

# Expression of keratin 19, Na-K-Cl cotransporter and estrogen receptor alpha in developing mammary glands of ewes

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**Summary.** Mammary gland remodelling is strictly related to intracellular signals and stem cell biology. Among the best candidates to identify the nature and development of mammary cells are cytokeratin 19 (CK19), the Na-K-Cl cotransporter (NKCC1) and receptor of estrogen alpha ( $ER\alpha$ ). In this study, we analyzed the expression of these genes in ewe mammary glands from prepubertal stage to involution. Using Real time PCR we showed that NKCC1 transcription was significantly down regulated during lactation and at involution in comparison to the expression measured in the prepubertal group. No significant differences were found in CK19 expression, whereas  $ER\alpha$  transcription was significantly down regulated before lambing, during lactation and at involution. *In situ* hybridization analysis confirmed quantitative data and localized the CK 19 transcript at basal and luminal compartment of terminal ductal unit (TDU) of prepubertal mammary glands. NKCC1 expression was also present in lactating glands and  $ER\alpha$  in connective tissue surrounding TDU. The characterization and identification of mammary developmental markers in the tissue of dairy animals is necessary to gain knowledge in mammary gland biology.

**Key words:** Mammary glands, Sheep, CK19, NKCC1,  $ER\alpha$

## Introduction

The sheep mammary gland is a complex tissue that proliferates under the control of systemic hormones during puberty, pregnancy and early lactation. Limited mammary growth is observed at late lactation, when the

gland gets ready for the involution period (Stefanon et al., 2002). However, although it has long been thought that terminally differentiated cells do not proliferate, milk secretory activity and cell division are not mutually exclusive. In fact, in a recent paper (Colitti and Pulina, 2010) casein gene transcription has been found to be up regulated during lactation, in comparison to prepubertal group of ewes, when the proliferation index was found at its highest (Colitti and Farinacci, 2009a). This remarkable regenerative capacity, which appears also during successive reproductive cycles, may involve recruitment of additional functionally differentiated cells from a population of pre-existing non secretory cells that, when necessary, can expand to generate a transiently amplified pool of progenitors to re-populate tissue (Woodward et al., 2005). Hence, the need to isolate molecular markers to identify mammary gland stem cells. In mouse and human, several investigators have employed a variety of methods including: non-adherent mammosphere cultures; 5-bromo-2-deoxyuridine (BrdU) label-retention studies that identify LRCs (Capuco, 2007); Hoechst dye efflux that identifies the side population cells (SP) (Zhou et al., 2001) and cell-surface markers, such as Sca1 and CD49f (Stingl et al., 2006). However, until now, no genetic marker has yet been found to positively identify mammary stem cells in dairy animals (Woodward et al., 2005). Histological examination of human tissue sections demonstrates that  $ER\alpha$ /PR-positive cells have an intermediate position in breast epithelium, large nuclei and express CK19 and, in smaller sub-populations, also p21CIP1 and Msi1 (Clarke et al., 2003).

In a previous paper Colitti and Farinacci (2009b) demonstrated that Msi 1 was expressed, at different degrees, in the developing, lactating and involuting mammary glands of ewes. Among the best candidates to identify the nature and development of mammary cells are cytokeratin 19 (CK19) and the Na-K-Cl cotrans-

porter (NKCC1) (Shillingford et al., 2002; Clarke et al., 2005). Relative quantification of these markers would be appropriate in order to correlate the expression pattern changes of stem cell markers already considered (Colitti and Farinacci, 2009b; Colitti, 2010).

Since Gudjonsson et al. (2002) have shown that the multipotent progenitor cells of the human breast reside in a predominantly keratin CK19 positive compartment, the expression of this marker was here analysed, together with its relationship to cells expressing steroid receptor of estrogen alpha (ER $\alpha$ ). In fact, as reported by Clarke et al. (2005) in their model of the cellular hierarchies in human breast, the scattered 'intermediate' CK19/Msi1/ER $\alpha$ /PR-positive stem cells are thought necessary for generating differentiated cells within a smaller patch of expression that may lead to cleavage of the Notch cytoplasmic domain as demonstrated in neural stem cells (Okano et al., 2002). Moreover, CK19 has been implicated in the differentiation of many different epithelial tissues, including mammary gland ducts, in which there is a transition between differentiated phenotypes (Stasiak et al., 1989).

NKCC1 has been shown to be expressed in a wide variety of tissues and is present at the basolateral membrane of secretory epithelial tissue, including mammary glands (Shillingford et al., 2002). It is involved in cell volume regulation (O'Neill and Klein, 1992) and cell proliferation (Panet et al., 2000). The NKCC1 protein was found to be present at high levels on ductal mammary epithelial cells in virgin mice and was down regulated in developing alveoli and ductal structures at pregnancy. During lactation, high levels of NKCC1 protein were only observed in a few cells within alveoli (Shillingford et al., 2002). These results demonstrated that NKCC1 is involved in ductal cell development in the virgin animal but not in alveolar cell development or function in the pregnant animal (Shillingford et al., 2003).

The estrogen receptor- $\alpha$  (ER $\alpha$ ) is a critical transcription factor that regulates epithelial cell proliferation and ductal morphogenesis during postnatal mammary gland development (Feng et al., 2007). Between the two isoforms  $\alpha$  and  $\beta$ , ER $\alpha$  is considered the primary receptor for mammary gland development and function, and it is significantly down regulated during pregnancy and lactogenesis (Schams et al., 2003; Colitti and Pulina, 2010). Moreover, it has also been reported that while ER $\alpha$  is expressed in luminal epithelial cells, dividing cells are steroid receptor-negative although they are often adjacent to steroid receptor-positive cells (Clarke et al., 1997; Capuco, 2007). In fact, the hormone-sensing and in vivo stem/progenitor activities of mammary epithelium are properties of distinct, separate cell populations (Sleeman et al., 2007).

In this study, genes that mark different developmental stages have been used to ascertain the timeline with regard to tissue architecture, mammary growth characteristics and function in dairy ewes and to

evaluate their relationship with the expression of ER $\alpha$  receptor also involved in stem cells biology.

## Material and methods

### Animals

Tissue was collected from mammary glands of twenty-five Sardinian sheep that were slaughtered at different developmental stages: prepubertal (30 $\pm$ 5 days, group P), 10 days before lambing (group LateP), at 30 and 60 days of lactation (groups 30L, 60L) and 8 days after the end of lactation (group 8IN, only for Real time PCR). At each sampling period, 5 animals were randomly selected from a flock of grazing sheep and a clinical examination of the selected animals was conducted in vivo to ascertain animal health and to exclude mastitis.

From each animal, two samples of tissue were collected, one was collected in TRIzol<sup>®</sup> (Invitrogen, Milan, Italy), frozen in liquid nitrogen and kept at -80°C till RNA extraction, the other one was fixed in 10% neutral formalin.

The experiment was carried out in accordance with state and local laws and ethical regulations (DL No. 116, 128 27/01/1992).

### RNA extraction and riboprobe

Total RNA was extracted from mammary tissues using TRIzol<sup>®</sup> Plus RNA Purification System (Invitrogen, Milan, Italy), following the manufacturer's instructions. The concentration of the extracted total RNA was quantified using a spectrophotometer (NanoDrop 1000 Spectrophotometer, ThermoScientific, Wilmington, DE, USA). The assessment of the purity of RNA samples was performed by a procedure referred to as the A<sub>260</sub>/A<sub>280</sub> ratio and A<sub>260</sub>/A<sub>230</sub> ratio, values ranged between 1.9-2.0. RNA integrity was evaluated through the observation of 18S and 28S ribosomal bands after electrophoresis on 1% agarose gel, in the presence of ethidium bromide. In sample analysis,  $\beta$ -actin (U39357) expression was used as an internal control, confirming thorough integrity of the RNA.

Primer3 Input software (Rozen and Skaletsky, 2000) was used to design the primer sequences encoding for the ER $\alpha$ , estrogen receptor alpha (AY033393), NKCC1, Na-K-Cl cotransporter (NM\_174782) and CK19, keratin 19 (NM\_001015600). Primers, product lengths and specific amplification conditions for each gene are listed in Table 1.

The CK19 and NKCC1 primers were designed on the bovine sequences. The RT-PCR products were then purified from agarose with Wizard<sup>®</sup> SV Gel Clean-Up System (Promega, Milan, Italy) and the sequence analysis was done by PRIMM (Milan, Italy). Blast analysis of the CK19 sequenced nucleotide showed 98% of identities with *Bos taurus* mRNA from 484 to 664 nucleotides. Blast analysis of the NKCC1 nucleotide

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sequence showed 99% of identities with *Bos taurus* mRNA from 3888 to 4064 nucleotides.

The RT-PCR products were then cloned using a dual promoter TOPO TA cloning kit containing pCRII-TOPO cloning vector (Invitrogen, Groningen, The Netherlands) and sequenced (Primm, Milan). To prepare sense and antisense RNA probes, the transcripts were digoxigenin-labeled by *in vitro* transcription using a DIG RNA Labeling kit (Roche Diagnostics, Milan, Italy) and T7 and SP6 polymerases. The riboprobe concentration was evaluated by spectrophotometric analysis and stored at -80°C.

### Real time PCR quantitation

Reverse transcriptions were performed with total RNA by using Improm-II Reverse Transcriptase (Promega, Milan Italy) as follows. Two micrograms of total RNA with 1  $\mu$ l random primers (0.5  $\mu$ g/ $\mu$ l MBI Fermentas, Italy) and nuclease free water to a final volume of 20  $\mu$ l were incubated at 70°C for 5 min in a PTC-100 thermocycler (MJ Research Inc. Waltham, MA USA). Then, a mix was prepared with 4  $\mu$ l of Improm-II Reverse Transcriptase buffer (5X Promega, Milan Italy), 1.2  $\mu$ l MgCl<sub>2</sub> (50mM), 1  $\mu$ l of Improm-II Reverse Transcriptase and 1  $\mu$ l of dNTP (10mM) was added to the reaction and incubated at 37°C for 90 min, and finally at 94°C for 5 min. (Bustin et al., 2009). The final concentration of cDNA was 100 ng/ $\mu$ l.

### Standard curves analyses

For each gene, an aliquot of cDNA samples were pooled and standard curves with serial dilution of pool were used to optimize PCR conditions and to calculate the efficiency, fluorescence baseline and threshold. The expression of target genes was normalized using the  $\beta$ -actin and 18S rRNA genes, which are known to be constitutively expressed (Robinson et al., 2007).

Real-time RT-PCR reactions were performed in triplicate using Platinum<sup>®</sup> SYBR<sup>®</sup> Green qPCR

SuperMix-UDG (Invitrogen, Milan Italy). For these reactions, a master mix with the following components was prepared to the indicated end concentration: 1  $\mu$ l of cDNA, 9.5  $\mu$ l water, 1  $\mu$ l of each primer and 12.5  $\mu$ l of 2X Platinum SYBR Green qPCR SuperMix-UDG for a total volume of 25  $\mu$ l. cDNA concentrations and primer molarities were different for each gene and determined with standard curves analyses performed before Real time PCR reactions (Table 1).

PCR amplification was conducted applying 45 cycles (1 sec at 95°C, 30 sec at the specific annealing temperature, 30 sec at 72°C) in a 96-well spectrofluorometric thermal cycler (DNA Engine Opticon 2; MJ Research, Inc. Waltham, MA USA). The melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

The expression level of a given target gene in each experimental group was analyzed using Real-time PCR and by the  $2^{-\Delta\Delta Ct}$  method (Pfaffl, 2001). The  $\beta$ -actin and 18S rRNA genes were used as internal controls in all Real time PCR experiments and  $\Delta Ct$ s were calculated from their geometric mean. Further,  $2^{\Delta\Delta Ct}$  represents the difference for a given target gene between each group after prepubertal stage (groups from LateP to 8IN) vs group P. More precisely, individual  $\Delta\Delta Ct$  were calculated for each sample of group (LateP-8IN) as  $\Delta\Delta Ct = \Delta Ct$  (sample group) - mean  $\Delta Ct$  (group P). The  $n$ -fold higher expression of a given target gene was calculated as  $\log_2(2^{-\Delta\Delta Ct})$ .

Correlation analysis was performed between these results and also with those previously obtained on the same samples for Ki67, a proliferation marker and the potential stem cells marker Musashi1 (Msi1), an RNA-binding protein (Colitti and Farinacci, 2009a,b).

### In situ hybridization

*In situ* hybridization was used to localize NKCC1, CK19 and ER $\alpha$  mRNAs in sheep mammary gland sections at P, LateP and 30-60L, using digoxigenin

**Table 1.** Real time PCR primer sequences and product specific amplification conditions.

Gene	Genbank accession number	Sequence (5'→3')	Product length, bp	cDNA ng	Primer, nM	Ta, °C	E
CK19	94574050	for: GATGACTTCGACCAAGTT rev: TCATTTCTCCTCGTGGTTC	181	0.5	200	59	1.95
NKCC1	31340942	for:GACATACCGGCAGATCAGGT rev:TGATTCCCACGGACTAGGAG	177	5	400	59	1.95
ER $\alpha$	15418804	for: CCACGATCAAGTCCACCTTT rev:ACGGAACCGAGACGATGTAG	193	5	200	58	1.9
18S rRNA	58760943	for: AAACGGCTACCACATCCAAG rev:TCCTGTATTGTTATTTTCGTCAC	90	0.1	100	58	1.97
$\beta$ -actin	2182268	for: TCCCTGGAGAAGAGCTACGA rev:AGCACCGTGTGGGATAGAG	102	5	200	58	2.0

for: forward; rev: reverse; Ta: annealing temperature; E: PCR amplification efficiency.

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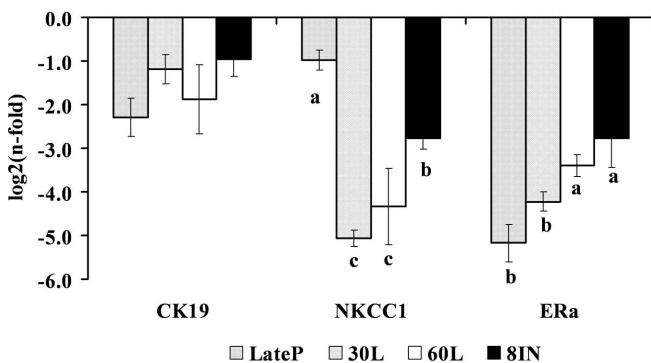
(DIG)-labelled sense and anti-sense NKCC1, CK19 and ER $\alpha$  probes.

The yields of labelled probes were determined by spotting a serial dilution of the probes on a nylon membrane, followed by an enzyme-linked immunoassay with anti-DIG AP and BCIP/NBT (DIG RNA labeling Kit, Roche).

Briefly, 5  $\mu$ m sections of the formalin-fixed and paraffin embedded tissues were heated at 65°C for 30 min and deparaffinized in two changes of xylene, followed by two washes in absolute ethanol. Sections were rehydrated in a graded series of ethanol and rinsed in distilled water. Subsequently, sections were digested with proteinase K (20  $\mu$ g/ml) for 20 min at RT. After several washes in TBS (100 mM Tris, pH 7.5, 150 mM NaCl), hybridization was performed for 16-18 h at 47°C in a solution containing 20% formamide (v/v), 6X SSC, 2X Denhardt's solution, 400  $\mu$ g/ml of denatured ssDNA, 5% dextran sulfate (w/v), 0.5% Tween 20, and 4ng/ml digoxigenin-labeled sense or anti-sense probe. Each section was hybridized with 20-50  $\mu$ l of hybridization solution. After hybridization, sections were washed in TBS to remove excess probe. Finally, sections were washed at high stringency in 0.5X SSC, 0.05% SDS for 7 min at 47°C. The sections were then incubated with a 1:200 dilution of anti-digoxigenin-alkaline phosphatase conjugate (Roche) in TBS for 1 h followed by chromogenic detection with 5-bromo-4-chloro-30-indolyphosphate p-toluidine (BCIP) and nitro-blue tetrazolium (NBT) for 3 h and mounted with aqueous medium (glycerol-gelatin). Controls included parallel step sections that were hybridized with sense riboprobe. No counterstain was performed on tissue slides.

*Statistical analysis*

All the recorded variables were submitted to analysis of variance using the ANOVA model to assess



**Fig. 1.** Expression analysis of target genes at 10 days from lambing, 30, 60 days of lactation and 8 days from dry-off, related to expression at prepubertal stage (P). Late P = 10 days from lambing; 30L = 30 days of lactation; 60L = 60 days of lactation; 8IN = day 8 after the dry-off. CK19, keratin 19; NKCC1, Na-K-Cl cotransporter; ER $\alpha$ , estrogen receptor alpha. Bars represent the mean plus/minus standard error (SE). Bars designated with different letters represent statistical differences between groups at  $p < 0.05$ .

significant differences between groups; Duncan's least significant difference test was used to compare the means (SPSS Inc., 1997).

Correlation analyses were performed by Pearson's test because the data were normally distributed as calculated by Shapiro-Wilk test (SPSS Inc., 1997).

**Results**

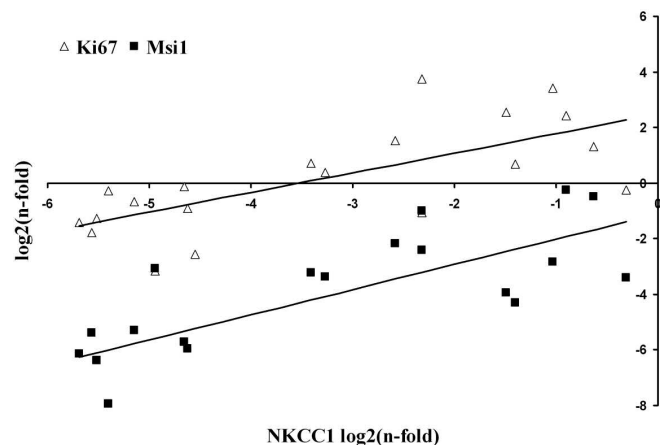
*Expression analysis*

Native agarose gel analysis of total RNA and  $\beta$ -actin amplification were used to verify the integrity of the mRNA extracted.

Quantitative measurements of mRNAs encoding CK19, NKCC1 and ER $\alpha$  in sheep mammary glands were obtained using Real time PCR on total mammary gland RNA samples.

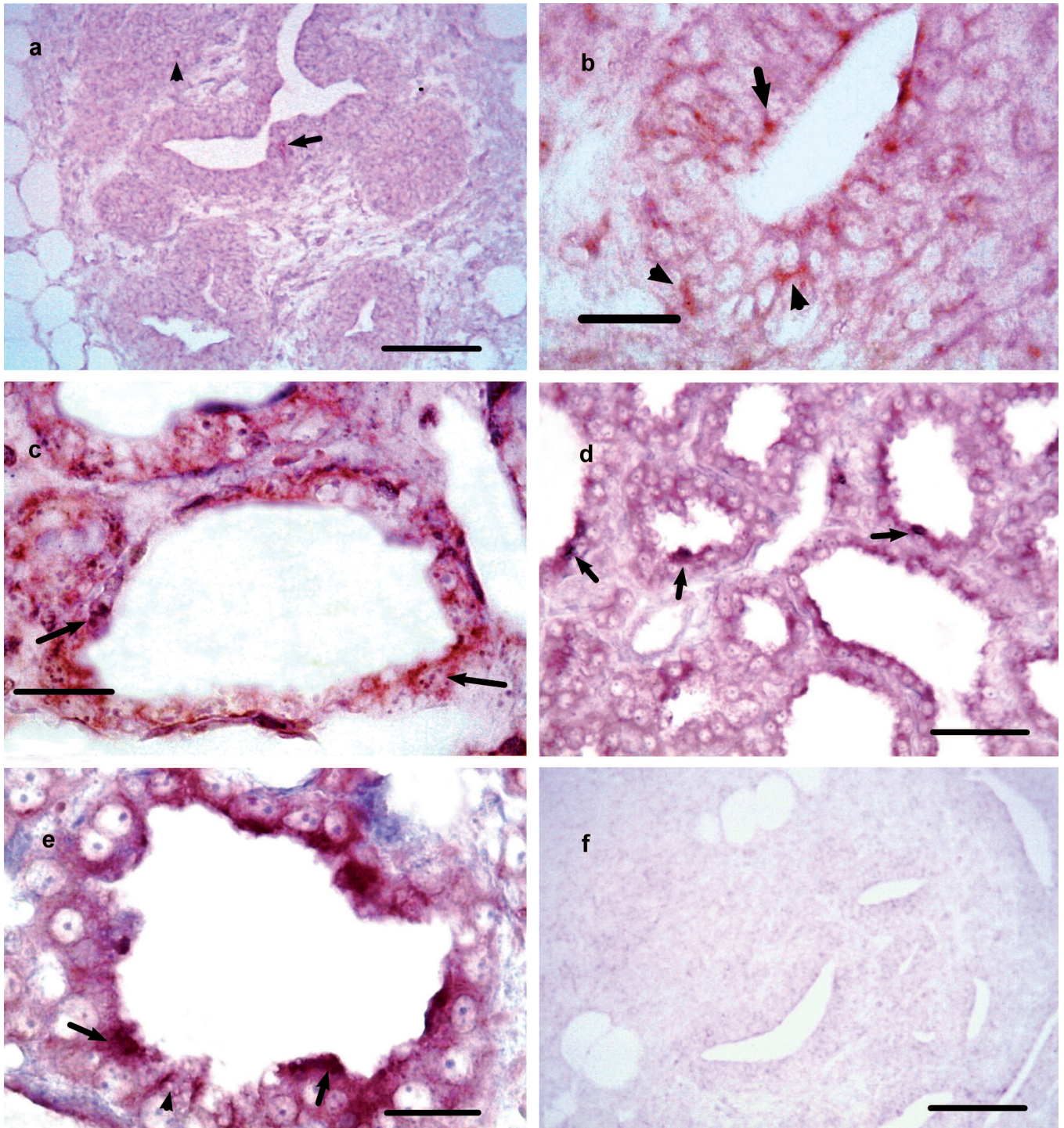
In terms of  $\Delta$ Ct, CK19 gene expression significantly differed ( $p < 0.05$ ) between groups at prepubertal stage (P), when it was at its lowest. The NKCC1 gene abundance was significantly different ( $p < 0.05$ ) between groups, being at its lowest at P and LateP and at its highest during lactation (30L, 60L groups). The expression of the ER $\alpha$  gene significantly changed ( $p < 0.05$ ), being at its lowest in P group and increasing during LateP and lactation (30L, 60L groups).

The  $n$ -fold values, reported in figure 1 as  $\log_2(n$ -fold), indicate the relative abundance of each target gene in comparison with the P group. The relative transcription of CK19 did not significantly differ between groups and was down regulated ( $p < 0.05$ ). The relative expression of NKCC1 significantly increased ( $p < 0.05$ ) at LateP and 8IN groups in comparison to 30, 60 days of lactation. ER $\alpha$  expression was significantly down regulated ( $p < 0.05$ ) in every group and it was at its lowest in LateP and 30L groups. The coefficient of correlation between CK19 and ER $\alpha$  was significant at 0.05 level and equal to 0.50. The NKCC1  $\log_2(n$ -fold) results were correlated with Ki67 and Msi1  $\log_2(n$ -fold)



**Fig. 2.** Correlation results between NKCC1 and Ki67 and Msi1 expression data ( $\log_2(n$ -fold)) significant at  $p < 0.01$ .

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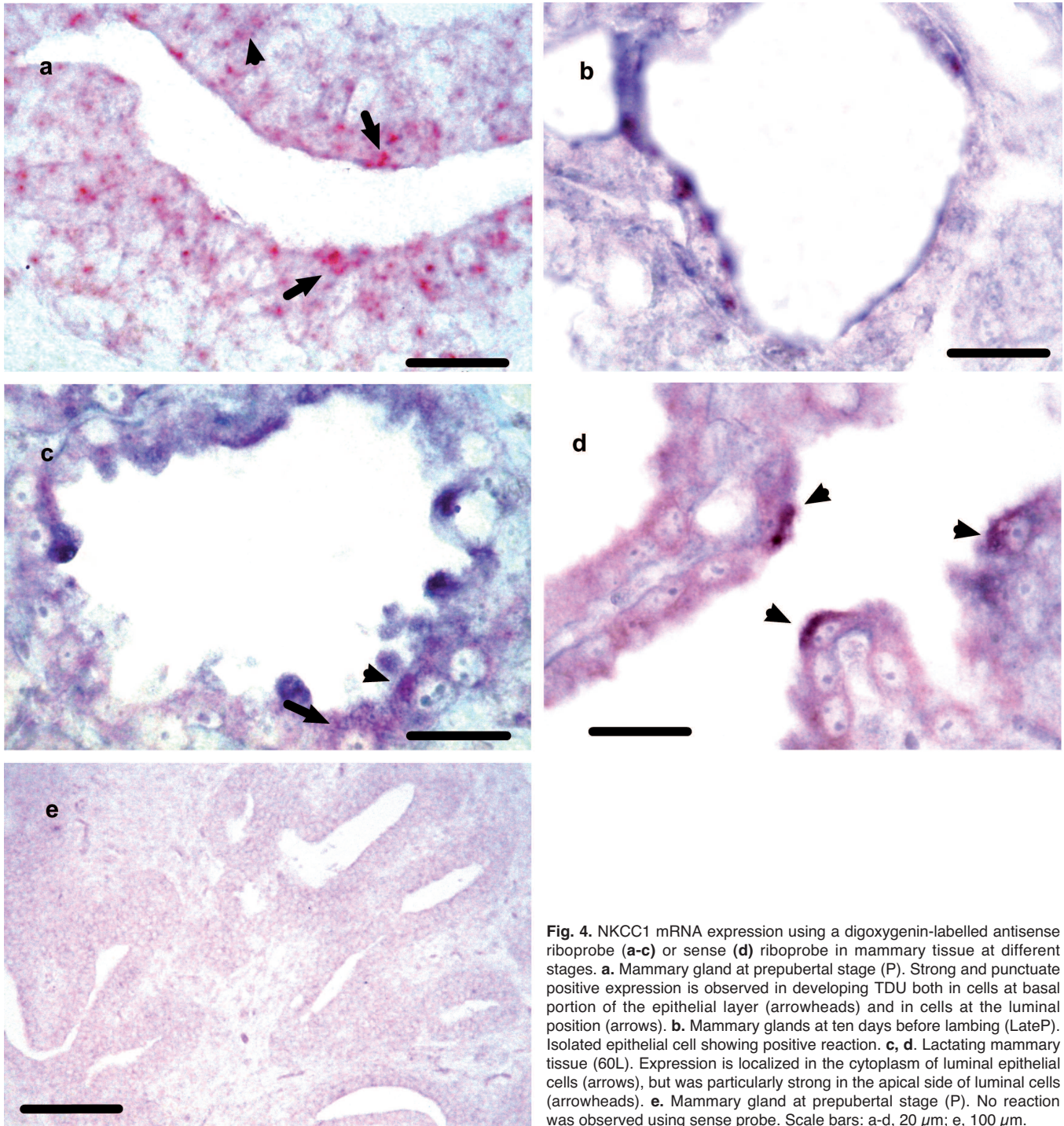
**Fig. 3.** CK19 mRNA expression using a digoxigenin-labelled antisense (a-e) or sense (f) riboprobe in mammary tissue at different stages. a, b. Mammary glands at prepubertal stage (P). Diffuse staining is observed throughout branching ductular unit (TDU) cells. Expression was localized in cells at apical (arrows) and intermediate position (arrowheads). c. Mammary glands at ten days before lambing (LateP). Detail of alveolus showing epithelial cells with positive reaction. c, d. Lactating mammary tissue (30L). c. Overview of lactating tissue with positive alveolar cells scattered within the tissue. d. Detail of positive reaction at the apical (arrow) and lateral (arrowhead) side of alveolar epithelial cells f. Negative reaction was observed with sense probe in prepubertal tissue. Scale bars: a, f, 100  $\mu$ m; b, c, e, 20  $\mu$ m; d, 50  $\mu$ m.

data at  $p < 0.01$  and the coefficient was equal to 0.74 and 0.70, respectively (Fig. 2).

#### *In situ hybridization*

*In situ* hybridization on histological sections was done using antisense and sense cRNA digoxigenin-

labeled probes directed against the 181bp CK19, 177bp NKCC1 and 193bp ER $\alpha$  sequences. The hybridization analysis was utilized to study the expression of the isolated mRNA in the mammary glands of sheep at P, Late P and 30-60L stages. Alkaline phosphatase-tagged RNA probes were revealed by a brownish to bluish colour (Figs. 3-6).



**Fig. 4.** NKCC1 mRNA expression using a digoxigenin-labelled antisense riboprobe (a-c) or sense (d) riboprobe in mammary tissue at different stages. **a.** Mammary gland at prepubertal stage (P). Strong and punctate positive expression is observed in developing TDU both in cells at basal portion of the epithelial layer (arrowheads) and in cells at the luminal position (arrows). **b.** Mammary glands at ten days before lambing (LateP). Isolated epithelial cell showing positive reaction. **c, d.** Lactating mammary tissue (60L). Expression is localized in the cytoplasm of luminal epithelial cells (arrows), but was particularly strong in the apical side of luminal cells (arrowheads). **e.** Mammary gland at prepubertal stage (P). No reaction was observed using sense probe. Scale bars: a-d, 20  $\mu$ m; e, 100  $\mu$ m.

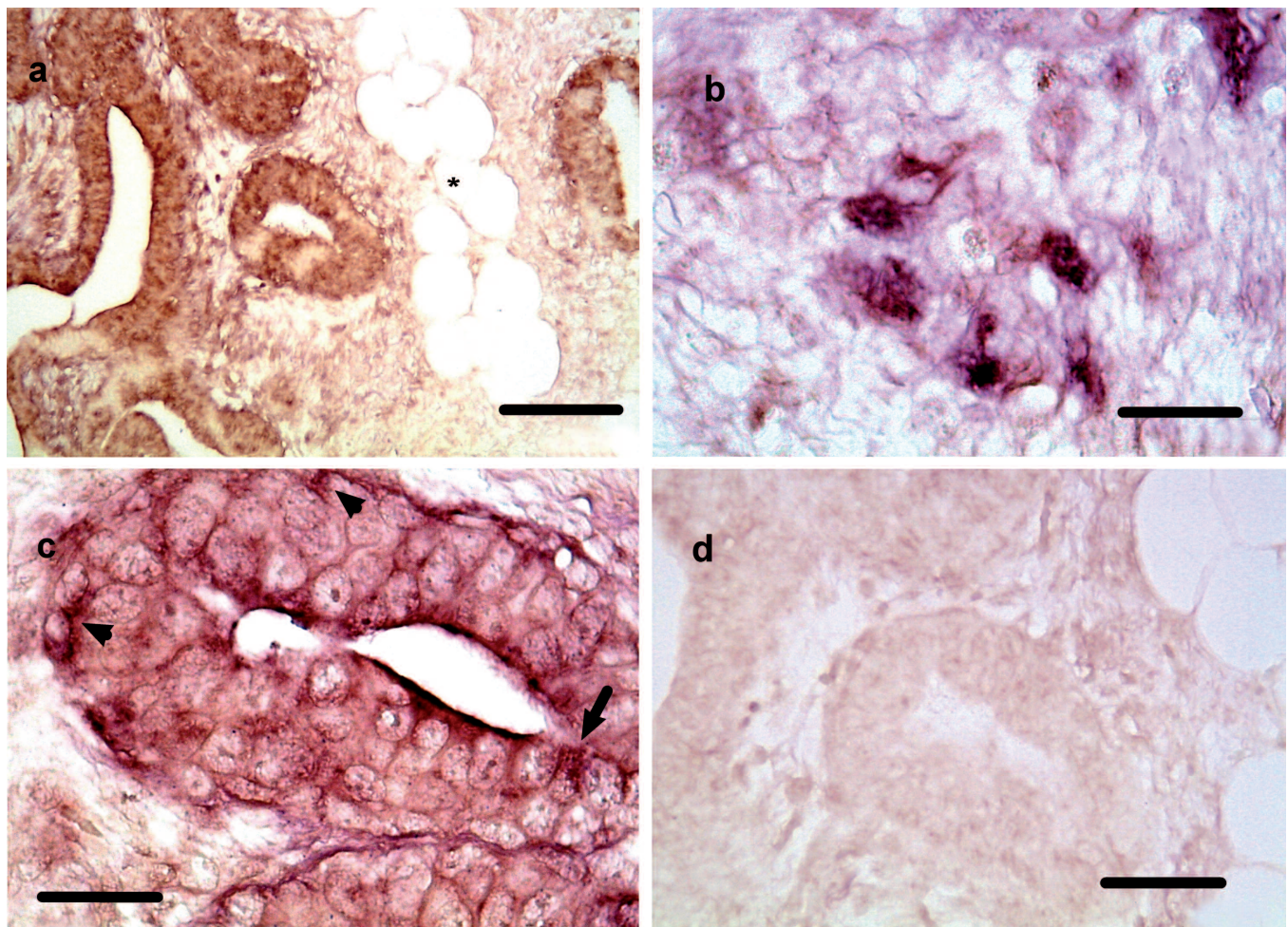
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In P mammary tissue the CK19 antisense probe labelled epithelial cells of differentiating ducts that formed the terminal ductal unit (TDU), the distal branching ductular structure, as referred by Capuco et al. (2002) in heifers (Fig. 3a,b). CK19 expression was scattered throughout the TDUs and marked cells at apical (Fig. 3a,b arrows) and intermediate position (Fig. 3a,b arrowheads). At LateP, CK19 expression labelled the cytoplasm of alveolar cells in some lobules (Fig. 3c). The staining at lactation was not homogeneous and was observed in just few epithelial cells throughout the alveoli (Fig. 3d). The staining was strong, cytoplasmatic, and was localized at the apical (arrow) and lateral side (arrowhead) of luminal epithelial cells (Fig. 3e). The specificity of CK19 antisense probe was ascertained using the sense probe as negative control at P stage (Fig. 3f).

Strong and diffuse NKCC1 expression was localised in epithelial cells of differentiating ducts in prepubertal

mammary tissue (Fig. 4a). The positivity was found in cells in the basal portion of the epithelial layer (Fig. 4a, arrowheads) as well as in the luminal cell layer (Fig. 4a, arrows). Lower and scattered NKCC1 expression was observed at LateP stage (Fig. 4b). NKCC1 expression was evident in some alveolar cells during lactation (Fig. 4c). The antisense probe labelled cytoplasm of luminal epithelial cells (Fig. 4c, arrows), but often NKCC1 expression was supranuclear at this stage (Fig. 4c,d, arrowheads). The sense cDNA probe did not label any epithelial cells in prepubertal tissue (Fig. 4e).

The staining indicative of ER $\alpha$  mRNA expression showed a strong to moderate intensity (Figs. 5-6) during mammary development. The predominant expression of ER $\alpha$  in P mammary glands of sheep, obtained by Real time PCR, was further confirmed by *in situ* hybridization (Fig. 5a,c) which demonstrated that ER $\alpha$  expression was clearly defined in the TDUs, but not in adipocytes surrounding the loose connective tissue that forms the



**Fig. 5.** ER $\alpha$  mRNA expression using a digoxigenin-labelled antisense (a-c) or sense (d) riboprobe in prepubertal mammary tissue. **a.** Overview of branching ductular unit (TDU) showing positive reaction and mammary outgrowth with no expression in the fat cells (asterisk). **b.** Strong positivity is also present in some stromal cells of connective tissue that enveloped the TDUs. **c.** Detail of reactive epithelial cell at the basal (arrowheads) and luminal layer (arrow) of TDU. **d.** Sense control showing lack of reactivity in prepubertal mammary tissue. Scale bars: a, 100  $\mu$ m; b, c, 20  $\mu$ m; d, 150  $\mu$ m.

intralobular stroma (Fig. 5a, asterisk). However, clusters of positive cells were observed dispersed in the mammary connective tissue that enveloped the TDUs (Fig. 5b). ER $\alpha$  expression was localized in epithelial cells of the basal portion of the TDU (Fig. 5c, arrowheads) as well as in the luminal cell layer (Fig. 5c, arrow). No reaction was seen with the sense probe (Fig. 5d).

Moderate intensity was observed at LateP (Fig. 6a), but a more intense bluish reaction was identified in lactating gland (Fig. 6b,c). At this stage the localization appeared both cytoplasmatic (Fig. 6c,d arrows) and nuclear (Fig. 6c,d arrowheads).

## Discussion

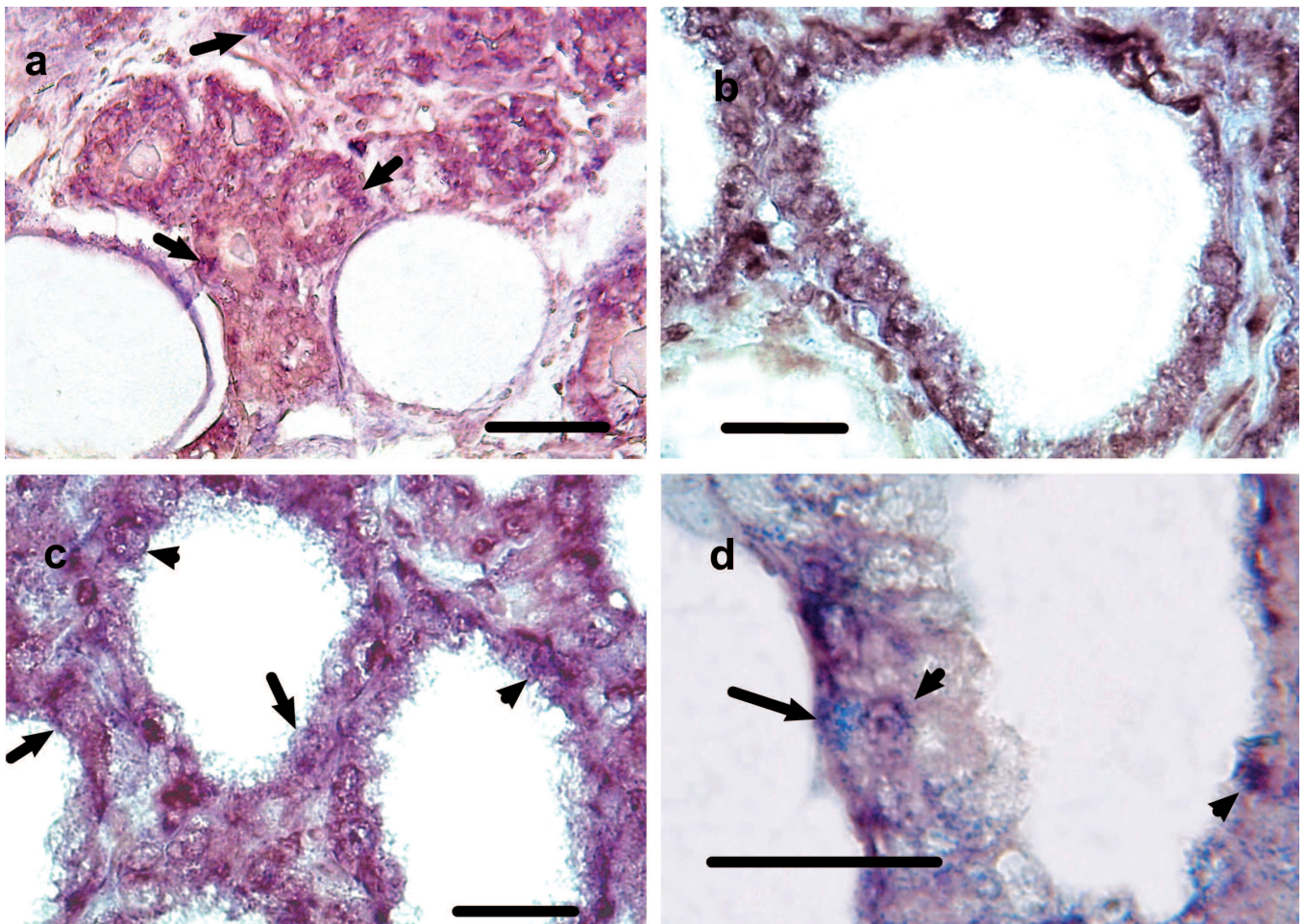
Mammary remodelling is determined by a combination of cell differentiation, proliferation and apoptosis. Sheep mammary glands are a dynamic tissue

in which considerable expansion of epithelial compartment takes place during pregnancy and lactation (Colitti and Farinacci, 2009a). As in human, this could be driven by a proliferation of differentiated epithelial

**Table 2.** Expression level of developmental markers in groups of sheep at different developmental stages.

Groups	CK19	NKCC1	ER $\alpha$
P	0.84 $\pm$ 0.07 <sup>a</sup>	-0.23 $\pm$ 0.24 <sup>a</sup>	0.78 $\pm$ 0.02 <sup>a</sup>
LateP	3.13 $\pm$ 0.44 <sup>b</sup>	0.74 $\pm$ 0.22 <sup>a</sup>	5.95 $\pm$ 0.43 <sup>b</sup>
30L	2.03 $\pm$ 0.34 <sup>ab</sup>	4.84 $\pm$ 0.18 <sup>b</sup>	5.00 $\pm$ 0.23 <sup>bc</sup>
60L	2.73 $\pm$ 0.79 <sup>ab</sup>	4.10 $\pm$ 0.88 <sup>b</sup>	4.17 $\pm$ 0.25 <sup>c</sup>
81N	1.80 $\pm$ 0.40 <sup>ab</sup>	2.55 $\pm$ 0.23 <sup>c</sup>	3.55 $\pm$ 0.67 <sup>c</sup>

mean  $\Delta$ Ct  $\pm$  standard error. CK19: cytokeratin 19; NKCC1: Na-K-Cl cotransporter; ER $\alpha$ : estrogen receptor alpha; <sup>a-c</sup>: different superscripts within a column indicate significantly ( $P < 0.05$ ) different means between groups.



**Fig. 6.** ER $\alpha$  mRNA expression using a digoxigenin-labelled antisense riboprobe in mammary tissue at different stages. **a.** Mammary glands at ten days before lambing (LateP). Overview of mammary tissue with moderate but diffuse staining in alveolar cells (arrows). **b-d.** Lactating mammary tissue (60L). **b.** Detail of alveolar cell positivity. **c-d.** Detail of positive expression in nuclei (arrowheads) and cytoplasm (arrows) of epithelial cells. Scale bars: a, 50  $\mu$ m; b, c, 20  $\mu$ m; d, 15  $\mu$ m.



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cells, as suggested by the expression of proliferation marker Ki67 (Suzuki et al., 2000; Colitti and Farinacci, 2009a), and/or may be a result of adult stem cells. In fact, the redevelopment of epithelial cells has been suggested to occur by self renewal, which claims the presence of stem cells in mammary tissue (Ellis and Capuco, 2000; Welm et al., 2003). Thus, the study of functional expansion and regression of mammary glands could not be analyzed without molecular and cellular events that consider the presence of progenitor cells, even in dairy species.

Very few markers were assigned directly to a candidate mammary stem cell pool in mammary glands of ruminants, due to the difficulty of identification and functional characterization of markers in these species. Nevertheless, the increasing knowledge of mechanisms influencing lineage choices in the mammary gland could produce beneficial effects on mammary gland development and milk production.

In this study, we investigated the expression of intracellular signals involved in mammary development of dairy ewes and that can affect stem cell biology. Hudson et al. (2001) in human prostate considered CK19 a useful marker for amplifying a non-stem-cell basal population and a marker of intermediate cell populations from *in vitro* studies.

Our mRNA expression results have demonstrated a relatively low expression of CK19, although not significant, in mammary tissue of sheep at all stages examined. In terms of  $\Delta Ct$ , the expression significantly increased during lactation in comparison to prepubertal stage. The mRNA data were also confirmed by the *in situ* localization with strong signal of staining on suprabasally located epithelial cells, as already shown by Fridriksdottir et al. (2005). The heterogeneous staining and the presence of stained epithelial cells in intermediate position in TDUs could be related to the short-term label-retaining cell (ST-LRCs) as described by Woodward et al. (2005), or to undifferentiated large light cells (ULLCs) described in ultrastructural studies (Smith and Chepko, 2001). Moreover, Clarke et al. (2005) in some breast lobules found scattered CK19 positive epithelial cells, which rarely contained the proliferation-associated nuclear antigen Ki67, but that were frequently ( $98.5 \pm 0.6\%$ ) ER $\alpha$  positive. Unfortunately, we did not perform a colocalization with double *in situ* hybridization, but steroid receptor positive cells were also found in basal and luminal position of TDUs of prepubertal ewes. Clearly, staining *in situ* without further experimentation has the disadvantage of being merely correlative. The localization of ER $\alpha$  transcript before lambing and at lactation in mammary tissue disagrees with the immunolocalization data reported by Schams et al. (2003). The reason for this discrepancy could be related to the fact that ER $\alpha$  is regulated at post-transcriptional level (Chang et al., 2005). In fact, in rhesus monkey mammary glands, these authors found that ER $\alpha$  mRNA level was unchanged during the menstrual cycle, whereas ER $\alpha$  protein levels

decreased as estradiol levels increased.

In prepubertal mammary glands of ewes ER $\alpha$  positive cells were also observed in the stromal compartment. They appeared dispersed in the connective sheath between TDUs and adipose tissue without any specific pattern of distribution, and often they were arranged in little clusters. This finding is in agreement with the results of a recent study conducted on human adult breast indicating that ER $\alpha$  immunoreactivity was detected in stromal cells, as well as in the inner layer of epithelial cell nuclei in the acini and intralobular ducts (Li et al., 2010). Moreover, the presence of stromal cells positive to ER nuclear staining has been observed in all developmental stages of murine mammary gland (Fendrick et al., 1998), while in mammary glands of sheep these cells were noted just at prepubertal stage. The nature of these cells have not yet been definitively identified, they were believed to be undifferentiated mesenchymal cells (Haslam and Nummy, 1992) but, most recently, ER $\alpha$  positive cells within the bovine mammary fat pad of prepubertal heifers have been identified as adipocytes and fibroblasts (Meyer et al., 2007). Authors agreed that additional research is needed to completely characterize the relationship between stromal ER $\alpha$  expression and epithelial cell growth in cattle, and that epithelial cells expressing ER $\alpha$  may mediate proliferation of ER negative cells via paracrine mechanisms (Capuco et al., 2002; Clarke, 2006).

NKCC1 expression was significantly down regulated at all stages examined in comparison to prepubertal mammary glands. These data are in agreement with the expression pattern of NKCC1 mRNA assessed during mammary gland development in mice (Shillingford et al., 2002), suggesting that the level of NKCC1 mRNA is regulated in a developmental-specific manner. Sheep NKCC1 expression in our study is in agreement with the results of Shillingford et al. (2002) which showed down regulation of mice NKCC1 expression during pregnancy and lactation. The mRNA was localized in mammary tissue of sheep in basal and luminal layers of TDUs at prepubertal stage. At lactation, NKCC1 expression exhibited strong reaction in few alveolar cells, but surprisingly the staining was localized in the cytoplasmic apical side of the cells. On the contrary, immunolocalization studies demonstrated that NKCC1 protein was present at the basolateral membrane of ductal epithelial cells throughout virgin development and it regressed as mouse mammary development proceeded during pregnancy and lactation (Shillingford et al., 2002). However, it is known that a divergence in polarization of the NKCC cotransport process may reflect differences in apical/basolateral targeting due to tissue specific functionality of cotransporter activity (D'Andrea et al., 1996). In this regard, NKCC1 could be differentially activated within selected membrane domains in the different physiological stages (prepubertal vs lactation) of the sheep mammary gland. Gamba et al. (1993) have demonstrated different targeting sequences which

ultimately determine the surface distribution of cotransporters between various epithelia, suggesting that distinctive “secretory” and “absorptive” forms of the cotransporter may exist. In mammary glands these differences could reflect the physiological state of the gland (growth vs secretion).

It is known that proliferation is dependent on NKCC activity in fibroblasts and endothelial cells since inhibition of NKCC activity suppresses cell growth (Panet et al., 2000). Ion transport across the cell membrane participates in regulation of cell volume, which in turn is indispensable in cell cycle progression. The proliferative state of the cells has been recently correlated with activity of the NKCC cotransporter in prostate cancer PC3 cells (Hiraoka et al., 2010), although it has also been suggested that the expression of NKCC1 was associated with nonproliferative cells in mouse mammary glands (Shillingford et al., 2002). In this respect, we found a significant ( $P < 0.01$ ) correlation between NKCC1 expression data with those of Ki67 previously reported in ewes (Colitti and Farinacci, 2009a) and also with Msi1 data (Colitti and Farinacci, 2009b) which is associated with increased activity of Noct and Wnt signalling, the net result being the stimulation of mammary progenitor cell proliferation (Wang et al., 2008).

In summary, the present findings demonstrated a specific mRNA localization of CK19, NKCC1 and ER $\alpha$  in the developing mammary glands of ewes. At prepubertal stage CK19 and NKCC1 are expressed in epithelial cells of the inner and outer layer of TDUs, ER $\alpha$  is also expressed in stromal cells. NKCC1 is also localized at lactation in few alveolar cells. All genes are down regulated in adult mammary glands in comparison to prepubertal stage and significant correlation is found between CK19 and ER $\alpha$  expression, and also between NKCC1 and Ki67 and Msi1 expression data. Different questions arise from these data, especially if compared with those observed in rodents: is the localization of CK19 related to progenitor cells? Does NKCC1 have a different role in lactating glands of ewes? Have the stromal ER $\alpha$  positive cells a paracrine activity in the development of mammary tissue? These questions can only be answered by isolation of cell subpopulations of the mammary gland of sheep and by characterization of their morphogenic potential in physiological relevant culture models. Moreover, the strong histological similarities in ruminant and human mammary development indicate that sheep could be used as a model for mammary research in human, even though the demonstration of functional and biochemical similarities have to be investigated.

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