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Markers and antibodies for characterization of goat mammary tissue and the derived primary epithelial cell cultures

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ABSTRACT - A selection of commercially available antibodies, targeted against markers employed in studies of mammary gland biology, was tested to determine their reactivity in goat mammary tissue and the derived tissue cultures. Expression of the markers smooth muscle actin (*SMA*), selected keratins (*KRT*) *5*, *14*, *18*, and *19*, CD24 molecule (*CD24*), epithelial cell adhesion molecule (*EPCAM*), mucin 1 (*MUC1*), integrin subunit alpha 6 (*ITGA6*; *CD49F*), integrin subunit beta 1 (*ITGB1*; *CD29*), cyclin dependent kinase inhibitor 1A (*CDKN1A*; *p21*), membrane metalloendopeptidase (*MME*; *CD10*), progesterone receptor (*PGR*), estrogen receptor 1 (*ESR1*), and vimentin (*VIM*) was first assessed on mRNA level, using reverse transcription PCR (RT-PCR). The reactivity of the antibodies in the tissue sections and the derived tissue cultures was determined using immunofluorescence. The result of this study is a list of commercially available antibodies, raised mostly against human antigens, which also recognize orthologous goat antigens and are useful for characterization of different mammary cell types. Additionally, primers that are functional in detecting expression of mammary lineage markers in goat mammary mRNA isolates were validated. The suggested antibodies, PCR primers, and the described methods are of practical value for researchers interested in characterization and isolation of cell types comprising mammary tissue of goats and probably other ruminants.

Keywords: antibody, cell culture, goat, immunofluorescence, mammary gland, marker

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Introduction

Fully developed mammary epithelium has the appearance of a tree of ducts and alveoli, which are comprised of luminal and basal cell layers, the latter including the myoepithelial cells and stem cells (Inman et al., 2015). After parturition, the alveolar epithelium starts to be fully functional, with mammary epithelial cells secreting milk proteins into the lumen of the alveoli. The ability of the mammary gland to undergo many cycles of lactation and involution suggests that the epithelial compartment contains multipotent stem cells (Siegel and Muller, 2010; Prpar et al., 2012; Visvader and Stingl, 2014). The differentiation to epithelial subtypes is accompanied by the expression of distinctive markers. Different epithelial subtypes have been recognized based on marker expression and isolated from mouse (Shackleton et al., 2006; Stingl et al., 2006), human (Eirew et al., 2008), and ruminant (Martignani et al., 2010; Prpar et al., 2012) mammary glands.

In our previous study, we showed that combination of different cytokeratins (e.g., *KRT5*, *KRT14*, *KRT18*), smooth muscle alpha actin (*SMA*), mucin 1 (*MUC1*), epithelial cell adhesion molecule

(*EpCAM*), several surface molecules (e.g., *ITGA6*, *ITGB1*), and mesenchymal marker vimentin (*VIM*) enables distinction between goat mammary epithelial and non-epithelial cells and characterization of different epithelial lineages (luminal, myoepithelial, and basal) (Prpar Mihevc et al., 2014). Additionally, we showed that in specific conditions, primary goat mammary epithelial cells are capable of expressing estrogen receptor 1 (*ESR1*), progesterone receptor (*PGR*) (Ogorevc and Dovč, 2016), and beta-casein milk protein (Ogorevc and Dovč, 2015).

While rodent mammary gland has been the most widely studied and has provided many biological insights, it does not fully represent the development and structure (e.g., terminal ductal lobular units organized within the connective tissue in humans vs. lobular-alveolar units in fat pads in mouse) of human or ruminant mammary gland (McNally and Stein, 2017). Morphological development of ruminant udder and human breast is much more alike; thus, ruminant mammary tissue and the derived cell cultures can serve as valuable models of human mammary development and pathology (Prpar Mihevc and Dovč, 2013). Additionally, genetically modified ruminants (especially goats) are useful for the production of recombinant proteins, which can be engineered for mammary expression and then simply isolated from milk (Maga et al., 2006; Batista et al., 2014; Wang et al., 2014). Besides bovine and caprine epithelial cell lines, the studies have been conducted on cell cultures isolated from mammary glands of other species, such as buffaloes (Kapila et al., 2016; Shandilya et al., 2016), yaks (Fu et al., 2014), pigs (Dahanayaka et al., 2015), dogs (Osaki et al., 2016), and cats (Borges et al., 2016).

Research of ruminant mammary gland biology requires the use of specific antibodies to characterize different cell types/lineages present in the mammary gland and to determine the plasticity potential of the cell populations. The majority of commercially available antibodies are raised against human or mouse antigens, while their reactivity in other species is mostly unknown. Our previous studies and literature review represented a rationale for the selection of markers, potentially useful for characterization of major cell types in goat mammary tissue and the derived primary cell cultures.

Our objective was the validation of markers, which would be suitable for the characterization of goat mammary cell cultures and isolation of specific mammary epithelial cell types in goats and probably in other ruminants.

Material and Methods

The mammary tissue was obtained in an abattoir as a slaughter by-product. The udders were removed from carcasses immediately after slaughter under the supervision of a representative of the Veterinary Services of the Slovenian National Health Service branch of the Ministry of Health. Caprine mammary tissue collection and experiments were performed according to the procedures and guidelines approved by the National Health Service branch of the Slovenian Ministry of Health.

A piece of tissue was dissected out of the area surrounding the teats and then minced with scissors and scalpels and further processed to obtain single cell suspensions. A small piece of the tissue was fixed in 10% neutral buffered formalin, followed by paraffin embedding. Another small piece of tissue was dissected and snap frozen in liquid nitrogen for RNA isolation.

Primary cell lines were established as described previously (Ogorevc et al., 2009; Prpar Mihevc et al., 2014). Briefly, a single cell suspension was obtained from mammary tissue samples of Saanen goats by initial enzymatic digestion with collagenase and hyaluronidase, followed by treatment with trypsin, dispase, and DNase I.

The cells were cultured for 7-9 days in EpiCult-B medium (StemCell Technologies), supplemented with 5% FBS, 10^{-6} M hydrocortisone, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Sigma-Aldrich) at 37 °C on a thin layer of Geltrex basement membrane matrix (Gibco, Life Technologies) that resembles the tissue basement membrane, in a humidified incubator at 37 °C and 5% CO_2.

TRI-reagent (Ambion) was used for RNA isolation from a piece of the mammary tissue dissected from the area surrounding the teats and the confluent primary cell cultures. Total RNA was isolated from

the aqueous phase, using a miRNeasy mini kit (Qiagen). RNA was reversely transcribed to cDNA with High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) according to the manufacturer's instructions. Then, the PCR reactions (20 µL), using the designed primer pairs, were performed in PCR GeneAmp 9700 Thermocycler (Applied Biosystems). The PCR program was as follows: initial denaturation at 95 °C for 1 min, 30 cycles [denaturation at 95 °C, 15 s; annealing at 56 °C, 35 s; extension at 72 °C, 30 s], final extension at 72 °C, 7 min. The melting temperatures of all primers were 60 °C; hence, the primer annealing temperature (Ta) was set to 56 °C. Due to poor annotation of the goat genome, primers for mRNA detection were designed against *Bos taurus* RefSeq (NCBI) mRNA and matched against the goat reference sequences (if available) for final adjustments (Table 1).

Five to seven micron sections of paraffin embedded mammary tissue from lactating goats were deparaffinized, rehydrated, and processed for immunofluorescence as described previously (Martignani et al., 2010). Primary cell lines were fixed rapidly (30-60 s) with acetone:methanol (1:1), permeabilized with 0.3% Triton X-100 for 10 min, blocked in 5% goat serum for 30 min, and incubated with primary antibodies overnight at 4 °C. Twenty-nine primary antibodies raised against antigens of the selected markers were purchased and tested (Table 2). After incubation with primary antibodies, cells/tissue slides were washed with PBS and incubated for one hour with secondary antibodies AlexaFluor 488-labeled goat anti-rabbit IgG and AlexaFluor 594-labeled goat anti-mouse IgG (both from Invitrogen) diluted to 1:500. Negative controls were performed for each antigen by replacing the primary antibody with a suitable isotype antibody (normal mouse IgG (sc-2025) or normal rabbit IgG (sc-2027) (both from Santa Cruz Biotechnology).

Table 1 - RT-PCR primers for the selected mammary-specific markers

Gene	Gene ID (GenBank)	Forward primer (5'->3')	Reverse primer (5'->3')	Product length (bp)
ACTB	28628620	CCAACCGTGAGAAGATGACC	CGCTCCGTGAGAATCTTCAT	247
KRT5	56710316	CCAAGCTGGCCCTGGACGTG	GCTGCTACTGCCGCCTCCAC	254
KRT14	262118300	GAGCTGGTGCAGAGCGGCAA	CAAGTGCTTGGGGGAGGCGG	552
KRT18	194667060	TGGCCATGCGCCAGTCTGTG	GACAGTGCGCCTCAGCTCCG	395
KRT19	284055298	TCCTTCGGGGGTATGGGCGG	CGCCAGTGTGCCTTCCAGGG	933
ACTA2 (SMA)	78045237	CCGCTGCCCTGAGACCCTGT	GATGGATGGCCCGGCTTCGT	343
CD24	73586562	GAATGGGCTGTGGAACAGAT	AAGCCCCAGAGGAAAAGTTC	250
EPCAM	78369401	ACGCACTCGGTCAGTGCCAG	GTGCCGTTGCACTGCTTGGC	217
MUC1	41386777	GGCCGAGTGGGTGAAGGCAC	GGCTGTGAGCAGCCCACCTG	550
ITGA6 (CD49F)	158341671	CTGTGGGGCGCCTAGTGGGA	CTCGCCTCCGAGTGCTTGGC	326
ITGB1 (CD29)	31342195	GCAACGAGGGACGTGTCGGG	AACAGTCACAGGCGCTGCCG	306
CDKN1A (p21)	149643100	CCCGAGACGACCTGGGAGGG	GGGGAGCGAGTCACGAGGGT	118
MME (CD10)	194663643	AGAGCAGAGGCGGGGAACCT	TTTGCGCAGCGCCTCCAAGT	790
VIM	110347569	GCCAGTCCGTGCTACCGCAG	TGCTGCTCCAGGAAGCGCAC	430

Results

The presence of mammary specific markers was first determined in mRNA extract of goat mammary tissue and primary cell cultures followed by testing reactivity of the selected antibodies.

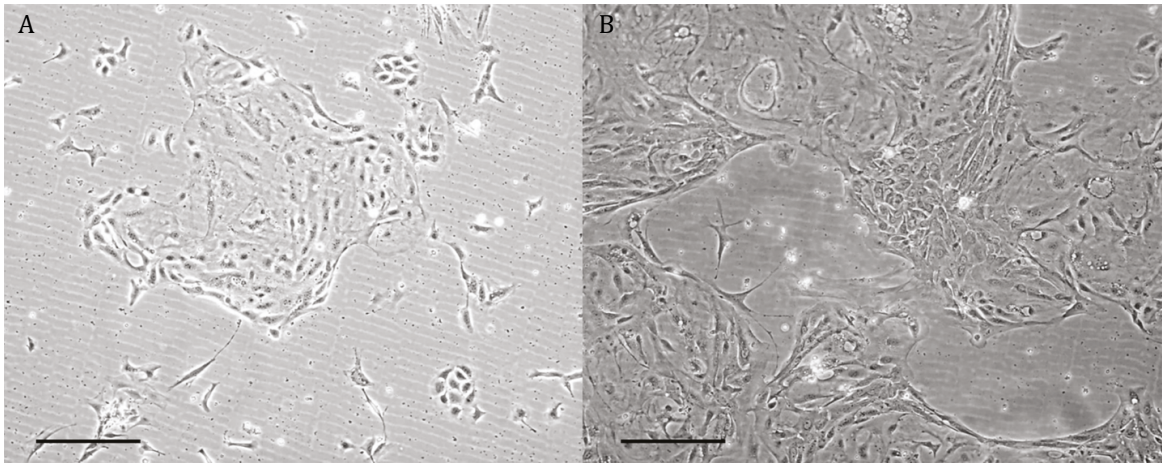
Tissue culture conditions favored the growth of mammary epithelial cells. Typical growth patterns were observed in the primary culture. Islands of densely packed epithelial cells were surrounded by larger stromal cells (Figure 1 A). After seven days in culture, cells expanded and the culture still consisted of two predominant cell types (Figure 1 B). For basic characterization, antibodies against cytokeratins (*KRT*) and vimentin (*VIM*) were used to distinguish mammary epithelial fraction from

mesenchymal cells. The distinction between different epithelial subtypes (myoepithelial and luminal) solely on cell morphology is difficult. However, myoepithelial cells typically stained for *KRT14* protein, whereas luminal epithelial cells for *KRT18* (Figures 2 AB and CD). Interestingly, when cells were grown at low confluence, the organization of the cells *in vitro* resembled organization in mammary acini, where myoepithelial cells surround luminal cells (Figures 2 AB and CD). Cells of mesenchymal origin (fibroblast-like cells) expressed vimentin (Figure 2 EF).

The mRNA expression of the markers was determined by RT-PCR and visualized in agarose gel after electrophoresis. The RNA was isolated from fresh goat mammary gland tissue and the derived primary cell cultures, the latter at two time points after three and seven days in culture as cells may alter

Table 2 - List of antibodies suitable for characterization of goat mammary cells by immunocytofluorescence (ICF) and immunohistofluorescence (IHF)

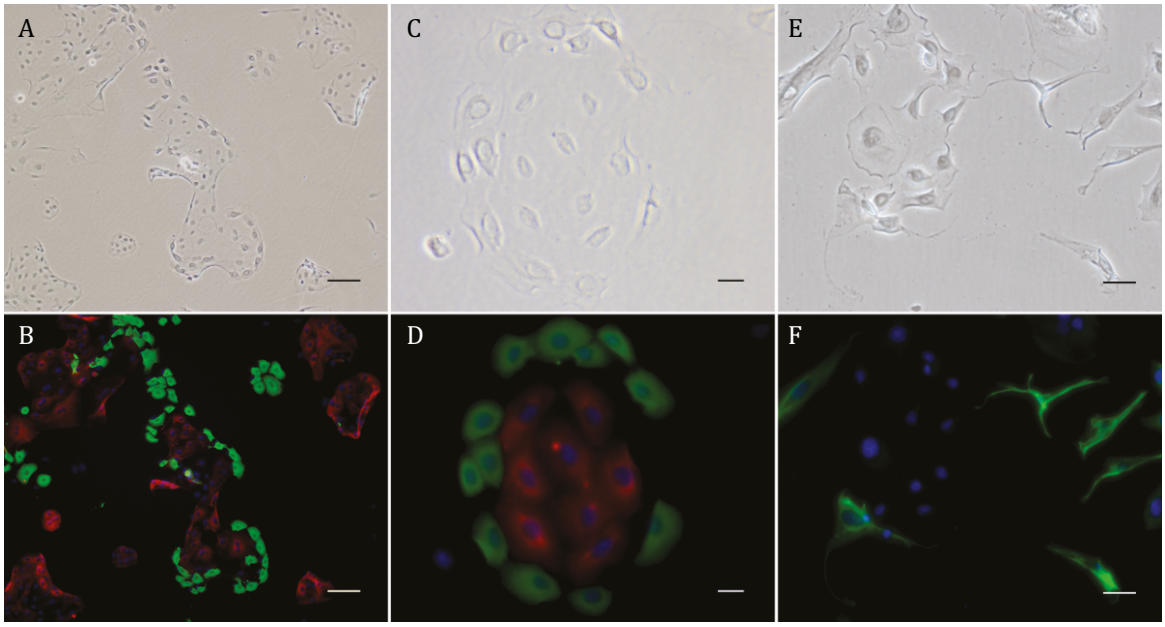
Marker, antigen, and antibody source	Application	Suggested dilution	Mammary lineage
ACTA2; α-Actin (0.N.5): sc-58669, Santa Cruz Biotechnology	ICF, IHF	1:200	myoepithelial
ACTA2; α-SMA, A5228, Sigma	ICF, IHF	1:500	
CSN2; #250558, Abbiotec	ICF	1:200	luminal
EPCAM; E144, ab32392, Abcam	ICF, IHF	1:200	epithelial
ESR1 (ERα); H-184: sc-7207, Santa Cruz Biotechnology	ICF	1:200	luminal
ITGB1 (CD29); cat. no.: 610467, BD Bioscience	ICF	1:200	basal (progenitor)
ITGA6 (CD49f); H-87 : sc-10730, Santa Cruz Biotechnology	IHF, ICF	1:200	basal (progenitor)
KRT18; C-04: sc-51582, Santa Cruz Biotechnology	ICF, IHF	1:200	
KRT18; C1399, KS-B17.2, Sigma	ICF, IHF	1:200	luminal
KRT18; KS18.04, 61028, Progen	ICF, IHF	1:200	
KRT5; H-40 : sc-66856, Santa Cruz Biotechnology	ICF	1:200	basal (myoepithelial)
KRT14; Cytokeratin 14, PRB-155P, Covance	ICF, IHF	1:500	myoepithelial
MUC1; ab37435, Abcam	IHF, ICF	1:200	luminal
PGR; Progesterone Receptor Ab-2 (Clone hPRa 2), Thermo Fischer Scientific	IHF, ICF	1:70	luminal
TP63; MA1-21871, 4A4, Thermo Fischer Scientific	IHF	1:200	basal (myoepithelial)
TP63 (clone 4A4), Thermo Fisher Scientific	IHF	1:200	
VIM; 2Q1035 : sc-73262, Santa Cruz Biotechnology	ICF, IHF	1:200 - 1:100	mesenchymal



A: three days in culture; B: seven days in culture.
Scale bars are 100 µm.

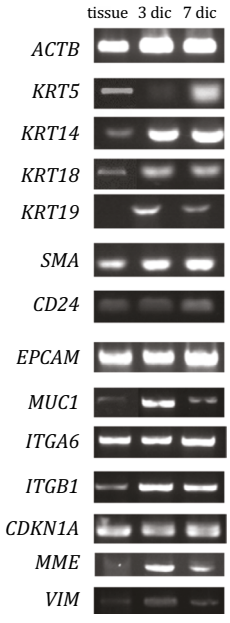
Figure 1 - Primary mammary cell culture growing on Geltrex.

metabolism and differentiate during *in vitro* growth, which can lead to variations in the expression of markers. The RT-PCR products for *KRT14*, *KRT18*, smooth muscle actin (*SMA*), CD24 molecule (*CD24*), epithelial cell adhesion molecule (*EPCAM*), mucin 1 (*MUC1*), integrin subunit alpha 6 (*CD49F*; *ITGA6*),



A and B: Colony of epithelial cells, consisting of KRT18-positive luminal (red) and KRT14-positive myoepithelial cells (green). Scale bars are 100 µm.
C and D: When grown at low density, the cells in some cases organized in alveoli-resembling manner, mimicking *in vivo* organization (myoepithelial cells (green), encircling luminal cells (red)). Scale bars are 50 µm.
E and F: Fibroblast-like cells stained positively against vimentin (green). Scale bars are 100 µm.
Nuclei in images B, D, and F (blue) were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI).

Figure 2 - Immunostained primary goat mammary epithelial cells under bright field (A, C, E) and fluorescent (B, D, F) illumination.



Representative photographs for housekeeping control β-actin (*ACTB*) and cytokeratins (*KRT*) *5*, *14*, *18*, and *19*, smooth muscle actin (*SMA*), CD24 molecule (*CD24*), epithelial cell adhesion molecule (*EPCAM*), mucin 1 (*MUC1*), integrin subunit alpha 6 (*ITGA6*; *CD49F*), integrin subunit beta 1 (*ITGB1*; *CD29*), cyclin dependent kinase inhibitor 1A (*CDKN1A*; *p21*), membrane metalloendopeptidase (*MME*; *CD10*), and vimentin (*VIM*).

Figure 3 - Reverse transcription-polymerase chain reaction (RT-PCR) products after agarose gel electrophoresis, expressed in tissue of lactating goat and the tissue-derived cell culture, grown for three (3 dic) and seven (7 dic) days in culture.

integrin subunit beta 1 (*CD29*; *ITGB1*), cyclin dependent kinase inhibitor 1A (*CDKN1A*; *p21*), membrane metalloendopeptidase (*CD10*; *MME*), and *VIM* were observed in all three RNA isolates (Figure 3). A very low amount of *KRT5* was detected after three days in culture. *KRT19* mRNA was not detected in the mammary tissue but was expressed in the primary culture at both time points, after three and seven days in culture. Beta-actin (*ACTB*) was used as a housekeeping control gene.

Twenty-nine antibodies, directed against twenty antigens, were purchased from different companies, of which fifteen adequately recognized twelve different goat antigens in immunostaining reactions of mammary tissue sections and/or fixed primary cells.

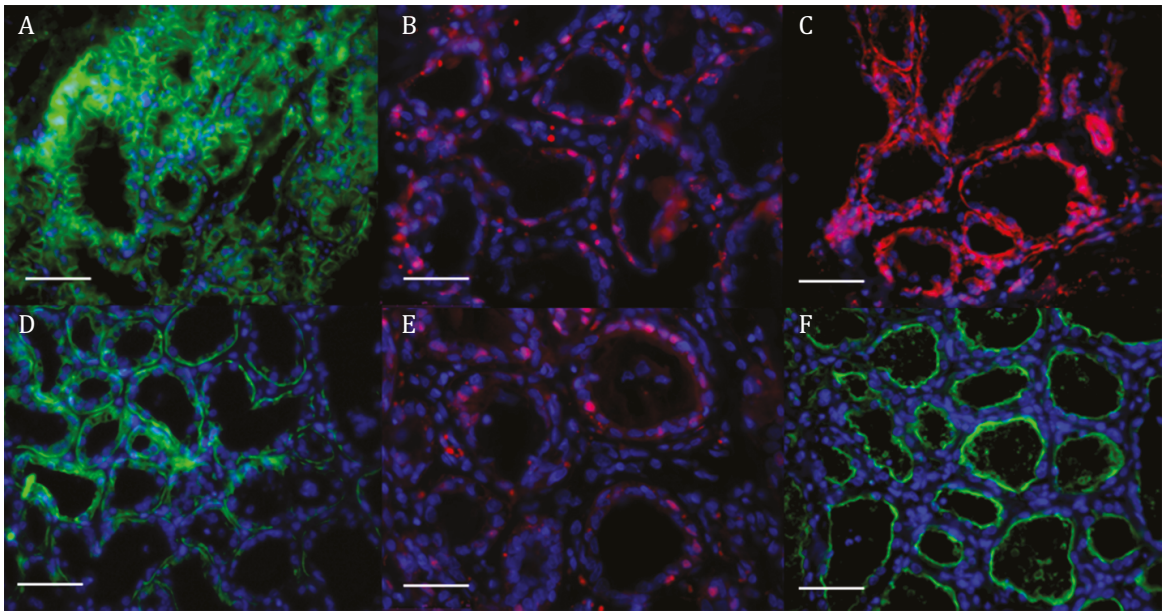
Antibodies directed against *KRT14*, *KRT18*, *ITGA6*, *SMA*, and *PGR* detected antigens in the tissue and the primary cultures, whereas *VIM*, *KRT5*, and *ESR1* were only detected in the cell cultures, and *EPCAM*, tumor protein p63 (*TP63*), *ITGB1*, and *MUC1* only in the tissue sections.

In goat mammary tissue, *EPCAM* was expressed in cytoplasm of the epithelial cells (Figure 4 A); *TP63* and *PGR* in cell nuclei of the luminal cells (Figures 4 B and E); *ITGB1* and *ITGA6* in cellular cytoplasm near the basal lamina (Figures 4 C and D); and *MUC1* near the apical membrane of luminal cells, facing the lumen of the mammary gland acinus (Figure 4 F).

Primary goat mammary gland cell cultures stained positively for cytoplasmic markers *KRT5* (Figure 5 A), *KRT18* (Figure 5 A, B, D), and *SMA* (Figure 5 C), nuclear luminal marker *ESR1* (Figure 5 D), and basal membrane marker *ITGA6* (Figure 5 B).

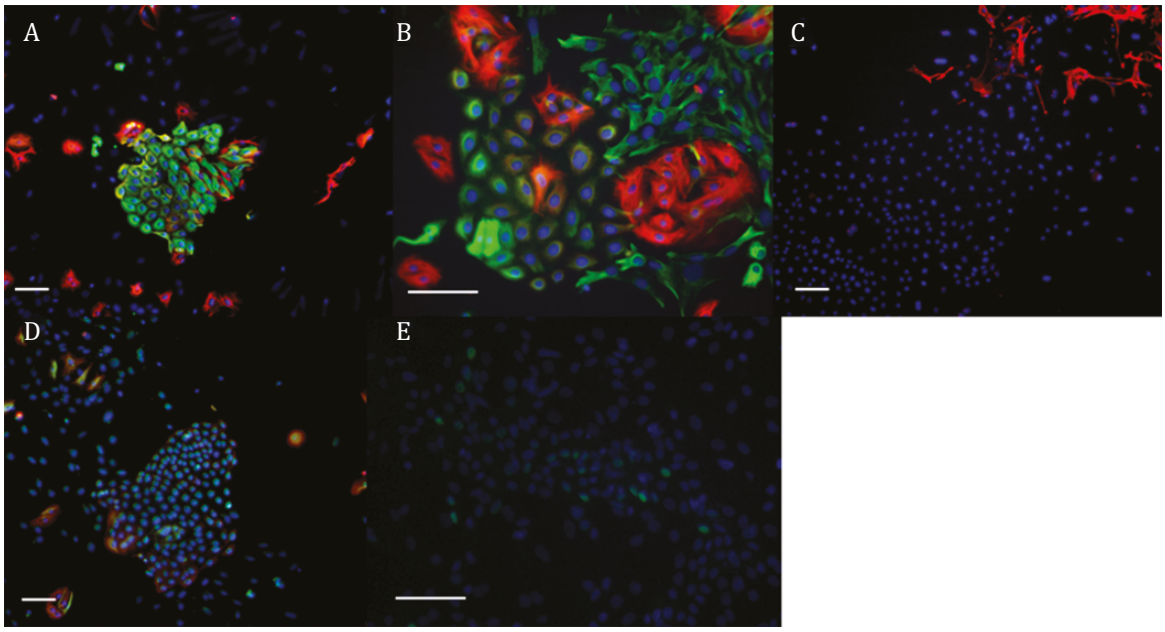
Discussion

Here we describe the methodology and provide a list of commercially available antibodies suitable for characterization of cellular types, comprising goat mammary tissue or the derived primary cell cultures. Of course, many other commercially available antibodies exist, which were not tested in this study, but may also be useful for characterization of goat (ruminant) mammary cells. We encourage producers to provide the information about reactivity of antibodies in species other than human or mouse.



Epithelial cell adhesion molecule (*EPCAM*) (A), tumor protein p63 (*TP63*) (B), integrin subunit beta 1 (*ITGB1*) (C), integrin subunit alpha 6 (*ITGA6*) (D), progesterone receptor (*PGR*) (E) and mucin 1 (*MUC1*) (F).
Nuclei were counterstained with DAPI (blue).
Scale bars in all images are 50 µm.

Figure 4 - Expression of immunofluorescently detected markers in lactating caprine mammary tissue.



Cytokeratins 5 (green, A) and 18 (red, A, B, D), integrin subunit alpha 6 (*ITGA6*) (green, B), smooth muscle actin (*SMA*) (red, C), estrogen receptor 1 (*ESR1*) (green, D), and progesterone receptor (*PGR*) (green, E).
Nuclei were counterstained with DAPI (blue).
Cells were grown for five days and were in the first passage.
Scale bars are 100 µm.

Figure 5 - Expression of immunofluorescently detected markers in primary goat mammary epithelial cells derived from lactating goat mammary gland.

The RT-PCR analysis on mRNA isolated from mammary gland tissue and primary cell cultures showed that most of the selected markers were expressed in all the samples. Very low levels of *KRT5* were detected after three days in culture, but a more intense RT-PCR band for *KRT5* was visualized after seven days in culture. Interestingly, luminal marker *KRT19* was detected only in the cell culture isolates but not in the tissue. As mammary tissue is comprised of heterogeneous cell populations and the cell cultures were enriched for epithelial fraction, it is possible that some of the epithelial markers would not be detected in the tissue because of their low expression and/or due to the tissue sampling, where compartments composed mostly of stromal tissue could be picked by chance. Additionally, expression of estrogen and progesterone receptors and beta casein was detected in primary goat mammary gland cell culture, which is known to be donor tissue and growth condition-dependent (Ogorevc and Dovč, 2015; Ogorevc and Dovč, 2016).

Immunostainings were mostly consistent with the mRNA expression patterns, except in cases of *MME*, *CD24*, and *CDKN1A*, where mRNA transcripts were detected, but the immunostainings did not result in positive signals. The possible reasons are the differences between human and caprine epitopes; thus, *MME*, *CD24*, and *CDKN1A* were not recognized by the antibodies directed against human epitopes. Additionally, expression of some markers in the tissue can vary during mammary gland development and lactation cycles or in cell cultures as a consequence of growth conditions and adaptations to *in vitro* growth. Tissue sections are subjected to rigorous chemical and physical treatment during sample preparation procedures, which might result in changed conformation and possible masking of antigens. On the other hand, cells grown in *in vitro* conditions adapt to the environment, which may alter the expression of markers. Thus, discrepancies in the reactivity of antibodies between the tissue sections and the cell cultures are expected to some degree and depend on many variables (e.g., physiological state of the mammary gland, tissue-culture conditions, sample preparation).

Therefore, it is difficult to suggest a universal set of markers that would be optimal for the characterization of the cell cultures and mammary tissues in all conditions. In general, the mammary gland is composed mostly of luminal (KRT18+) and myoepithelial (KRT14+) cell types, their respective progenitors, and

stromal cells. For basic characterization of goat mammary tissue and primary cells, we recommend a combination of markers *SMA*, *KRT14*, *KRT18*, *ITGA6*, and *VIM*, as it allows identification of both of the major cell types, progenitor and epithelial cells and the cells of mesenchymal origin (considered as contaminants of epithelial cultures). *SMA* marks both – myoepithelial cells and mesenchymal fibroblast-like cells (possibly myofibroblasts). Cells of mesenchymal origin are usually present from the beginning of the cell culture, but can also emerge in the process of epithelial-to-mesenchymal transition, which is commonly observed in *in vitro* conditions and sometimes associated with acquiring stem/cancer cell characteristics (Sikandar et al., 2017). Vimentin is a typical marker of mesenchymal tissue and stains fibroblasts in the stromal compartment of the mammary gland and fibroblast cells, contaminating epithelial cell cultures. Luminal epithelial lineage distinctively expresses *KRT18*. *ESR1* and *PGR* are expressed by cells of epithelial origin, not exclusively by luminal *KRT18*-positive cells (Ogorevc and Dovč, 2016).

Conclusions

The proposed antibodies enable identification of major cell types comprising goat mammary gland (i.e., luminal, myoepithelial, basal, and mesenchymal) or the derived cell cultures and could be employed for characterization and isolation of particular cell types using cell sorting or other antibody-based assays. The described methodology and the results are of practical value for researchers working with ruminant mammary tissue and cell cultures.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: S. Prpar Mihevc, J. Ogorevc and P. Dovč. Data curation: S. Prpar Mihevc and J. Ogorevc. Funding acquisition: J. Ogorevc and P. Dovč. Investigation: S. Prpar Mihevc and J. Ogorevc. Methodology: S. Prpar Mihevc and J. Ogorevc. Resources: P. Dovč. Supervision: P. Dovč. Visualization: S. Prpar Mihevc and J. Ogorevc. Writing-original draft: S. Prpar Mihevc, J. Ogorevc and P. Dovč. Writing-review & editing: S. Prpar Mihevc, J. Ogorevc and P. Dovč.

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