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

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% CO₂.

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
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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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID (GenBank)</th>
<th>Forward primer (5’-&gt;3’)</th>
<th>Reverse primer (5’-&gt;3’)</th>
<th>Product length (bp)</th>
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</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>28628620</td>
<td>CCAACCGTGAGAGATGACC</td>
<td>CGCTCCGGTGAAGATCTTCAT</td>
<td>247</td>
</tr>
<tr>
<td>KRT5</td>
<td>56710316</td>
<td>CCAAGCTGGCCCCTGACGTCG</td>
<td>GCTCGTACTGGCCTTCAC</td>
<td>254</td>
</tr>
<tr>
<td>KRT14</td>
<td>262118300</td>
<td>GACCTGTCAGGAGCCGCAA</td>
<td>CAAGTGTCAGGAGCCGCAA</td>
<td>552</td>
</tr>
<tr>
<td>KRT18</td>
<td>194667060</td>
<td>TTGCCATGGGACCTGCCTGTG</td>
<td>AGACTGTGCTACAGTCCG</td>
<td>395</td>
</tr>
<tr>
<td>KRT19</td>
<td>284055298</td>
<td>TCTCCTGGGATTCGGGACG</td>
<td>CCGAGGCTGCCTCAGG</td>
<td>933</td>
</tr>
<tr>
<td>ACTA2 (SMA)</td>
<td>78045237</td>
<td>CGCCTGGCTGACAGGGTGT</td>
<td>GATGGATTGCGGCTTGCTG</td>
<td>434</td>
</tr>
<tr>
<td>CD24</td>
<td>73586562</td>
<td>GAATGGGTTGGAGAAAG</td>
<td>AAGGCCAGAGGAAAGTTT</td>
<td>250</td>
</tr>
<tr>
<td>EPCAM</td>
<td>78369401</td>
<td>ACGCAGTGGCTGCTGCGA</td>
<td>GTCCGCGCTGACTGGTGGC</td>
<td>217</td>
</tr>
<tr>
<td>MUC1</td>
<td>41386777</td>
<td>GGGCAGAGGACGTGCTGGAG</td>
<td>GGGCTGGCTGACGAGCAGC</td>
<td>550</td>
</tr>
<tr>
<td>ITGA6 (CD49F)</td>
<td>158341671</td>
<td>CTCTGGGGGCTCTGGCTGGGA</td>
<td>CTCGGCTGACTGGTGGC</td>
<td>326</td>
</tr>
<tr>
<td>ITGB1 (CD29)</td>
<td>31342195</td>
<td>GCAACAGAGGACGTGCTGGAG</td>
<td>AAGCTGACGGCGGCCTGGC</td>
<td>306</td>
</tr>
<tr>
<td>CDKN1A (p21)</td>
<td>149643100</td>
<td>CCGCAGACGAGCTGGGAGAGG</td>
<td>GGGAGGACGAGCTGGGAGG</td>
<td>118</td>
</tr>
<tr>
<td>MME (CD10)</td>
<td>194663643</td>
<td>AGAGCAGACGAGCCCCAGCT</td>
<td>TTGGCGAGCGGCCGACCT</td>
<td>790</td>
</tr>
<tr>
<td>VIM</td>
<td>110347569</td>
<td>GCCACTCGGTTGCTACCGAG</td>
<td>TGCTGCTGCCAGAGGCGAC</td>
<td>430</td>
</tr>
</tbody>
</table>

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)

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

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*), *CDKN1A* (p21), membrane metalloendopeptidase (*MME; CD10*), and vimentin (*VIM*). 

![Figure 2](image2.png)

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

![Figure 3](image3.png)

**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.
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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 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).

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

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

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


Acknowledgments

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