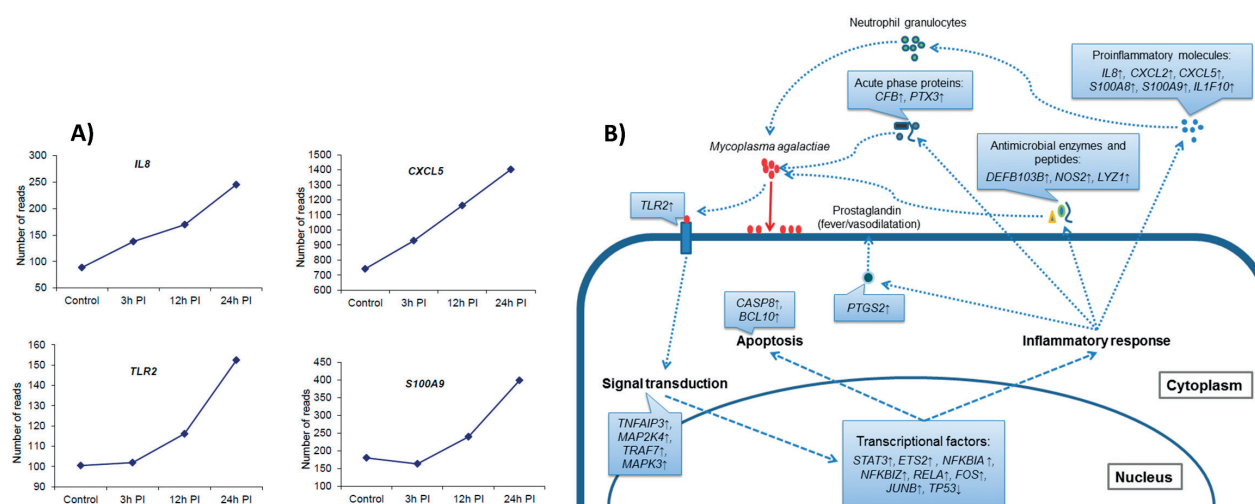


Figure 7. Transcriptomic studies on *Mycoplasma agalactiae*-infected pgMECs (modified from Ref. [25]). (A) Induction of immune-associated genes interleukin 8 (IL8), chemokine (C-X-C motif) ligand 5 (CXCL5), Toll-like receptor 2 (TLR2), and S100 calcium-binding protein 9 (S100A9). (B) Possible immune response mechanisms in pgMEC, suggested based on differential expression of genes and analysis of genetic networks and metabolic pathways.

neutrophils, activation of complement system and apoptotic pathways, as well as expression of several bactericidal molecules (**Figure 7B**) [25].

5.2. Mammary differentiation and lactation model

Expression of milk proteins and appearance of milk drops containing lipids are signs of lactogenic differentiation of the mammary cell culture. Lactogenic differentiation is dependent on multiple factors. To determine if the derived pgMECs are capable of lactogenic differentiation, we performed screening for beta-casein expression, the most abundant protein in goat milk, and stained putative milk drops for the presence of lipids, using Oil Red O.

Several pgMEC lines from mammary tissue of animals in different physiological states (different stages of lactation and juvenile goats) were established and grown under different growth conditions (basic and lactating). We observed different morphology, expression of steroid receptors (estrogen and progesterone), and expression of beta casein (CSN2), and tried to evaluate the effect of different growth conditions (medium, growth surface, and cell density) and donor tissue on characteristics of the derived cell lines. It was shown that primary mammary cells rapidly loose expression of steroid receptors [26] and casein [27] when grown in monolayer on plastic, whereas growth on extracellular matrices should provide the basal-apical polarization to the epithelial cells, needed for maintaining certain characteristics or to enable proper differentiation and milk component synthesis [28, 29].

5.2.1. Morphology

Morphology differed between the derived primary cultures established from mammary tissue of goats in different physiological states. Cells of the same primary cell line, grown under different growth conditions, also exhibited morphological differences (**Figure 8**). When cells were grown to confluency in lactogenic medium, dome-like and acini-like structures were formed in pgMECs, derived from the lactating tissue. No such structures appeared in pgMECs derived from the tissue of juvenile goats, grown under the same conditions. However, lumen-like and milk drop-like structures were formed only in pgMECs derived from juvenile goats, grown in lactogenic medium.

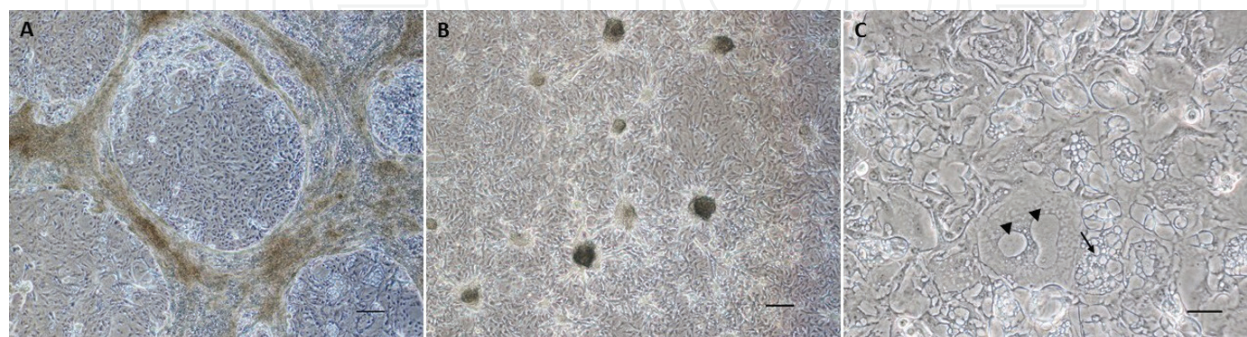


Figure 8. Morphology of different pgMEC lines in lactogenic conditions (photo: J. Ogorevc). Dome-like (A) and acini-like structures (B) were formed in pgMECs, derived from lactating mammary tissue (40× magnification; scale bars = 200 μm). Vacuoles resembling milk drops (arrow) and lumen-like structures (arrowheads) were formed in pgMECs, derived from juvenile goats (200× magnification; scale bar = 50 μm).

5.2.2. Expression of the steroid receptors

Terminal differentiation of mammary epithelial cells is required for luminal cells to secrete milk. Estrogen and progesterone are important hormones in mammary development and mammary cell differentiation. The roles of estrogen and progesterone during morphogenesis are well known [12], but their role during lactation is not clear. The function of the hormones is mediated through estrogen (ESR) and progesterone receptors (PR) that in ligated form migrate to nucleus and act as transcription factors for various genes. The studies suggest that ESR1 and PR are (co)expressed in mammary epithelial cells [30] where they participate in regulation of differentiation and control balance between luminal and basal mammary epithelial cells, mediated through paracrine signaling between the neighboring cells [31, 32]. Most of the ESR1- and PR-positive cells express markers of the luminal lineage [33], whereas the lack of ESR and PR is typical for undifferentiated mammary progenitor cells [34]. Unclear mechanisms by which hormonal action regulates lineage commitment and cell proliferation hamper our understanding of mammary differentiation, potentially useful for boosting milk production, as well as for better understanding of malignant transformations in mammary cells.

We quantified the expression of the steroid receptors in different pgMEC lines, grown in different conditions [35]. The cell lines derived from the mammary tissue of nonlactating doelings exhibited higher relative expression of ESR1 (approximately 50-fold) and PGR (approximately 8-fold), compared to cells derived from tissue of lactating goats. The response to lactogenic conditions was variable upregulation (from 1.4- to 12-fold) of ESR1 and consistent (approximately 3-fold) downregulation of PGR. Using immunostainings, we identified epithelial cells negative for both receptors, positive solely for ER- α or PR, and cells coexpressing both receptors. ER- α and PR were mainly localized in the nuclei and partly in cytoplasm of the cells. Multiple staining with luminal (CK18) or basal (CK14) markers revealed that not all of the ER- α -positive or PR-positive markers belonged to the luminal lineage and that not all of the luminal cells are ER- α and/or PR-positive (**Figure 9**). It seems that lactogenic conditions caused differentiation and proliferation of the luminal lineage and that ER- α could be involved in functional differentiation of the luminal mammary cells.

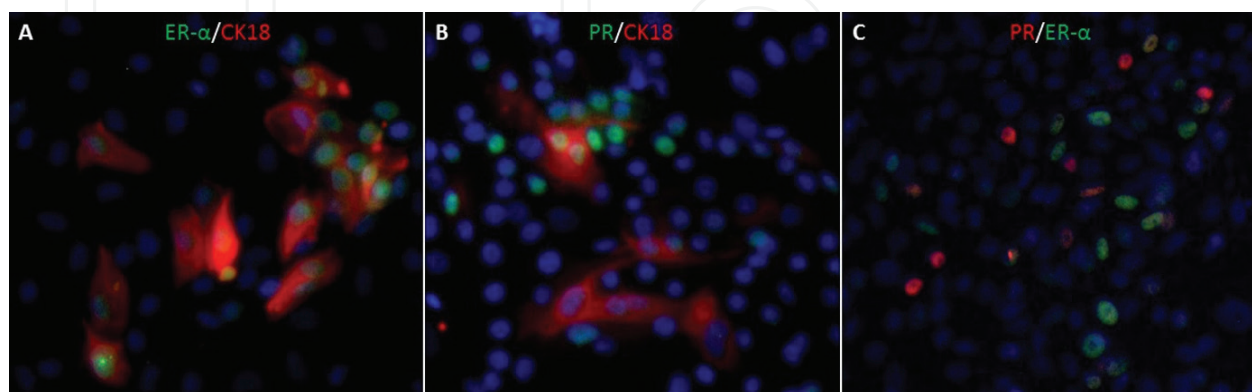


Figure 9. Immunofluorescent double stainings of the pgMEC lines, under 200 \times magnification (photo: J. Ogorevc). Nuclei were counterstained with DAPI (blue). (A) ER- α (green) localized mainly in nuclei of CK18-positive (red) cells. CK18-negative cells with nuclear staining against ER- α can also be observed. (B) PR-positive (green) and PR-negative luminal (red—CK18-positive) cells. (C) Nuclear colocalization of ER- α and PR (orange) and cells positive solely for ER- α (green) or PR (red).

5.2.3. Expression of beta casein (CSN2)

Expression of caseins was detected in various primary and immortalized mammary cell lines from different species. For example, mouse HC11 cells [36], several bovine mammary cell lines [15, 28, 37–39], and goat primary mammary cells [8, 18, 40] are able to express caseins. Transcription of CSN2 was studied in mouse HC11 cells and the authors [41] found that its expression is induced synergistically by combination of lactogenic hormones, local growth factors, and cell-cell and cell-substratum interactions.

We evaluated how the starting tissue material, addition of hormones (insulin, hydrocortisone, and prolactin) to the growth medium, and growth on a commercially prepared extracellular basement membrane matrix affect relative expression of beta casein [42], determined using RT-qPCR (**Figure 10A**). The CSN2 transcripts were detected in all of the samples, including cells originating from nonlactating goat, grown in basal medium. However, the expression of CSN2 and response to different growth conditions were different in individual cell lines. Interestingly, we found that CSN2 expression was the highest in pgMECs derived from juvenile goats, grown in lactogenic medium. Addition of hormones in most cases induced expression of CSN2. The effect of extracellular membrane matrix-covered growth surface (Geltrex in our case) was variable. We found that extracellular membrane matrix growth surface was not indispensable for casein expression in the cell lines. In some of the cell lines, membrane matrix significantly increased CSN2 expression, whereas in others, it did not have a statistically significant effect (in several cases, expression of CSN2 was even lower [nonsignificantly] in pgMECs grown on membrane matrix). We speculate that some of the cell types present in a heterogeneous mixture of the cell cultures, are capable of extracellular matrix production in quantities sufficient for luminal cells to achieve lactation competency. It was reported previously that mouse HC11 cells can produce extracellular matrix [43] and do not require additional matrix for CSN2 expression [36].

Complex regulatory mechanisms are required for the onset of lactation, which involve functional differentiation/proliferation of the mammary cells and considerable anatomical and physiological tissue perturbations during gestation. Interestingly, milk proteins can be detected in pgMECs already after several days of growth in cell culture, even if derived from the tissue of nonlactating animals. It seems that pgMECs are capable of terminal differentiation from basal to secretory cells in a short period of time, when grown in environment enabling lactogenic differentiation. CSN2 was localized mainly in circular (lumen-like) structures, formed only by pgMECs grown in lactogenic conditions (**Figure 10B**). To conclude, expression of CSN2 in pgMECs is variable and depends mostly on starting tissue material and growth conditions.

5.2.4. Lactating versus juvenile mammary tissue-derived pgMECs

The expression of the steroid receptors and beta casein was the highest in juvenile mammary tissue-derived pgMECs, which could indicate a possible role of ER and PR in lactogenic

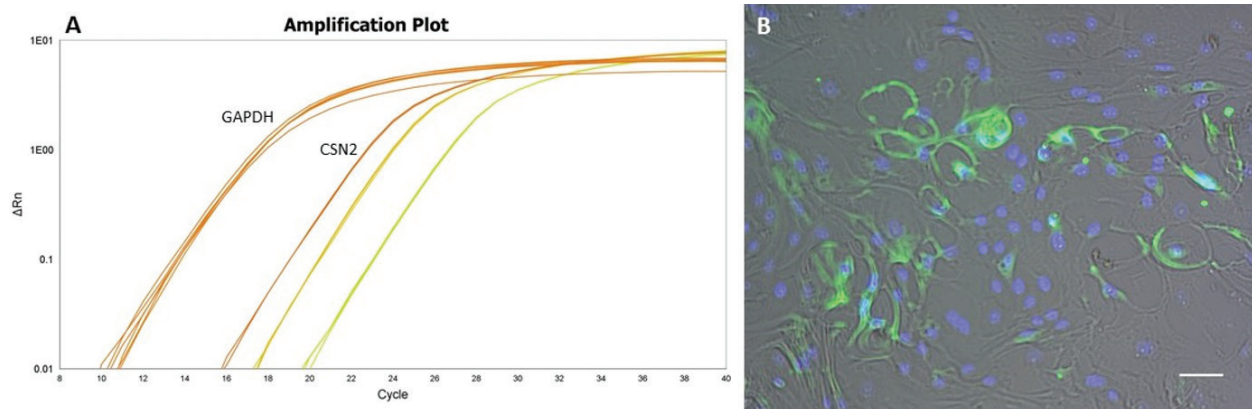


Figure 10. Expression of CSN2 mRNA and localization of the protein in pgMECs (photo: J. Ogorevc). (A) RT-qPCR amplification plot for housekeeping GAPDH and CSN2 in three different pgMEC lines. (B) Beta casein was present in pgMEC lines and localized mainly in vacuoles (lumen-like structures), formed only under lactation-inducing conditions (magnification 200×; scale bar = 50 μ m).

differentiation and proliferation of mammary epithelial cells. Research on humans shows that ER-/PR-positive mammary cells represent early mammary progenies and regulate differentiation of ER-negative mammary progenitors, from which basal/myoepithelial cells arose, and to ER-positive bipotent progenitors, which can give rise to luminal and myoepithelial lineage [33, 44]. It would make sense that progenitor fractions are enriched in juvenile mammary tissue (and in derived pgMECs) where lactogenic differentiation had not occurred yet. Prpar and colleagues [10] reported existence of different mammary progenitors in goats, luminal-restricted, myoepithelial-restricted, and bipotent and showed that the tissue from animals at the peak of lactation and from a juvenile animal contained the highest number of luminal progenitors, whereas the tissue at the onset of involution contained mostly myoepithelial progenitors. In our experience, tissue from young, nonlactating (juvenile) goats seems more suitable for preparation of lactation-competent cell cultures than tissue from lactating or involuting animals. Similar was also suggested in case of bovine primary mammary cells and attributed to a better proliferation capability of such cells [38].

6. Conclusions

The development of primary cell lines from lactating, juvenile, and involuting goat mammary tissue has been described. The derived pgMECs were maintained in cell culture for several passages without signs of further differentiation or senescence. The extensive characterization of established cell lines was performed and the main cell types in the mammary culture were determined. The pgMECs were capable of innate immune response and remained hormone responsive. Under lactogenic conditions, the cells successfully change morphology, synthesize milk proteins, and form lumen-like and milk drop-like structures. The established cell lines represent an adequate model of goat mammary tissue, useful for basic and applied research in mammary gland biology and biotechnology.

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