Italian Journal of Animal Science 2010; volume 9:e55

SHORT COMMUNICATION

Expression profile of caseins, estrogen and prolactin receptors in mammary glands of dairy ewes

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Abstract

In this study, we analyzed the developmental expression of estrogen receptors (*ESR1* and *ESR2*), prolactin receptors (*PRLR*) and casein genes (*CSN1S1, CSN1S2, CSN2* and *CSN3*) in the ewe mammary glands from prepubertal stage to involution. Using Real-time PCR we showed that the activation of casein genes transcription was up regulated during lactation and significantly down regulated before lambing and at involution in comparison to the expression measured in the prepubertal group. The highest expression of *ESR1* and *ESR2* genes occurred in prepubertal group compared to adult group. The *PRLR* expression of the short and long forms was up regulated before lambing and down regulated during lactation and involution.

Thus, the mRNA expression data for *ESRs* and *PRLR* show clear regulatory changes suggesting involvement of these receptors in sheep mammary glands during development to involution. Casein genes transcription could be primed through *PRLR* signal, but other factors may be necessary for milk protein long-term expression during lactation.

Introduction

In dairy sheep, genetic selection has caused deep morphological changes in the udder and physiological changes in the whole body of the animal. The former is identified by the higher mammary cistern volume, the latter by neuro-hormonal changes. These changes allow the alveoli to have a longer life span and maintain a metabolic status that favours the switch of energy and nutrients to the mammary gland instead of body reserves (Pulina *et al*., 2007). The pattern of the lactation curve is influenced by the number of secretory cells in the mammary gland at each day in milking (DIM) and by the synthetic activity of each secretory cell. Growth and differentiation of the glandular epithelium during puberty and pregnancy are important determinants of the total area of secretory epithelium and consequently of milk yield (Pulina *et al*., 2009).

These physiological changes are orchestrated by systemic and local factors, which control synthetic and secretory mechanisms by transcriptional control of key mammary genes (Stefanon *et al*., 2002).

In dairy species, it is generally believed that there is normally little mammary growth during established lactation (Akers 2002); however, in human breast the onset of secretory activity is accompanied by glandular-alveolar growth and expansion of acini (Battersby and Anderson, 1988). Moreover, in many tissues it exists a dividing transit population of cells in which signs of proliferation, phenotypic differentiation and functional differentiation are displayed simultaneously (Potten and Loeffler, 1990). In a recent paper, we reported for the first time the turnover of mammary cells and the interaction of their signals during the complete lactation cycle in sheep (Colitti and Farinacci, 2009); we concluded that mammary glands of dairy ewes seem to operate in a much more dynamic state than other lactating animals and this is particularly important in the construction of mechanistic models of lactation. In general, these models are based on the assumption that milk production at each time of lactation depends on the number of active cells and on the secretory activity (Dimauro *et al*., 2007). Therefore, mechanistic models of lactation could represent a useful tool to evaluate possible effect of selection for increasing lactation persistency in different breed and production scenarios. In a complementary paper (Pulina *et al*., 2009), based on the same experimental units, we concluded that the milk production around lactation peak (30 L) is sustained by the higher epithelium volume and higher milk secretion rate per secretory tissue unit.

The estrogen receptor-α (*ESR1*) 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 α and β (*ESR1* and *ESR2*, respectively), *ESR1* is considered the primary receptor for mammary gland development and function; it induces proliferation of the mammary tissue, but the mechanism is not clear, since the proliferating mammary cells do not contain this receptor (Clarke *et al*., 1997). Moreover, *ESR2* modulates *ESR1* action in tissues where they are co-expressed (Hall and McDonnell, 1999).

The role of prolactin in milk synthesis is probably related to the fact that it inhibits mammary apoptosis by suppressing the actions of IGF binding protein (*IGFBP-5*), which antagonizes the effects of IGF-I on the survival of mammary epithelial cells (Tonner *et al*., 2000). Proliferation and differentiation of secretory mammary epithelium are also dependent on the presence of the prolactin receptor (Ormandy *et al*., 1997) and the downstream Jak2-Stat5 pathway (Liu *et al*., 1998). The prolactin receptor (*PRLR*) belongs to the superfamily of cytokine receptors (Kelly *et al*., 1991) and exists in different isoforms, generated by alternative splicing, that are identical in their extracellular ligand-binding domain, but differ in the length and sequence of their intracellular domain (Bole-Feysot *et al*., 1998). cDNAs encoding a long and a short form of *PRLR* have been isolated from different species (Bignon *et al*., 1997; Shirota *et al*., 1990) and are differentially expressed in different tissues, suggesting that they can activate distinct signalling pathways (Schuler *et al*., 1997). The long form of *PRLR* actives Jak2, a cytoplasmic protein tyrosine kinase, which in turn can serve as docking sites for the SH2 domains in *STAT5* (Groner, 2002). Activated *STAT5* binds to DNA sites in the nucleus known as GAS elements and modulates the activity of target genes, as the β-casein gene (Kazansky *et al*., 1995; John *et al*., 1999). However, the lack of correspondence of *STAT5a* gene expression and β-casein gene expression suggests that *STAT5* activation may facilitate the interaction of other factors binding within composite response elements identified recently in the milk protein gene promoters. Responsive

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Key words: Mammary gland, Dairy sheep, Expression of casein, Estrogen receptors, Prolactin receptors.

Received for publication: 1 February 2010.
Accepted for publication: 5 May 2010.

Italian Journal of Animal Science 2010; 9:e55
doi:10.4081/ijas.2010.e55

elements are responsible for the stable expression of milk protein genes in terminally differentiated mammary epithelial cells (Kazansky *et al*., 1995). In lactating animals, *STAT5a* induces expression of milk protein genes, largely in response to prolactin (Nevalainen *et al*., 2002) together with laminin-1, which is a major basement membrane component required for milk protein expression (Streuli *et al*., 1995; Xu *et al*., 2009).

Moreover, in a recent paper it has been demonstrated that in ruminants, the increased milking frequency enhances the expression of the long and short isoform of prolactin receptors and β casein on the mammary epithelial cells and reduces cell apoptosis by modulating hormone sensivity (Bernier-Donner *et al*., 2010).

Caseins comprise a group of four proteins (αs1, αs2, β, and κ) resulting from the expression of four structural genes (*CSN1S1, CSN1S2, CSN2* and *CSN3*, respectively) (Bevilacqua *et al*., 2006). These proteins represent on average 82% of sheep milk Total Nitrogen (N x 6.38; Pulina and Nudda, 2004), but there is a large variability from one species to another (Miranda *et al*., 2004).

Since a surprisingly very high proliferation index, measured by Ki-67 immunostaining, was observed during lactation in mammary glands of Sardinian sheep (Colitti and Farinacci 2009), the goal of this study was to investigate in the same mammary tissues the expression of *CSN1S1, CSN1S2, CSN2* and *CSN3*, markers of functional differentiation. The expression patterns of *ESR1* and *ESR2*, and *PRLR* long and short forms, which play an active role in morphogenesis, growths and functional differentiation, were also studied in mammary glands of sheep prior to lambing to involution.

Materials and methods

Animals

Tissue was collected from mammary glands of thirty Sardinian sheep that were slaughtered at different developmental stages: prepubertal (30±5 days, group P), 10 days before lambing (group LateP), 30, 60, 150 DIM (groups 30L, 60L, 150L, respectively) and 8 days after the end of lactation (group 8IN). At each sampling periods, five animals were randomly selected from a flock of grazing sheep and a clinical examination was conducted in vivo to ascertain animal health and to exclude mastitis. Sardinian sheep are a breed primarily used to produce milk; the typical breeding system implies one lambing per year, with the mating season starting in late spring for mature ewes and in early autumn for maiden ewes and with lactation starting in autumn and in late winter, respectively. Dry-off occurs simultaneously in mid summer for yearlings and mature ewes (lactation length 150 DIM and 240 DIM, approximately) when nutritional value of pastures collapses due to lack of rain in this season. In this study milk yield ranged from 1600 g/d to 900 g/d at 150 DIM. Ewes at 30 DIM were allowed to suckle their lamb; the other groups (60L, 150L) were mechanically milked twice daily and manually ten minutes before slaughtering, therefore just before tissue collection.

Samples of tissue were collected in TRIzol® (Invitrogen, Milano, Italy), frozen in liquid nitrogen and kept one week at -80°C till RNA extraction. The experiment was carried out in accordance with state and local laws and ethical regulations (Italian Regulation, 1992).

RNA extraction and primer design

Total RNA was extracted from about 30 mg of mammary tissues using TRIzol® Plus RNA Purification System (Invitrogen, Milano, Italy), following the manufacturer's instructions. The concentration of the extracted total RNA was quantified using a spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo-Scientific, Wilmington, DE, USA) and the assessment of the purity of RNA samples ranged between 1.8-1.9 The 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, the β-actin (U39357) expression was used as an internal control, confirming thorough integrity of the RNA.

A Primer3 Input software (Rozen and Skaletsky, 2000) was used to design the primer sequences encoding for: *CSN1S1* (X03237), *CSN1S2A* (X03238), *CSN2* (X79703), *CSN3* (AY237637), PRLR long form (AF041257), *PRLR* short form (AF041977), ESR1 (AY033393), *ESR2* (AF177936) and *18S rRNA* (AY753190). Primers and product lengths for each gene are listed in Table 1 according to the HUGO Gene Nomenclature Committee.

Reverse transcription

Reverse transcriptions were performed with 2 μg of extracted total RNA by using Improm-II Reverse Transcriptase (Promega, Milano, Italy) as following described. Two micrograms of total RNA with 1 μL oligo(dT)18 primers (0.5 μg/μL MBI Fermentas, Italy) and nuclease

Table 1. Oligonucleotide primer sequences and reaction conditions for SybrGreen qRT-PCR.

Gene	Primer sets	Product length, bp	cDNA concentration, ng	Primers, nM
CSN1S1	F: 5'AGCACCAAGGACTCTCTCCA 3'			
	R: 5'CACTTGACGAACTGCTTCCA 3'	186	0.1	300
CSN1S2A	F: 5'AAGAACGCAGATGAAGAGGA 3'			
	R: 5 ATCCCATGGGTTCAAAACAA'3'	198	0.1	200
CSN2	F: 5' ACAGCCTCCCACAAAACATC 3'			
	R: 5'AGGAAGGTGCAGCTTTTCAA 3'	206	0.1	300
CSN3	F: 5'ATTTATGGCCATTCCACCAA 3'			
	R: 5' GCAATCGATTCTGAGGAAGC 3'	156	0.1	200
PRLR long	F: 5' TTCCCAGTGAAGGATACAAGC 3'			
	R: 5'GTTCTTTGGAGGGGTGTGG 3'	310	10	200
PRLR short	F: 5' TTCCCAGTGAAGGATACAAGC 3'			
	R: 5'CTATTAAAACACAGACACAAGG 3'	207	10	600
ESR1	F: 5' CCACGATCAAGTCCACCTTT 3'			
	R: 5'ACGGAACCGAGACGATGTAG 3'	193	10	200
ESR2	F: 5' TCTTTGCTCCAGACCTCGTT 3'			
	R: 5'GACTGTTGCTGGGAGGACAT 3'	292	50	300
18S rRNA	F: 5'AAACGGCTACCACATCCAAG 3'			
	R: 5' TCCTGTATTGTTATTTTTCGTCAC 3'	90	0.1	100
ACTB	F: 5' TCCCTGGAGAAGAGCTACGA 3'			
	R: 5' AGCACCGTGTTGGGATAGAG 3'	102	5	200

CSN1S1, α-S1-casein; *CSN1S2A*, α-S2-casein; CSN2, β-casein; *CSN3*, k-casein; *PRLR long*, prolactin receptor long form; *PRLR short*, prolactin receptor short form; *ESR1*, estrogen receptor α; *ESR2*, estrogen receptor β; 1*8SrRNA*, 18S subunit rRNA, *ACTB,* actin, β. F, forward primer; R, reverse primer.

free water to a final volume of 20 μL, 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 μL of Improm-II Reverse Transcriptase buffer (5X Promega, Milano, Italy), 1.2 μL $MgCl_2$ (50 mM), 1 μL of Improm-II Reverse Transcriptase and 1 μL of dNTP (10 mM) was added to the reaction and incubated at 37°C for 90 min and finally at 94°C for 5 min. The final concentration of cDNA was assumed as 100 ng/μL.

Standard curves analyses

For each gene, an aliquot of cDNA samples was 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 18S rRNA gene, which is known to be constitutively expressed (Robinson *et al*., 2007) and was retro transcribed also with 1 μL random hexamers (100 μM, MBI Fermentas, Milano Italy).

Real time PCR quantitation

Realtime PCRs were performed in triplicate form using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Milano, Italy). For these reactions, a master mix with the following components was prepared to the indicated end concentration: 1 μL of cDNA, 9.5 μL water, 1 μL of each primer and 12.5 μL of 2X Platinum SYBR Green qPCR SuperMix-UDG for a total volume of 25 μL. cDNA concentrations and primers molarities were different for each gene and determined with standard curves analyses performed before Real time PCR reactions. cDNA and primers concentrations are showed in Table 1.

PCR amplifications were 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 by the $2^{-\Delta\Delta Ct}$ method (Bustin, 2000; Pfaffl, 2001) where $2^{-\Delta\Delta Ct}$ represents the difference of a given target gene between each group before lambing and during lactation (groups from LateP to 8IN) vs. the group P. More precisely, individual ΔΔCt was calculated for each sample of group (LateP to 8IN) as ΔΔCt=ΔCt (sample group) – mean ΔCt (group P). The n-fold expression of a given target gene was calculated as $\log_2 (2^{-\Delta\Delta Ct})$ (Figure 1).

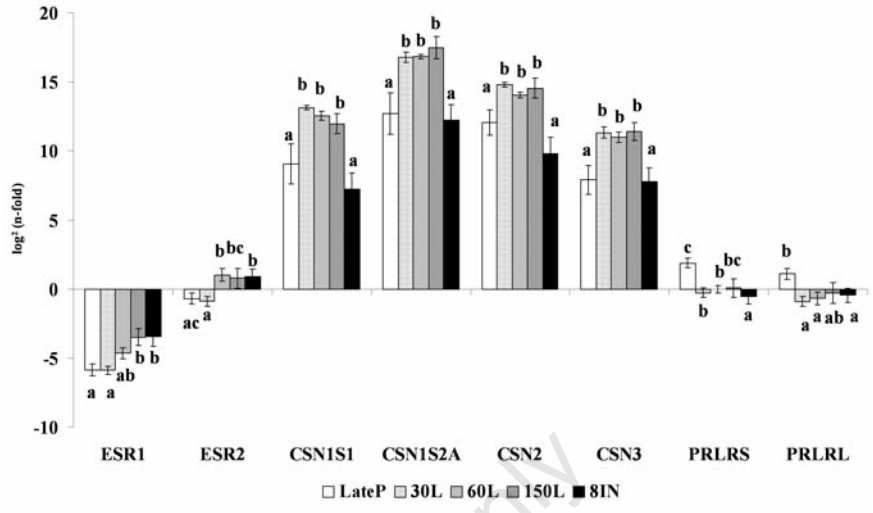

Figure 1. Expression analysis of target genes at 10 days before lambing, 30, 60, 150 days of lactation and 8 days from dry-off, related to the expression at prepubertal stage.

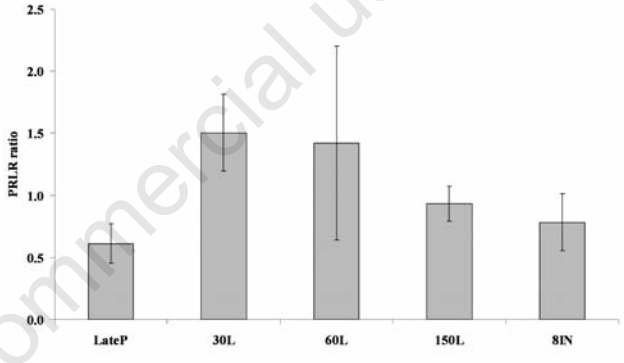

Figure 2. Ratio of the long to the short form of PRLR expression analysis at different developmental stages of ovine mammary glands.

Statistical analysis

All the recorded variables were submitted to analysis of variance using the ANOVA model to assess significant differences between groups; Duncan's least significant difference test was used to compare the means (SPSS Inc., 1997).

Results

18S rRNA expression was quantified in all samples and resulted in constant expression levels. No significant differences between the groups could be shown in the investigated ovine mammary tissues. Expression of *ERS1, ERS2, PRLRs* and caseins mRNA were normalised according to the relative *18S rRNA* expression of each sample.

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 (prepubertal).

The relative expression of *CSN1S1, CSN1S2A, CSN2* and *CSN3* genes in the sheep mammary glands indicated the same significant pattern of difference ($P<0.05$) among groups. These genes were down-regulated at LateP and 8IN and up-regulated during lactation (30L, 60L, 150L).

The relative transcription of *ESR1* significantly increased ($P<0.05$) from LateP to 8IN; the same parameter for *ESR2* significantly increased at 60L and remained constant and up regulated (even if not significantly for 150L) until 8IN ($P<0.05$). The *PRLR* expression of the short and long isoforms showed similar trend, being significantly up regulated

at LateP and down regulated during lactation and involution. Statistical analysis of the ratio between the long and the short form of *PRLR* did not significantly differ among groups, but showed a trend in which the mRNA encoding the long form of the ovine *PRLR* predominated on the short one, in every group of sheep (Figure 2).

Discussion

In a previous paper (Colitti and Farinacci, 2009), where cell turnover and gene activity in mammary gland of Sardinian sheep was evaluated, we suggested that sheep mammary glands seem to operate in a much more dynamic state than those of other domestic ruminants. In particular, to explain the high apoptosis to proliferation ratio we suggested that, as reported by Potten and Loeffler (1990), there was a dividing transit population of cells in which signs of proliferation, phenotyopic differentiation and functional differentiation were displayed simultaneously. This was in agreement with the results reported by Suzuki *et al*. (2000) that also found cells, in the breast tissue of pregnant women, positive to marker of proliferation, Ki-67 and to markers of mammary functional differentiation, β-casein and κ-casein. We evaluated markers of functional differentiation like *CSN1S1, CSN1S2A, CSN2, CSN3* by Real time PCR analyses.

In this study, we showed that activation of casein genes transcription, relatively to the prepubertal group (P), is up regulated during lactation and significantly down regulated before lambing and at involution. This is concomitant to the enhancement, although not significantly, of the ratio between the long and short form of prolactin receptors (Figure 2). This is in agreement with the data found by Cassy *et al*. (1998) in which they suggested that the short form of the ovine *PRLR* may have a dominant negative action in the activation of milk protein gene transcription. In fact, the authors reported that the activation of caseins gene transcription was concomitant with the enhancement of the ratio of the long to the short form of the ovine *PRLR*, which may play a key role in the shift between growth and differentiation of the mammary gland.

Compared to P group, the long form of *PRLR* is up regulated before lambing and this is in agreement with the trend of *STAT5a* expression that was lower during lactation and higher after the end of lactation (Colitti and Farinacci, 2009). As already reported, *STAT5a* expression resulted negatively related to that of lactalbumin, a major milk protein gene for ruminants, which significantly increased from lambing to lactation and it is also related to the expression of caseins. Therefore, in agreement with Kazansky *et al*. (1995) the activation of *STAT5a*, induced by *PRLR*, may prime milk gene expression, but other factors are necessary for milk protein long-term expression during lactation.

Our mRNA expression results demonstrated a high expression of *ESR1* and *ESR2* genes in prepubertal group. In fact, at the beginning of lactation period and during lactation (lactogenesis and galactopoiesis) the *n*-folds were significantly lower for *ERS1* gene and also for *ESR2* gene (Figure 1). These is in agreement with Schams *et al*. (2003), who found the presence of high *ERS1* and *ERS2* levels before the start of lobulo-alveolar development and *ESR1* significantly lower expression during pregnancy and lactogenesis. This is due to distinct regulatory mechanisms that involved the receptors, being the *ESR1* regulated at post-transcriptional level and *ESR2* at transcriptional level (Chang *et al*., 2005). In fact, the receptors present opposite expressions in presence of estradiol (Schams *et al*., 2003). This is in agreement with our results that showed a higher espression of *ESR2* during lactation. Moreover, *ESR2* is expressed, in mammary glands, not only in the luminal cells but also in myoepithelial and stromal cells, suggesting different roles for this gene within the glands (Speirs *et al*., 2002). In fact, the colocalization of *ESR1* and *ESR2* expressions with that of proliferation marker (Ki-67) could be remarkable to clarify the nature of cells in which they are expressed and the pathways by which hormones modulate proliferation.

These observations suggest a possible and important role of these receptors for the initiation of alveolar development, maybe in cooperation with proliferative growth factors.

Conclusions

This study, designed to investigate in mammary tissues the expression of markers of functional differentiation as αs1, αs2, κ, β caseins, the expression patterns of α and β receptors for estrogen and prolactin receptors long and short forms, showed that: i) activation of casein genes transcription, relatively to the P group, is upregulated during lactation and significantly downregulated before lambing and at involution; ii) a high expression of of α and β receptors for estrogen genes occurs in P group compared with adult group; iii) the prolactin receptor expression of the short and long isoform are upregulated at LateP in comparison with P group, and down-regulated during lactation and involution.

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