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Activin-related proteins in bovine mammary gland: Localization and differential expression during gestational development and differentiation

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ABSTRACT

Bovine mammary gland morphogenesis and differentiation are regulated by actions of growth factors including members of the transforming growth factor β superfamily. Activins A and B, which are members of the transforming growth factor β superfamily, bind selectively to ActRIB and ActRIIA receptors and their biological effects are antagonized by inhibins and follistatins. In the present paper we evaluated gene and protein expression of the activin and inhibin subunits βA, βB, and α-inhibin and follistatin and ActRIB and ActRIIA receptors in the mammary gland of nonpregnant and pregnant heifers. Mammary glands were obtained from nonpregnant Nelore (*Bos indicus*) heifers (n = 9) and from primigravid Nelore heifers during early (n = 9), mid (n = 6), and late (n = 5) pregnancy. Specimens of mammary tissue were analyzed by real-time PCR and immunohistochemistry. The βA and α-inhibin subunits and ActRIB and ActRIIA mRNA expression was higher in the early-pregnancy group compared with the nonpregnant group. In the mid-pregnancy group, the subunits βA, βB, and α-inhibin as much as follistatin mRNA expression was higher compared with the nonpregnant group, whereas ActRIB transcripts were absent in the late-pregnancy group. Immunostaining of these proteins, with the exception of ActRIB, was observed in the mammary tissue sections at all time points analyzed; these findings are in agreement with the observed pattern of mRNA expression. Staining and mRNA expression for ActRIB were undetected in the late-pregnancy group. In summary, the present study demonstrated that the activin-related proteins, βA, βB, and α-inhibin subunits, as much as follistatin and ActRIB and ActRIIA receptors display different patterns of expression regarding time of gestation in the bovine mammary gland. The modulation of the expression pattern during gestation suggests that activin-related proteins may play a key role in regulating bovine mammary branching morphogenesis and epithelial differentiation.

Key words: activin, inhibin, follistatin, mammary gland

INTRODUCTION

Transforming growth factor β (**TGF-β**) superfamily members play a key role in regulating mammary epithelium proliferation and are important mediators of mammary gland morphogenesis, development, and differentiation (Robinson and Hennighausen, 1997; Daniel et al., 2001; Akers, 2006; Manickam et al., 2008; Montesano et al., 2008; Bierie et al., 2009). The TGF-β superfamily contains more than 30 members including TGF-β, bone morphogenetic proteins, growth and differentiation factors, activins, and inhibins. These molecules regulate several cellular functions such as growth, adhesion, migration, apoptosis, and differentiation and have remained well conserved during evolution (Feng and Derynck, 2005). Activins in particular are vital for embryonic development and morphogenesis of most organs, including the mammary glands (Robinson and Hennighausen, 1997).

Activins and inhibins display 97% of homology among large domestic animal species (Phillips, 2005). Activins are dimers comprising 2 β subunits (βA or βB); therefore, 3 possible activins exist: A (βA/βA), B (βB/βB), and AB (βA/βB) (Muttukrishna et al., 2004). They bind initially to their type II receptor, ActRIIA or ActRIIB, which leads to the recruitment, phosphorylation, and subsequent activation of the type I receptor, ActRIB. Upon activation, ActRIB binds and then phosphorylates a subset of the cytoplasmic signaling proteins of the Smad family (Smad 2, Smad 3, and Smad 4), forming a complex that translocates toward the nucleus and modulates gene expression (Harrison et al., 2005; Schmierer and Hill, 2007). Importantly,

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activins bind selectively to the ActRIB and ActRIIA receptors; ActRIA and ActRIIB in turn, bind indiscriminantly to other TGF-β superfamily members (Lin et al., 2006; Komatsu et al., 2007). Activin bioactivity is regulated by various extracellular proteins such as inhibins and follistatins. Inhibins antagonize activins by binding to ActRII receptors and are formed by 1 α and 1 βA (inhibin A) or βB (inhibin B) subunit (Harrison et al., 2005; Bilezikjian et al., 2006). Whereas follistatin (**FS**) is an activin-binding single-chain glycoprotein that exists in 3 isoforms that differ in cell surface proteoglycans binding properties, FS-288 and FS-315 are formed by alternative splicing and FS-303 is generated from the proteolytic cleavage of FS-315 (Sidis et al., 2002; Schneyer et al., 2004; Saito et al., 2005).

In mice, activins and inhibins play a pivotal role in mammary gland organogenesis. Actually, βB subunit knockout mice do not produce milk. Instead, they exhibit impaired ductal branching and epithelium differentiation during gestation, and at parturition alveolar lumina fail to expand because of the absence of secreted milk (Robinson and Hennighausen, 1997). Moreover, activin-related proteins have been detected during nongestational and lactational mammary gland development in mice, with a growth regulatory role in lactation being suggested (Jeruss et al., 2003). Regarding the physiological functions of activins in the mammary gland, experiments performed in isolated cultured acini demonstrated that activin A inhibited cell proliferation and downregulated the expression of β-CN by blocking prolactin/signal transducer and activator of transcription 5 (STAT5) signaling, thus showing a role in modulating an important lactogenic pathway (Bussmann et al., 2004; Cocolakis et al., 2008). Such antiproliferative effect was also identified in human breast cancer cell lines, where activin A arrested cells in the G0/G1 (gap 0/gap 1) cell cycle phase (Liu et al., 1996; Burdette et al., 2005). Similarly, in the bovine species, in vitro experiments have demonstrated that TGF-β treatment has an inhibitory effect on bovine epithelial cell growth (Akers, 2006). Such effect might modulate branching morphogenesis because cultured mouse mammary epithelial tubules start branching at sites with minimum local concentrations of TGF-β growth factors (Nelson et al., 2006; Montesano et al., 2007). In accordance with an inhibitory growth effect, TGF-β1 transcripts are higher during developmental phases in which a great tissue remodeling is occurring in the mammary gland (Plath et al., 1997). Altogether, TGF-β superfamily members have been related to mammary growth and function and therefore are important growth factors that may influence milk production.

Based on the βB subunit knockout mouse model and previous activin expressional studies, along with the fact that TGF-β members are associated with normal development, differentiation, and function of the mammary gland, we hypothesize that activins, inhibins, and their related proteins are expressed in the bovine mammary gland. Therefore, we determined the expression profile of the activin-related proteins, βA, βB, and α-inhibin subunits, and follistatin and ActRIB and ActRIIA receptors in the developing mammary gland during different stages of pregnancy that are characterized by important morphophysiological changes that will determine milk production.

MATERIALS AND METHODS

Animals and Sample Collection

All tissue samples used in the present study were collected from healthy animals that were killed routinely in the local slaughterhouse (Betim, Minas Gerais, Brazil) in compliance with all the Ministry of Agriculture regulations for animal care.

Mammary tissue from beef Nelore heifers (*Bos indicus*; n = 29) was collected and subsequently processed. Tissue samples were collected from ovary-intact nonpregnant heifers that were showing signs of diestrus (age = 18–20 mo; n = 9). The identification of the stage of the estrous cycle was based upon the appearance of the corpus luteum (color, size, and vasculature) as described previously (Ireland et al., 1979). Mammary tissue of primigravid Nelore heifers, bred to purebred sires and carrying a single fetus, was also collected in 3 different phases: early pregnancy (d 60–120 of gestation; n = 9), a phase during which a massive ductal branching morphogenesis occurs; mid pregnancy (d 150–210 of gestation; n = 6), a phase during which an increased pattern of ductal branching and alveolar proliferation is in progress (Hovey et al., 1999, 2002); and late pregnancy (d 250–260 of gestation; n = 5), a phase during which the developed lobular-alveolar structures differentiate from a quiescent to a secretory stage (Hovey et al., 2002; Neville et al., 2002).

With the purpose of classifying the gestational age of the pregnant heifers so their mammary tissue could be collected, the fetal crown-rump length (**CRL**) was calculated by measuring the length from the fetal nose tip to the base of the tail. Results were graphically correlated with gestational age (Rexroad et al., 1974). Heifers carrying fetuses that displayed CRL between 9.3 and 27 cm were considered to be in the early-pregnancy stage. If the measurement of fetal CRL was between 45.4 and 78.6 cm, heifers were considered to be in the mid-pregnancy stage. To evaluate the late-pregnancy stage, mammary tissue was collected from heifers with fetuses with CRL between 104 and 117 cm. The Brazil-

Table 1. Summary of fetal crown-rump length and gestational age

Fetal crown-rump length	Mean (cm)	SEM	Mean calculated gestational age (d)	n
Early pregnancy (9.3–27 cm)	16.96	1.7	87.8	9
Mid pregnancy (45.4–78.6 cm)	51.10	1.3	184.3	6
Late pregnancy (104–117 cm)	107.84	1.3	282.8	5

ian Association of Zebu Breeds (ABCZ, Uberaba, Minas Gerais, Brazil) accepts a gestation length of 275 to 310 d for Zebu cattle breeds (Nasser et al., 2008), whereas the estimated gestational average for the Nelore breed has been described as 284.73 ± 3.65 d (Cavalcante et al., 2001). Summary of the fetal CRL and gestational age is listed in Table 1.

At slaughter, the mammary gland was immediately removed and dissected so 2 parenchymal samples from each animal could be collected from the right rear quarter. Subsequently, approximately 1 cm^3 of parenchymal tissue was frozen in liquid nitrogen and stored at −80°C for real-time PCR analysis. Samples for immunostaining were fixed overnight in 10% phosphate buffered formalin, dehydrated, and embedded in paraffin.

Real-Time PCR

Total RNA was extracted from mammary tissue by the acid guanidinium thiocyanatephenol-chloroform method (Trizol Reagent, Invitrogen, Carlsbad, CA) in a proportion of 1 mL/100 mg of tissue. Concentration of RNA was determined by measuring absorbance at 260 nm. The DNA contamination was removed by the use of DNase I, amplification grade (Invitrogen), and the SuperScriptTM III First-Strand Synthesis Super Mix Kit (Invitrogen) was used according to manufacturer's instructions to reverse transcribe 3 μg of total RNA. Integrity of RNA was evaluated by gel electrophoresis under denaturing conditions; intact bands were visualized by ethidium bromide staining. All PCR primers were designed with the Real Time PCR Primer Design program (Genscript Corp., Piscataway, NJ) based on sequences obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/Database/index.html; Table 2). Primers were designed to span intron-exon borders whenever possible in order to avoid any accidental amplification of genomic DNA.

Real-time PCR was performed using ABI Prism 7000 SDS (Applied Biosystems, Carlsbad, CA) in a final volume of 25 μL and all samples were run in duplicate. Also, 1.7 μL of cDNA was added to 2 μL of each primer plus 17 μL of SYBR Green Master Mix (Applied Biosystems) and completed with 4.3 μL of RNase-free water. Control reactions were performed omitting either the reverse transcriptase enzyme or template RNA to test for contamination with genomic DNA or nonspecific amplification. The PCR runs were programmed as follows: stage 1, 1 cycle of 52°C for 2 min; stage 2, 1 cycle of 95°C for 10 min; stage 3, 40 cycles of 95°C for 15 s and 50°C for 1 min. Expression levels of β-actin were used as endogenous controls within each sample (Suchyta et al., 2003; Li et al., 2006) given that target and housekeeping genes had similar slopes. Positive controls consisted of cDNA from bovine ovary that is known to express activin-related proteins (Izadyar et al., 1998). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method, where CT is cycle threshold (Livak and Schmittgen, 2001).

Statistical Analysis

For real-time PCR analysis, the ΔCT values obtained for different stages of mammary gland development

Table 2. Oligonucleotide sequences used for real time PCR amplification

Gene	Oligonucleotide	Sequence 5′→3′	bp	GenBank no.[1]
βA subunit[2]	Sense	ggaatcagcacagccaggaa	117	NM_174363
	Antisense	tcctgttggccttgggaact		
βB subunit[2]	Sense	cagcttcgccgagacagatg	98	NM_176852
	Antisense	ctggcctgcaccacaaacag		
α-Inhibin subunit	Sense	cacgtatgtgttccagccat	86	NM_174094
	Antisense	gtctgtccagtcctgtgtgg		
Follistatin	Sense	tttctgtccaggcagctcta	126	L21716
	Antisense	gtcactccatcattcccaca		
ActRIB	Sense	gcatcaagaagaccctctcc	137	AY185302
	Antisense	agaggtaggcctccatcgta		
ActRIIA	Sense	aaagtttgaggctggcaagt	108	U43208
	Antisense	gcatccctttggaagttgat		
β-Actin	Sense	agcagatgtggatcagcaag	82	AY141970
	Antisense	taacagtccgcttagaagca		

[1]Source: http://www.ncbi.nlm.nih.gov/Database/index.html

[2]Primers spanning intron-exon borders.

were normally distributed and therefore expressed as means ± standard deviation and compared using 1-way ANOVA followed by Student-Newman-Keuls post hoc test for multiple comparisons. The relative mRNA concentrations are summarized as fold increase over the nonpregnant group ($2^{-\Delta\Delta CT}$; Yuan et al., 2006).

Immunohistochemistry

Paraffin-embedded bovine mammary tissue samples were sliced into 6-μm serial sections and mounted on gelatinized slides. The sections from all animals were run at the same time for each antibody. After deparaffinization and rehydration through graded series of ethanol, endogenous peroxidases were then quenched with 3% (vol/vol) hydrogen peroxide solution in absolute methanol (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature. After being washed in PBS (Sigma-Aldrich), slices were incubated with normal rabbit or normal horse serum (Vector Laboratories Inc., Burlingame, CA) for 1 h to block nonspecific binding sites. Slides were then incubated with the primary antibodies in 0.1% BSA/PBS (Sigma-Aldrich) at 4°C overnight.

The primary antibodies for βA subunit, follistatin, ActRIB, and ActRIIA were kindly donated by Wylie Vale (Salk Institute, La Jolla, CA) and were used as described previously (Ciarmela et al., 2004; Florio et al., 2005; Torres et al., 2007; Ferreira et al., 2008). They were all rabbit polyclonal antisera raised against human antigens; however, activin-related proteins display 97% of homology between human and large domestic species (bovine included), which allowed us to use them in this study (Phillips, 2005). Aliquots of lyophilized whole serum were reconstituted to the original volume with distilled water and used at a final dilution 1:200 or 1:400 (for anti-βA subunit) in PBS. The primary antibodies for βB and α-inhibin subunits were affinity purified goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) used at final dilution 1:200 and final concentration 1 μg/mL.

Between each step, sections were washed in PBS for 10 min. All incubations were carried out in humidified chambers to prevent evaporation. Subsequently, biotinylated secondary antibodies were added to the slices and incubated for 30 min followed by peroxidase-streptavidin incubation in ABC reagent (Vector Laboratories Inc.). The immunolocalization of the primary antibodies was determined with the addition of diaminobenzidine (Sigma-Aldrich) that stained the reacting sites in a brownish color. Tissue slices were counterstained with hematoxylin (Sigma-Aldrich). Negative control slices were incubated with normal serum instead of primary antibodies (for each specific antibody) and human mammary tissue was used as positive control (Reis et al., 2002; Burdette et al., 2005; Bloise et al., 2009).

Assessment of Staining

Images were acquired and analyzed on an Olympus BX-40 imaging microscope (Olympus, Center Valley, PA) and captured by a Spot camera (version 3.4.5; Sterling Heights, MI) and Corel Draw software (version 7.468; Corel Inc., Mountain View, CA). Sections were examined at 400× magnification by 3 different researchers who were blinded to groups. The percentage of epithelial and stromal cells with positive staining was graded semiquantitatively as described previously (Bloise et al., 2009; Lavalle et al., 2009) on a scale of 0 to 3 arbitrary units as follows: 0 = less than 5% staining; 1 = 5 to 10% staining; 2 = 10 to 50% staining; and 3 = more than 50% of the cells stained positively. The possible association between staining scores and gestational age (surrogated by CRL) was assessed by nonlinear regression analysis (Prism 4.0, GraphPad, La Jolla, CA).

RESULTS

To address the question of whether activin-related proteins are expressed in the bovine mammary tissue at multiple phases of gestational mammary development and differentiation, we examined βA, βB, and α-inhibin subunits, follistatin, and ActRIB and ActRIIA receptors mRNA and protein expression using real-time PCR and immunohistochemistry. Their transcripts profiles are shown in Figure 1 and their tissue localization is shown in the Figure 2. Real-time PCR control reactions were negative, ruling out genomic DNA contamination. The mammary tissue of nonpregnant heifers displayed profuse adipose tissue with small rudimentary branched structures, as expected, whereas the mammary parenchyma in primigravid heifers progressed from organized undifferentiated ductal units to enlarged lobuloalveolar glands. Positive control slices showed the expected staining, whereas the negative control tissue sections showed no detectable staining (data not shown).

Activin and Inhibin Subunit mRNA and Protein Expression

The mRNA expression of βA and α-inhibin subunits was significantly higher during early pregnancy (3.6- and 2.9-fold increase over nonpregnant group for βA and α-inhibin subunits, respectively; $P < 0.05$) and mid pregnancy (4.3- and 3.0-fold increase; $P < 0.05$). Conversely, βB subunit transcripts were higher only in

the mid-pregnancy group (2.3-fold increase; $P < 0.05$; Figure 1).

Activin and inhibin subunits immunostaining was cytoplasmic and nuclear. Signals of βA subunit staining were present mainly in luminal epithelial cells in nonpregnant heifers (Figure 2A), and the area of βA subunit immunostaining increased across gestation (Table 3).

On the other hand, βB and α-inhibin subunit signals were present in cells surrounding luminal epitheliums in nonpregnant heifers (Figures 2E and I). In mammary glands from pregnant heifers, however, staining signals were found mainly in luminal epithelial cells and stromal region but not in cells surrounding luminal epithelial cells (Figure 2F–H and J–L). Similar to βA subunit, the area of immunostaining for the subunits βB and α-inhibin increased across gestation (Table 3). The increase of all activin and inhibin subunits across gestation was confirmed by nonlinear regression analysis, showing a positive association between the stained area for βA, βB, and α-inhibin subunits and the CRL ($R^2 \approx 0.70$ for each of the 3 subunits vs. CRL).

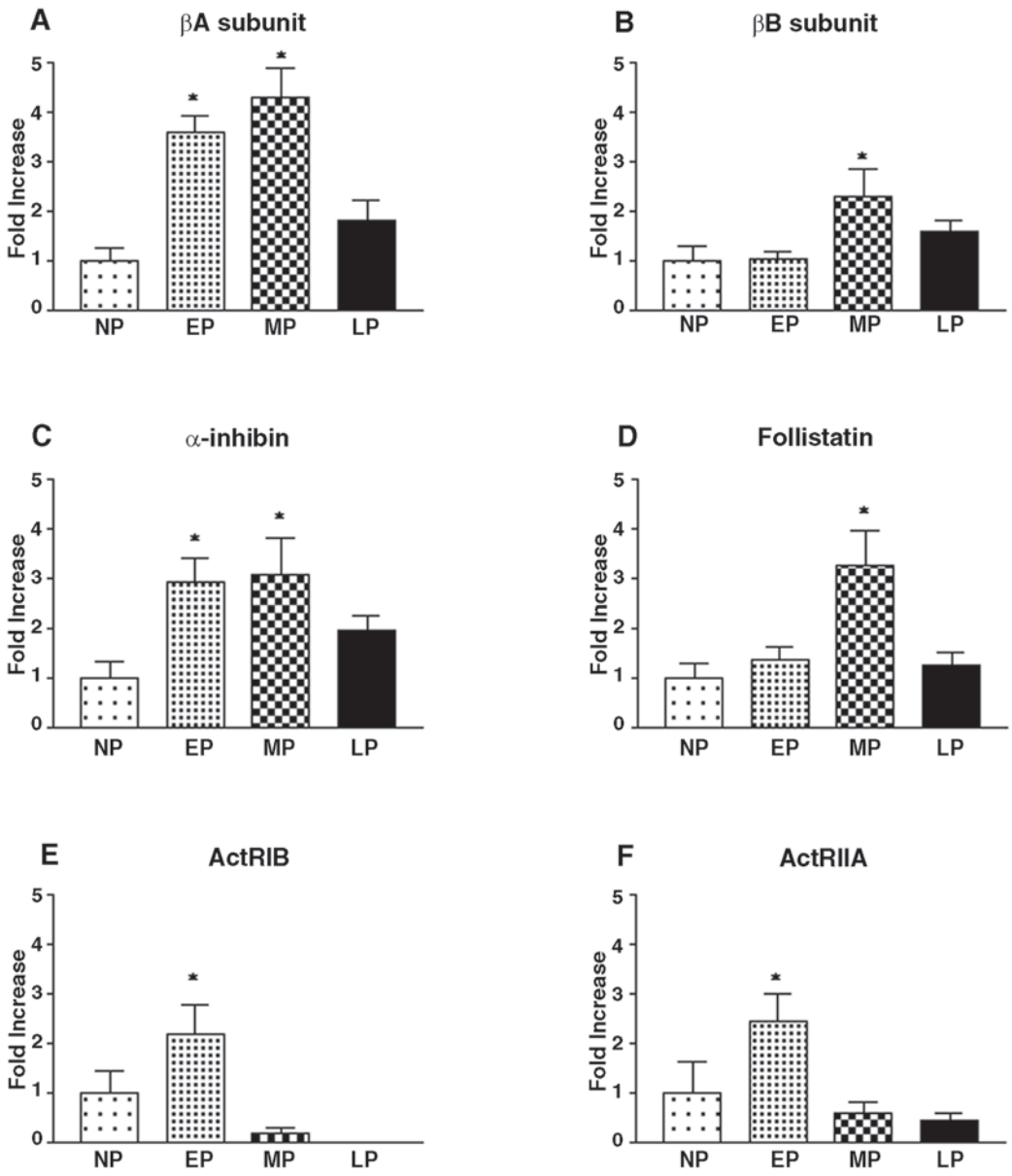

Figure 1. Messenger RNA expression of βA (A) and βB subunits (B), α-inhibin subunit (C), follistatin (D), and activin receptors ActRIB (E) and ActRIIA (F) in the bovine mammary gland in nonpregnant (NP) heifers and primigravid heifers during early pregnancy (EP), mid pregnancy (MP), and late pregnancy (LP). $^*P < 0.05$ compared with NP stage (1-way ANOVA and Student-Newman-Keuls test).

Follistatin mRNA and Protein Expression

Follistatin mRNA expression showed a significant increase during mid pregnancy (3.7-fold increase; $P < 0.05$; Figure 1D). Immunostaining for follistatin was present both in the cytoplasm and nucleus at all stages investigated and staining signals were present mainly in luminal epithelial cells in nonpregnant heifers (Figure 2M) and during all gestational phases analyzed (Figures 2N–P). Follistatin area of immunostaining to the same

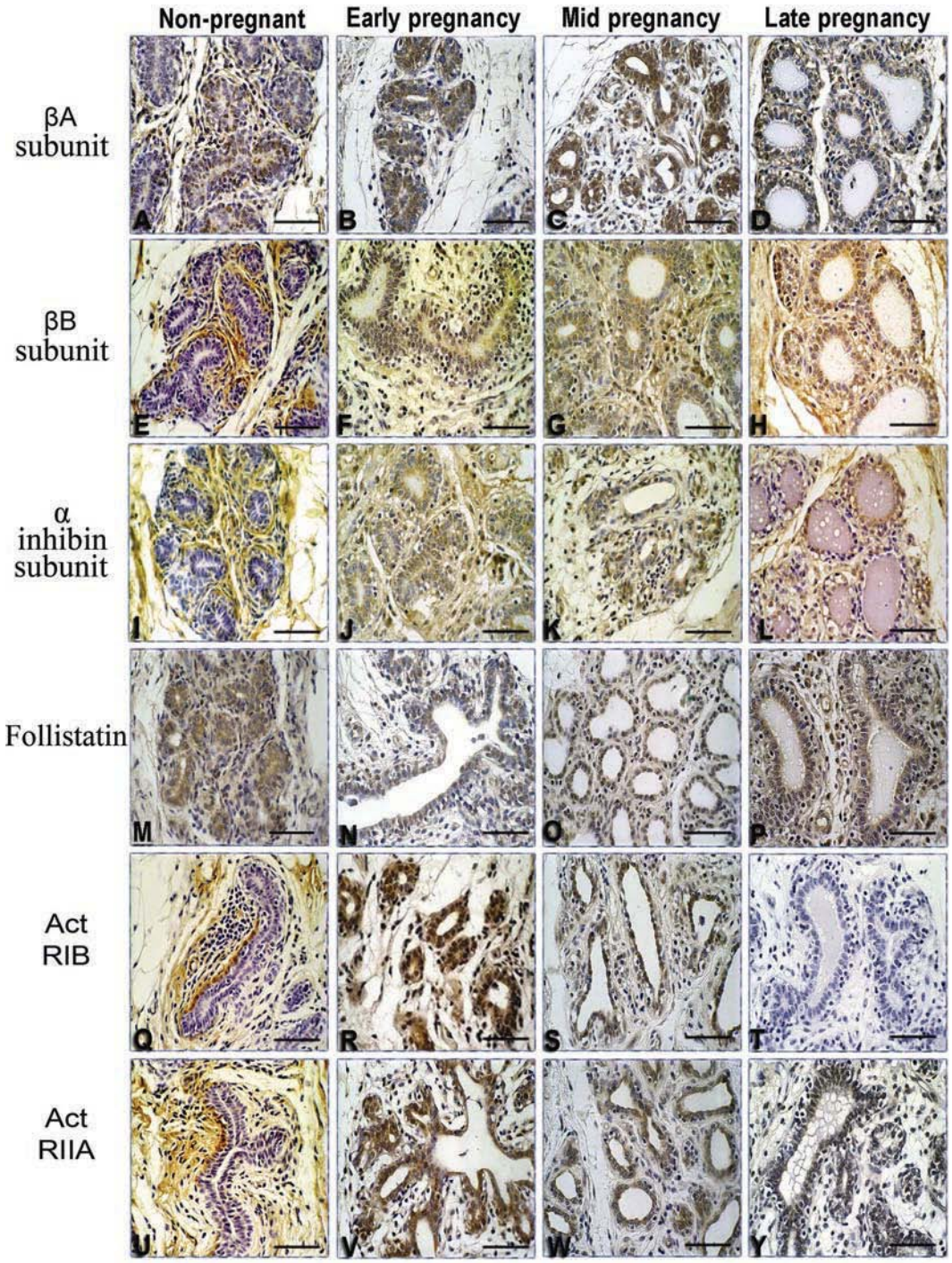

Figure 2. Immunohistochemistry for βA (A–D), βB (E–H), and α-inhibin (I–L) subunits, follistatin (M–P), ActRIB (Q–T), and ActRIIA (U–Z) in the bovine mammary gland in nonpregnant heifers and in primigravid heifers during early, mid, and late pregnancy. Bar = 50 μm. Color version available in the online PDF.

Table 3. Summary of activin-related protein area of immunostaining for the mammary gland of nonpregnant (NP) heifers and primigravid heifers during early pregnancy (EP), mid pregnancy (MP), and late pregnancy (LP)[1]

	Epithelial cells				Stromal cells			
Antibody	NP	EP	MP	LP	NP	EB	MP	LP
βA subunit	+	++	+++	+++	+	++	++	++
βB subunit	+	++	+++	+++	+	++	++	++
α-Inhibin subunit	+	++	+++	+++	+	++	++	++
Follistatin	+	++	++	+ ++	+	++	++	++
ActRIB	+	++	++	−	+	++	++	−
ActRIIA	+	++	++	+	+	++	++	+

[1]Area of immunostaining was graded as follows: less than 5% staining (−, score 0); up to 10% (+, score 1); 10–50% (++, score 2); or more than 50% (+++, score 3). The table shows the median values for each protein evaluated.

extent as for activin and inhibin subunits increased across the gestational progression (Table 3).

Activin Receptor mRNA and Protein Expression

Transcripts of ActRIB and ActRIIA were both significantly higher in the early-pregnancy group (2.2- and 2.6-fold increase, respectively; $P < 0.05$; Figures 1E and F); ActRIB mRNA was not detected in the late-pregnancy group (Figure 1E). In the mammary tissue of nonpregnant heifers, activin receptors were localized mostly in the cytoplasm of the luminal epithelial cells (Figures 2Q and U); during gestation, they were present in both cytoplasm and nucleus of the epithelial cells and in the cytoplasm of the stromal cells. Signals were localized mainly in the luminal cells during gestation (Figures 2R–S and V–W), whereas, in accordance with the transcripts analysis, no staining signal was found for the ActRIB receptor in the late-pregnancy group (Figure 2T).

The ActRIB area of immunostaining increased in early- and mid-pregnancy stages and was absent in the late-pregnancy stage (Table 2). The ActRIIA area of staining followed the same pattern as described for ActRIB but was present in less than 10% of the epithelial cells during late pregnancy (Tables 2 and 3).

DISCUSSION

The present study showed for the first time the expression of activin-related proteins in the mammary gland of nonpregnant and pregnant heifers during early, mid, and late pregnancy. Activin-related proteins mRNA expression was higher in the mammary tissue of early- and mid-pregnant animals, suggesting a role for activin in the establishment of the mammary tree-like structure. Their staining pattern demonstrated that they are expressed mainly in luminal epithelial cells across gestation. The proportion of luminal epithelial cells increases during gestational processes and achieves full development during late pregnancy, which could explain, at least in part, their increased mRNA expression observed in early or mid pregnancy or both. However, activin-related proteins expression is down-regulated during late pregnancy even though their area of immunostaining is present in more than 50% of the cells. These data suggest that an increased stained population of luminal epithelial cells is not responsible for an increased pattern of mRNA expression found in the early- and mid-pregnancy stages, raising the question of how those growth factors are regulated in the bovine mammary gland across gestation.

Mammary activin-related proteins expression could be influenced by hormonal changes that take place during gestation. Pregnancy steroid hormones such as estrogen and progesterone coordinate multiple stages of mammary growth, differentiation, secretory activity, and involution (Connor et al., 2007). In cattle, blood serum estrogen levels increase approximately 10-fold 1 mo before parturition, with the most dramatic increase occurring during the last 5 d of gestation (Barth et al., 1978). Progesterone levels, in turn, are higher throughout gestation and decline about 10 d before parturition (Stabenfeldt et al., 1970). Estrogen plays a role in regulating activin's downstream pathways, as shown in MCF7 breast cancer cells, where a crosstalk between estrogen and activin has been demonstrated (Burdette and Woodruff, 2007). In fact, estrogen and activin signaling cascades intersect and repress each other's transcription pathways. Additionally, estrogen is able to reduce the amount of βB subunit mRNA and protein produced by the same cells (Burdette and Woodruff, 2007). Altogether, in the bovine mammary gland, estrogen may play a role regulating βA and βB subunits expression because, during gestation, both are down-regulated in stages where estrogen levels are increased the most. Progesterone in turn plays a tissue-specific role in regulating βA subunit expression. In cultured

human endometrial stromal cells, βA subunit mRNA expression is upregulated after progesterone exposure (Florio et al., 2007), whereas in the myometrium of ovariectomized and steroid-replaced rats, progesterone decreased βA subunit mRNA expression (Ciarmela et al., 2009). However, here we demonstrate that during gestational phases in which blood progesterone levels are present in high concentrations, βA and βB subunits are overexpressed, suggesting then a role for progesterone in regulating βA and βB subunits expression in the mammary tissue of pregnant animals. Therefore, steroid hormones might regulate expression of activin-related proteins in the mammary gland, a hypothesis that will require further investigation.

The observation that βB subunit pattern of mRNA expression does not follow those from βA and α-inhibin subunits (i.e., βB subunit transcript levels are higher only in mid-pregnant animals) suggests that activins and inhibins A and B may play a different regulatory role during epithelial branching and proliferation.

Regarding activins physiological antagonists, α-inhibin subunit mRNA expression was increased in early- and mid-pregnant animals, whereas follistatin was increased only in mid-pregnant animals. The observation that α-inhibin subunit and follistatin staining localization matched those from βA and βB subunits (i.e., the staining signals were mainly found in luminal epithelial cells during all gestational phases analyzed) suggests a potential interaction of these regulatory factors in regulating mammary growth. Furthermore, these findings suggest that both inhibins and follistatin may function as activin antagonists in different time points during mammary epithelial proliferative processes.

Recently, it has been described that follistatin is overexpressed in breast proliferative diseases such as breast fibroadenoma, demonstrating a role in regulating mammary gland proliferation (Bloise et al., 2009). Moreover, follistatin also binds and neutralizes another TGF-β superfamily growth factor member named myostatin (Hill et al., 2002). This molecule regulates skeletal muscle mass (Lee and Mcpherron, 2001) and its expression has been characterized in different stages of porcine mammary gland development (Ji et al., 1998). In bovine adipocytes, activins and myostatin impair preadipocytes differentiation (Hirai et al., 2007), which is essential for the growth of the mammary tree-like structure (Hovey et al., 1999; Hinck and Silberstein, 2005).

CONCLUSIONS

The present study provides the first report of the activin-related proteins presence in the bovine mammary gland. Different patterns of expression during bovine gestational mammary gland development and differentiation were detected and their tissue and cellular localization changed depending on the protein and on the gestational phase analyzed. The modulation of the expression levels suggests a role for activins in regulating mammary branching morphogenesis and epithelial differentiation, which could be highly relevant to the molecular mechanisms leading to milk production.

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