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Immunolocalization of estrogen alpha and progesterone beta receptors in goat mammary gland

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ABSTRACT

Steroid receptors particularly estrogen receptor alpha and progesterone receptor beta are essential for mammary gland development. Objective of this study was to explore transcript and protein expression profile of steroid receptors in goat mammary glands. A varied expression of ER-alpha and PR-B was observed during lactation, non-lactating/dry, mastitic and mammary pre-cancer/cancer in goats. During lactation, immunopositivity of ER-alpha was observed only in the nuclei of mammary epithelial cells (MEC) and stromal cells. However, in non-lactating stage, ER-alpha immunopositivity was observed both in nucleus and cytoplasm of MEC. In mammary pre-cancer (based on aberrant expression of CD10, FNDC3B and MUC1) immunoreactivity of ER-alpha (38±12.5%) varied from non-lactating (14.8±3.1%) and lactating (7.9±2.6%) glands. During naturally infected mastitis, a reduction in the expression of ER-alpha and PR-B was observed.We observed similar expression patterns of ER-alpha and PR-B as that of their protein expression. Transcripts of these receptors were highest in mammary precancer. In comparison to lactating glands, expressions of ER-alpha and PR-B was upregulated in mammary precancers by 17-folds and 9.2-folds, respectively. These results showed a reduction in expression of steroid receptors in mastitic glands and upregulation in mammary precancer indicating role of these receptors in cell proliferation.

Key words: ER-alpha, Goat mammary gland, Mammary precancer, PR-B

Ovarian steroid hormones, estrogen and progesterone are the key regulators of mammary gland growth and proliferation. Estrogen elongates mammary ducts (Brisken *et al.* 1998, Mallepell *et al.* 2006) whereas, progesterone causes branching of ducts (Mueller *et al.* 2002). Estrogen and progesterone bind to their specific receptor to mediate their actions. Estrogen receptors are the transcription factors that regulate mammary epithelial cell, stromal cell proliferation and alveolar morphogenesis in mice (Feng *et al.* 2007), bovine (Capuco *et al.* 2002) and ewe mammary glands (Colitti and Pulina 2010). Although, the expression patterns of estrogen receptor-alpha (ER-alpha) and progesterone receptor beta (PR-B), in mammary glands of cow, mice, human and ewe are known in the literature (Feng *et al.* 2007, Martin *et al.* 2008, Colitti and Pulina 2010, Hefti *et al.* 2013). Nonetheless, we cannot extrapolate these results on goat mammary gland due to species differentiation and difference in mechanistic models of lactation. Mammary gland growth is little during lactation in bovine (Capuco *et al.* 2002) but is dynamic in ewe (Colitti and Farinacci 2009). Likewise, there is paucity of information on regulation of goat mammary gland development at histological and molecular levels (Rowson *et al.* 2012) especially the lack of information about histochemical localization on of ER-alpha and PR-B. The aim of this study was to evaluate expression pattern of ER-alpha and PR-B in goat mammary glands during various physiological stages of the animals including naturally diseased state like mastitis and mammary cancer.

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MATERIALS AND METHODS

Animal tissue collection: Mammary tissue was collected from mammary parenchyma region of the glands of goats (N=19) that were brought to the slaughterhouse. A 5×5×5 $mm^3$of mammary parenchyma from each animal was collected and fixed in 10% (w/v) neutral formalin for overnight at room temperature. Subsequently, fixed tissues were processed for embedding in paraffin as per routine and standard procedures.

Physiological stages of animals were accessed by gross morphological examination of mammary glands by veterinary anatomist (Devendra Pathak) and histological examinations of hematoxylin and eosin (H&E) stained tissue sections. Broad classification of animal stages namely, two different methods of mammary gland examinations were used to access physiological stage and normal ormastitic or precancerous glands.

This study was conducted on slaughterhouse samples and permission to conduct the research was taken from Head

of the School of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.

Immunohistochemistry: Immunohistochemical staining was performed according to procedures previously described (Choudhary *et al.* 2014). List of primary antibodies and their dilution and incubation time is given in Table 1.

Table 1. List of primary antibodies and their dilutions used to characterize goat mammary tissues

Antibody	Dilution	Incubation time
ESR1 (Santa Cruz)	1:2000	Overnight
ERα (Santa Cruz)	1:2000	Overnight
PR (Santa Cruz)	1:2000	Overnight
CD10 (Biogenex)	Ready to use	Overnight
FNDC3B (Santa Cruz)	1:1000	Overnight
MUC1 (Biogenex)	Ready to use	Overnight
Ki-67 (Biogenex)	Ready to use	Overnight

Quantitation of ER-alpha and PR-B positive mammary epithelial cells: For each section, 6–10 photomicrographs were captured at 400-magnification (40×) using bright field microscopy (Eclipse 80i, Nikon, Japan). Images were processed and counted using cell counter plugin of ImageJ (version 1.49d) (Schneider *et al.* 2012).We counted 2500 to 3000 MEC/animal in randomly chosen microscopic fields of each animal tissue regardless of the intensity of reactivity. The mean percentage of immune-labeled (ER-alpha and PR-B-positive) MEC was expressed as labeling index as per our previous protocol (Kaur *et al.* 2016).

RNA isolation and quantitative real time qPCR: Total RNA of mammary tissue was isolated using a combination of Trizol (Qiagen, Germany) and GenElute Mammalian Total RNA Miniprep Kit (Sigma, USA) with on-column DNase digestion (Sigma) (Choudhary *et al.* 2017). DNase-treated RNA (~1000 ng) was reverse transcribed using the Affinity Script qPCR cDNA Synthesis Kit (Agilent). Transcript abundance was determined by SYBR green RT-PCR master mix (iQ SYBR Green Supermix; Bio-Rad Laboratories, CA, USA). A 20µl of the RT reaction was used in the 0.20 ml PCR tube strips in duplicates. PCR amplifications were performed in 96-well spectrofluorometric thermal cycler (CFX 96, BioRad, CA, USA). Melt curve analysis and non-denaturing gel electrophoresis was performed at the end of each reaction to confirm single PCR product. Primers of target genes, ER-alpha and progesterone receptors (Connor *et al.* 2005) were used to evaluate abundance of transcripts which was normalized using ribosomal protein 23 (RPS23) gene (Table 2). RPS23 is one of the appropriate reference gene in mammary tissue of water buffalo for RT-qPCR analysis (Kapila *et al.* 2013). The expression level of ER-alpha and PR-B genes in lactating and non-lactating goats (in comparison to precancer) were analyzed by $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). No template controls (NTC) had been used as negative control for the PCR amplification.

Table 2. List of qPCR primers, annealing temperatures and their product size

Gene name	Primer pairs (5'to 3')	T_m (°C)	Product size
ER-alpha	F: TTGCTGGCTACT TCGTCTC R: GGTGGATGTGGTC CTTCTC	56	148 bp
PR-B	F:CAGTGGTCAAGT GGTCTAAATC R: TCTCCATCCTAGT CCAAATACC	56	116 bp
RPS23	F: CCCAATGATGGT TGCTTGAA R: CGGACTCCAGGA ATGTCACC	60	101 bp

Immunohistochemical expression data were analyzed using a one-way ANOVA with Dunnett's multiple comparison tests (GraphPad Software, ver. 6.00, San Diego California, USA). The mean percentage of ER-alpha- and PR-B-positive cells in groups was estimated as mean percentage of MEC. Pearson's correlation coefficient was used to evaluate correlation between mean expression of ER-alpha and PR-B.

RESULTS AND DISCUSSION

Estimation of physiological stages: Animals were visualized and their glands were examined after slaughter. Mammary glands of nulliparous animals (may include prepubertal and pubertal animals) were under developed having less parenchymal tissue with prominent and glistening fat pad (Fig. 1A left panel). We were not knowing if these animals had precancer/cancer mammary glands. Lactating glands, were morphologically well developed, swollen and milk was oozing out from parenchymatous tissue (Fig. 1A, mid panel), while non-lactating glands were sunken and parenchymal tissue was dry while dissection (Fig. 1A, right panel). Histomorphological examinations of H&E stained sections of nulliparous glands revealed tree-like structures of terminal ductal units (TDU) that invaded fat pad (Fig. 1B). Distinguished features of mammary cancer included solidification of lumen, loss of mammary cytoarchitectures and multiple layers of epithelial cells near the bifurcation of TDU. Presence of three layers of mammary epithelium in TDU namely, basal, embedded and luminal layers of epithelium were evident. Presence of three layers of mammary epithelia cells are the characteristics of ruminant like bovine mammary glands (Berry *et al.* 2003, Meyer *et al.* 2006). The tissue having no to minimal secretions and regressed or poorly developed alveoli (Fig. 1C) characterized non-lactating mammary glands. Lactating mammary glands of goat had moderate to well-developed alveoli, alveolar lumen and reduced occupancy by the stromal tissue (Fig. 1D). Loss of alveolar epithelial cells, abundance of polymorphonuclear cells (PMN) and

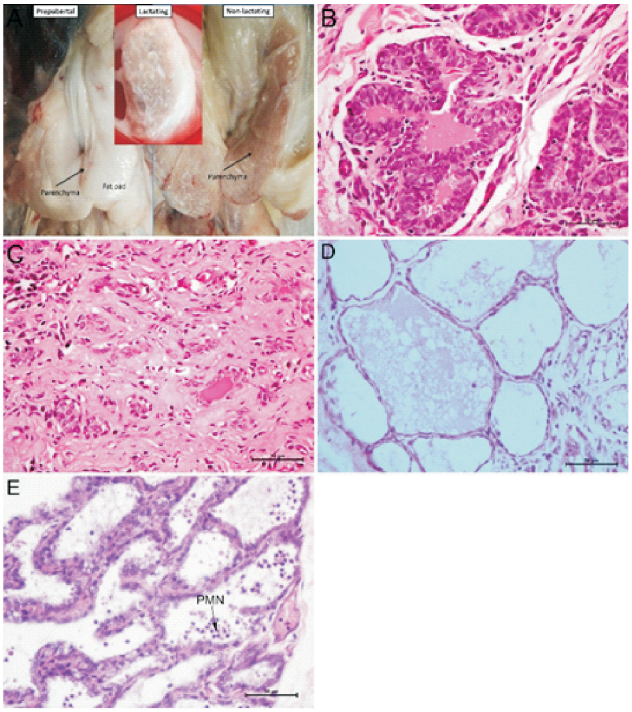

Fig. 1. Estimation of physiological stages of mammary glands using two different methods namely, gross examination of glands at the time of slaughter and histomorphological examinations on hematoxylin stained tissue sections. Nulliparous mammary glands of goats were identified with scanty amount of mammary parenchyma (arrow) and prominent glistening fat pad (A, left panel), whereas in lactating glands (A, mid panel), milk was oozing out from the cut surfaces of the glands. In non-lactating but parous glands (A, right panel) mammary parenchyma was abundant but appeared to be dry while dissection. Histomorphological evaluations of Hematoxylin and Eosin (H&E) stained mammary tissue sections of nulliparous (B), non-lactating (C), lactating (D) and mastitic (E) glands, were distinct under the bright field microscope. Scale bar = B, C, D, E: 50 µm.

cellular debris in alveoli were evidenced in mastitic glands (Fig. 1E).

Localization of ER-alpha: The immunohistochemical profile of ER-alpha, ER- beta and PR-B in goat mammary is summarized in Table 3.

Distribution patterns of ER-alpha in cancer tissue revealed a strong positive staining. Immunoreactivity of ER-alpha was in the nuclei (arrows) and cytoplasm of epithelial cells of terminal ductal units (TDU) (Fig. 2A) and in the ducts (Fig. 2B). Weak expression of ER-alpha was also seen in few stromal cells (arrow head). In non-lactating glands, epithelial cells showed strong nuclear (arrows) and weak cytoplasmic staining (Fig. 2C), while stromal cells showed no to very faint staining of ER-alpha. Ducts showed moderate nuclear staining (arrows) and weak cytoplasmic (arrow head) staining of luminal epithelial cells (Fig. 2D). In lactating glands, a weak ER-alpha immunoreactivity was observed in alveolar cells (arrows) (Fig. 2E). In mastitic glands, expression of ER-alpha was minimal (Fig. 2F). Localization of ER-beta was weak and cytoplasmic staining in cancer tissue whereas; moderate nuclear staining was in alveolar cells of lactating MEC (not shown).

Localization of PR-B: Localization of PR-B was observed in cytoplasm and nuclei of MEC and stromal cells of cancer, non-lactating, lactating and mastitic glands. In cancer tissue, a strong nuclear (arrow) and weak cytoplasmic (arrow head) staining of PR-B was seen in TDU (Fig. 3A) and ducts (Fig. 3B). Staining pattern of PR-B in non-lactating glands were exclusively nuclear (arrows) (Fig. 3C) in regressing acini and apparently no staining were observed in the ducts (Fig. 3D). In lactating glands, expression of PR-B was limited to nuclei of epithelial cells of alveolar epithelium (arrows; Fig. 3E). Apparently, no PR-B positive cells were found in the stroma. In mastitic glands, expression of PR was minimal like that of ER-alpha (Fig. 3F).

Scoring of ER-alpha and PR-B immunoreactivity and

Table 3. Immunohistochemical staining patterns of estrogen receptor alpha, estrogen receptor beta and progesterone receptor B in epithelial and stromal cells of goat mammary glands

Group	Estrogen receptor alpha	Estrogen receptor beta	Progesterone receptor B
Cancer			
Epithelial cells			
Cytoplasm	+ + + +	+ + +	+ + +
Nuclei	+ + + +	++	+ + +
Stromal cells			
Cytoplasm	+	++	+
Nuclei	-	++	-
Non-lactating			
Epithelial cells			
Cytoplasm	+ +	+	-
Nuclei	+ +	+	+ +
Stromal cells			
Cytoplasm	-	+	-
Nuclei	+	+	+
Lactating			
Epithelial cells			
Cytoplasm	-	+	+/-
Nuclei	+	++	+ +
Stromal cells			
Cytoplasm	-	-	-
Nuclei	+/-	-	+/-
Mastitis			
Epithelial cells		NA	
Cytoplasm	-		-
Nuclei	+/-		+/-
Stromal cells		NA	
Cytoplasm	-		-
Nuclei	-	NA	-

Nulliparous (may include prepubertal and pubertal), lactating and non-lactating goat mammary glands based upon morphological and histological observations of mammary glands collected from slaughterhouse. "+" and "-" indicates staining intensity from no staining (-) to weak (+), moderate (+ +), strong (+ + +) to very strong staining (+ + + +).

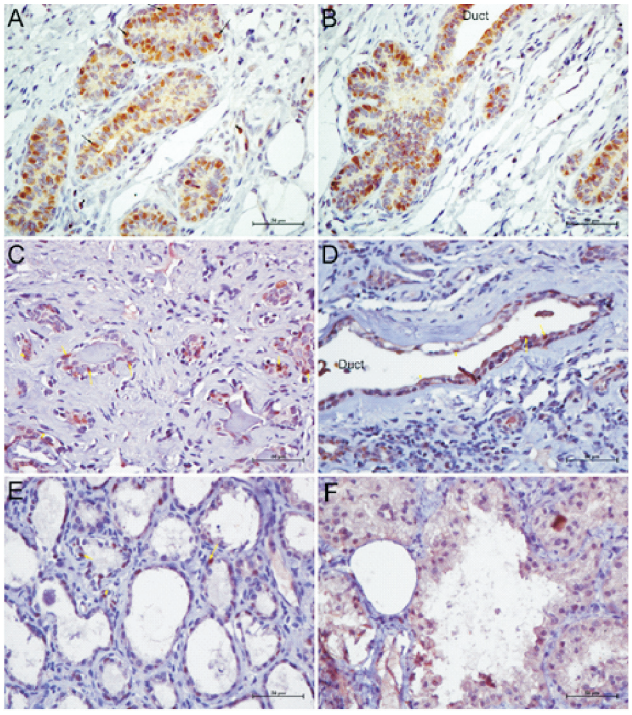

Fig. 2. Immuno-localization of ER-alpha in goat mammary glands. ER-alpha positive cells (epithelial and stromal) of terminal ductal unit (A) and duct (B) of nulliparous, non-lactating (C-D), lactating (E) and mastitic (F) mammary glands. Scale bar = 50 µm.

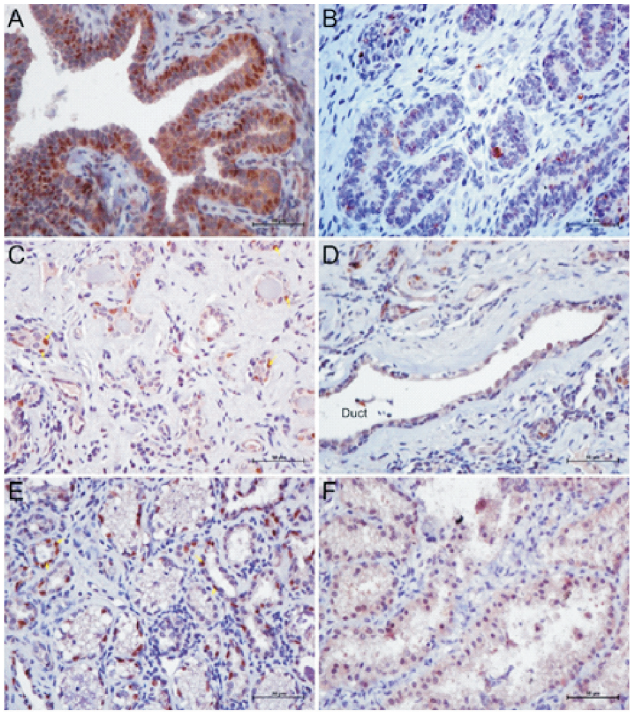

Fig. 3. Immuno-localization of PR-B in goat mammary glands. PR-B positive cells (epithelial and stromal) of terminal ductal units ranges from very high (A) to low expression (B) in nulliparous glands. In non-lactating, PR-B expression was limited to regressing alveoli and stroma (C) but not in the duct (D). Lactating glands showed nuclear staining (E) while mastitic glands (F) showed reduced expression in the epithelial cells. Scale bar = 50 µm.

qPCR quantification: Labeling index of ER-alpha was expressed in mean percentage of ER-alpha-positive MCE. In total, we counted ~30000 MEC from animals (n=19) grouped into three categories namely, cancer, non-lactating, lactating and mastitis. One-way ANOVA with non-repeated measures indicated significant (P=0.01) difference among mean expression of ER-alpha-positive MEC. Dunnett's multiple comparison tests indicated difference in expression of ER-alpha in non-lactating, lactating and mastitic glands in comparison to cancer glands (Fig. 4A). In goat mammary cancer, >38% of MEC were ER-alpha-positive in comparison to non-lactating, lactating and mastitic glands. High percentage of ER-alpha positive MEC and extent of staining suggest goat mammary cancer were ER-alpha positive. As per the recommended guidelines of American pathologist for immunohistochemical testing of estrogen and progesterone receptors in breast cancer, more than 1% of tumor cells should be immunoreactive (Hammond *et al.* 2010). Likewise, we counted ~ 26600 MEC for quantification of PR-B localization. Results indicated highest expression of PR-B in cancer tissue (14.38±10.84) with large variation in extent of staining. Extent of ER-alpha staining was highly variable in this group, which could be due of different stages of mammary cancer of these animals. Pattern of PR-B expression was consistent with the pattern of ER-alpha expression being highest in cancer followed by non-lactating, lactating and mastitic glands. Although, one-way ANOVA test produced non-significant (P=0.34) difference in the mean expression percentage of PR-B in the MEC of non-lactating, lactating and mastitic glands in comparison to cancer, likely due to high variation in PR-B expression (Fig. 4B). No correlation was observed between expressions of ER-alpha and PR-B (Person's correlation coefficient = -0.075, P=0.78) in goat mammary glands.

Duncan least square significance test was applied to compare means of each group normalized to *RPS23* expression. Ratio of fold change expression was calculated. Expression of *RPS23* was quantified in all the samples and resulted a constant expression level. Expression of *ER-alpha* and *PR-B* were normalized relative to the expression of *RPS23* of each sample. Fold changes of *ER-alpha* in cancer vs. non-lactating glands were significantly different in comparison to lactating glands (cancer vs. lactating, 8.6-fold up; P = 0.01; non-lactating vs. lactating, 9.84-fold up; P = 0.016). Likewise, *PR-B* was upregulated in cancer in comparison to lactating (9.2-fold up; P = 0.01) and non-lactating glands (4.2-fold up; P=0.032) (Fig. 4C).

Evaluation of CD10, MUC1 and FNDC3B markers in mammary precancer/cancer tissue: We validated our observations of goat mammary precancer/cancer tissue cyto-architecture with histopathological observation using various protein markers that identfies basal epithelial cells (CD10), luminal epithelial cells (MUC1) and putative bovine mammary epithelial cells (FNDC3B). These markers, however, have been suggested to be abundantly and aberrantly expressed in various carcinomas (Rahn *et*

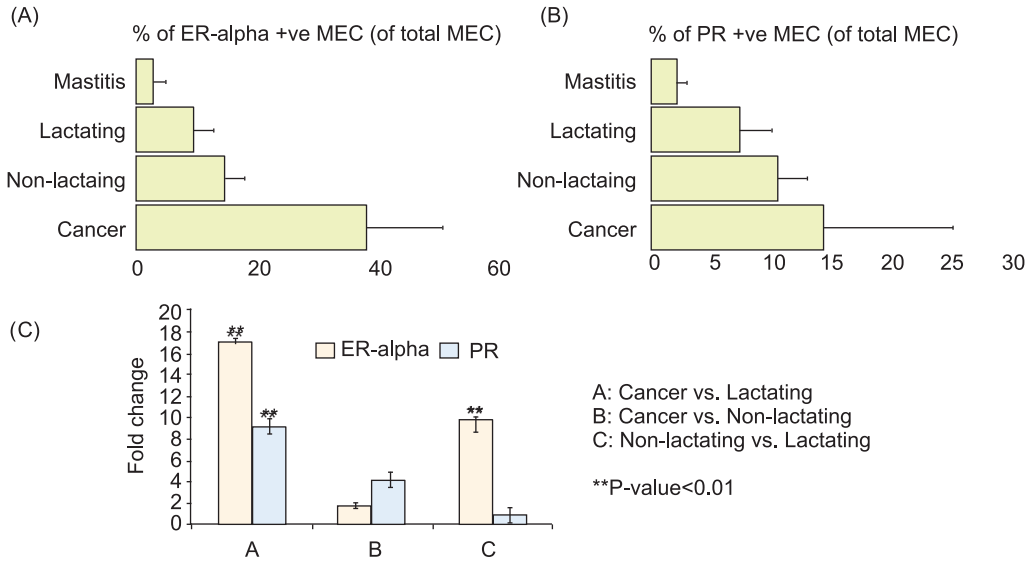

Fig. 4. Quantitation of ER-alpha and progesterone receptors in goat mammary glands. Cancer, non-lactating, lactating and mastitic mammary glands of goats were quantified for ER-alpha positive cells (A) and PR-B positive cells (B). Real time qPCR quantification of *ER-alpha* and *PR-B* transcripts in cancer, non-lactating and lactating mammary glands and their fold change expression (C).

al. 2001, Mohammadizadeh *et al.* 2012, Cai *et al.* 2012, Choudhary and Choudhary 2016). Histological observations of these mammary tissues by veterinary pathologist, indicated low to medium grade of mammary cancer. Cytoarchitecture of precancer/cancer tissues showed solidification of ductal lumen, *in situ* proliferation of MEC and sometimes complete loss of organization of MEC (Fig. 5D). Overexpression and aberrant expression of these protein markers in such tissue, provided second line of evidence of being mammary cancer. In normal and healthy goat mammary glands, CD10 protein is expressed in basal mammary epithelial cells only. Overexpression of CD10 in basalstroma (Fig. 5A) was observed in these mammary carcinoma. Likewise, expression of FNDC3B in goat mammary cancerous tissue was aberrant and abundant (Fig. 5B). MUC1, a marker of luminal epithelial cells, showed expression in luminal, basal and all over the stroma as well, indicating its aberrant expression (Fig. 5C), a characteristics features of cancer. Interestingly, varied expression of Ki-67 (high expression, Fig. 5D and for low expression, Fig. 5E), showed that these tumors were of different grades. In all the experiments, negative control did not show tissue staining of either DAB or NovaRed signals (Fig. 5F). Additionally, in normal goat mammary tissues as control tissues, expression of CD10 was limited to basal epithelium, MUC1 to luminal and FNDC3B was nuclear in prepubertal and mainly cytoplasmic in lactating animals (data not shown).

Distribution of ER-alpha: ER-alpha is required for elongation of mammary ducts. Loss of ER-alpha was associated with compromised ductal branching in mouse mammary glands (Mallepell *et al.* 2006). High and strong nuclear staining of MEC in cancer which were nulliparious animals were observed in the present study. Highest mRNA expression of ER-alpha was found in non-pregnant heifers (Schams *et al.* 2003) but protein localization was undetectable during pregnancy and lactation. In contrary to this, we found protein localization of ER-alpha during lactating and non-lactating period in goat mammary glands.

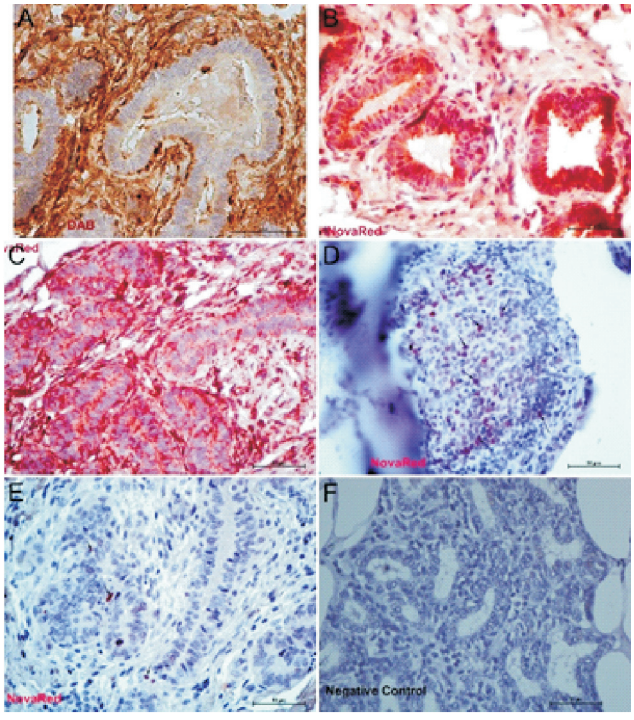

Fig. 5. Immunohistochemical characterization of goat mammary tumors using panel of cancer markers. Overexpression of CD10 (A), FNDC3B (B) and MUC1 (C) validate our results that these tissues are mammary cancer. Proliferation index of these pre-cancer/cancer tissue varied from high (D) to low (E), based on expression of Ki-67, a cell proliferation marker. Negative control, omission of primary antibody, showed no staining (F). Scale bar = A: 100 µm; B-F: 50 µm.

We failed to observe localization of ER-alpha in basal/ myoepithelial cells and that was in agreement with lack of ER-alpha expression in myoepithelial cells (Shao *et al.* 2000, Anderson and Clarke 2004). In bovine, ER- beta expression lacks during lactation thus permitting PR expression (Connor *et al*. 2005), however, ER-beta expression was observed in present study.

Distribution of PR-B: Progesterone is required for branching of mammary ducts (Atwood *et al.* 2000) and for proliferation of MEC (Beleut *et al*. 2010) including proliferation of mammary stem cells (Joshi *et al.* 2010). Progesterone acts through binding with its cognate receptors called progesterone receptor. In PR-B null mice, the development of secretory alveoli was impaired (Brisken *et al.* 1998), suggesting its role in alveolar development as well. We observed PR-B expression being highest in cancer stage was highest among non-lactating and lactating stages and report was consistent with PR-B expression in sheep (Colitti and Farinacci 2009). PR-B expression during lactating stage suggests that functional differentiation of MEC to secrete milk does not interfere with cell proliferation. This consistent observation was noted in bovine (Capuco *et al.* 2002) as well as in sheep mammary glands (Colitti and Pulina 2010).

In goat mammary precancer mammary precancerous cancer/cancer, we observed a solidification of duct (arrow) and excessive growth of MEC. High expression of ER-alpha in nuclei and weak expression in the cytoplasm of MEC were noticed. It appears that cytoplasmic localization of ER-alpha in majority of the cells is a consistent feature of cancer cells. Cytoplasmic localization of ER-alpha is similar to aberrant localization of receptor proteins, a hallmark of abnormal cell. Detail investigation of these mammary tissues with CD10, MUC1 and FNDC3B revealed aberrant and overexpression of all the markers. CD10 or Common Acute Lymphoblastic Leukemia/Lymphoma Antigen (CALLA) is a metalloproteinase that cause degradation of extra-cellular matrix. Although, CD10 positive cells has been detected in the basal layer of normal goat mammary gland (Safayi *et al.* 2012), its overexpression was associated with various malignancies including breast cancer (Bachelard-Cascales *et al.* 2010) and goat mammary cancer (Choudhary *et al.* 2016). Majority of the ductal carcinoma *in situ* (DCIS ~ 60%) showed strong immunoreactivity to estrogen receptor in rat mammary gland (Moise *et al.* 2013). Our results showed strong immunoreactivity to ER-alpha and inward growth of ER-alpha-positive mammary epithelial cells towards the lumen, indicating that progression of mammary tumors is firmly connected with estrogen profile. PR-B expression was at moderate level and its weak cytoplasmic expression in majority of the MEC appears to be consistent with hallmarks of mammary gland tumors (Moise *et al.* 2013).

Our mRNA expression pattern results showed a high expression of ER-alpha and PR-B genes in cancer group was consistent with the observation of ER-alpha expression observed in prepubertal sheep mammary glands (Colitti and Pulina 2010). Transcripts of ER-alpha was downregulated in lactating stage but upregulated in non-lactating stage. This is in agreement with the idea that estrogen mainly required for ductal branching during prepubertal stage and therefore expression of ER-alpha was high in precancer tissue. Expression of ER-alpha goes down during lactation as it occurs in primates (Cheng *et al.* 2005) and ewes (Colitti and Pulina 2010). Estrogen is required for elongation of mammary ducts whereas progesterone is required for ductal branching (Atwood *et al.* 2000). High expression of estrogen receptors has been shown in many incidences of breast cancer with its wide dynamic range of expression (Aitken *et al*. 2010). These observations suggest that steroid receptors have important roles in growth and development of goat mammary glands and their pattern of expression depends upon physiological stages of the animal. There is emerging evidence that steroid receptors have a role in regulation of mammary cancer stem cells and mammary cancer in mice. Future comprehensive studies are required to analyze steroid receptors expression in large population (goat farms) to evaluate the gland health and its possible association with mammary cancer.

The present findings demonstrated cell-specific localization and quantification of ER-alpha and PR-B in lactating, non-lactating, mastitic and precancer mammary glands of goat. ER-alpha and PR-B expressed primarily in luminal layer of epithelium in the lobules and in the outer layer of epithelium in the interlobular ducts, reflecting their regulatory roles in mammary gland growth and development. The mRNA expression of ER-alpha and PR-B was highest in mammary precancer than the lactating and non-lactating tissue. At mRNA and protein level, an increased expression of ER-alpha was noticed in mammary cancer, indicating dysregulation of estrogen signal. Further studies are required to evaluate role of ER-alpha and PR-B in the regulation of mammary cells in goat and correlate their expression profile at various physiological stages.

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