To increase our understanding of the mechanisms by which growth hormone (GH) and insulin-like growth factor (IGF)-I influence bovine mammary gland development, the potential roles of T-box2 (TBX2) and T-box3 (TBX3) were investigated. Although no information regarding expression of either transcription factor in the bovine mammary gland exists, it is known that TBX3 and its closely related family member, TBX2, are required for mammary gland development in humans and mice. Additionally, TBX3 mutations in humans and mice lead to ulnar mammary syndrome. Evidence is present in bone that TBX3 is required for proliferation and its expression is regulated by GH, an important regulator of mammary gland development and milk production. We hypothesized that TBX2 and TBX3 are expressed in the bovine mammary gland, and that GH, IGF-I, or both increase TBX2 and TBX3 expression in bovine mammary epithelial cells (MEC). Bovine mammary gland tissue, MAC-T cells, primary MEC, and fibroblasts were obtained and TBX2 and TBX3 expression was determined by real-time reverse transcription PCR. In addition, TBX2 and TBX3 expression was examined in cells treated with 100 or 500 ng/mL of GH or 100 or 200 ng/mL of IGF-I for 24 or 48 h. Both TBX2 and TBX3 were expressed in bovine mammary tissue. Surprisingly, expression of TBX2 was only detected in mammary fibroblast cells, whereas TBX3 was expressed in all 3 cell types. Growth hormone did not alter TBX3 expression in MAC-T cells or MEC. However, IGF-I increased TBX3 expression in MAC-T, but not in primary MEC. We did not observe a change in TBX2 or TBX3 expression in fibroblasts treated with GH and IGF. Therefore, we concluded that (1) TBX2 and TBX3 are expressed in bovine mammary gland, (2) their expression is cell-type specific, and (3) IGF-I stimulates TBX3 expression in MAC-T cells.

Key words: MAC-T, mammary gland, T-box2, T-box3

Mammary gland development is a complex process regulated by several factors, including hormones, growth factors, and transcription factors. Evidence exists in humans and mice that T-box3 (TBX3) is required for mammary gland development (Carlson et al., 2002; Platonova et al., 2007). During embryonic development, TBX3 is a constitutive gene involved in the establishment of the rudimentary mammary gland (Hens and Wysołomski, 2005). Specifically, TBX3 is needed for the formation of the mammary placodes from the mammary line of the developing fetus. Ulnar mammary syndrome, which results from a haploinsufficiency of the TBX3 gene, alters placode development, resulting in hypoplasia of the mammary gland (Carlson et al., 2002; Platonova et al., 2007). Alternately, TBX2 is expressed in the mammary mesenchyme, which will become the stroma of the mammary gland (Rowley et al., 2004). It is clear that these transcription factors are important in embryonic development and the mammary gland in humans and mice, but it is not known if they are expressed in the bovine mammary gland or what role they play in mediating key growth factors required for bovine mammary gland growth and lactation.

Growth hormone (GH) and IGF-I are essential for mammary gland growth and development. Growth hormone is important for ductal development and milk production by partitioning nutrients to the mammary gland for lactation (Bauman, 1999; Kleinberg and Ruan, 2008). However, within the mammary gland, many of the actions of GH are mediated by IGF-I (Kleinberg and Ruan, 2008). Insulin-like growth factor-I regulates cell cycle, increases proliferation, regulates hypertrophy and prevents apoptosis of mammary epithelial cells (MEC; Cohick, 1998; Akers et al., 2000). Changes in
concentrations of IGF-I occur during different stages of mammary development, and these changes are positively correlated with MEC proliferation (i.e., pregnancy and lactation; Baumrucker and Erondu, 2000). Therefore, based on the knowledge that TBX2 and TBX3 are important regulators of mammary gland development, TBX3 expression is stimulated by GH in bone (Govoni et al., 2006), and many of the effects of GH are mediated by IGF-I in the bovine mammary gland, we hypothesized that TBX2 and TBX3 are expressed in the bovine mammary gland and that GH, IGF-I, or both increase TBX2 and TBX3 expression in bovine MEC. To test this hypothesis, mammary tissue was obtained from mammary parenchymal tissue of 3 lactating dairy cattle at slaughter. Tissue isolated for RNA extraction was excised, snap-frozen in liquid nitrogen, and stored at −80°C. Isolation of primary MEC and fibroblasts was performed as described herein.

The MAC-T cells, an immortalized cell line derived from bovine MEC (Huynh et al., 1991), were used for cell culture experiments. Primary MEC were isolated and cultured as previously described (Wellnitz and Kerr, 2004). Specifically, immediately following slaughter, mammary gland parenchymal tissue (100 to 200 g) was excised and transported to the laboratory on ice. In order to remove the fat and connective tissue from mammary parenchymal tissue of 3 lactating dairy cattle, tissue was ground in a mortar cooled with liquid nitrogen before the addition of 1 mL of TriReagent (Sigma-Aldrich). Genomic DNA was removed from samples using a Turbo DNA Free kit (Ambion, Foster City, CA). The quality of RNA was determined by using an Experion analysis system (Biorad, Hercules, CA).

Reverse transcription (RT) PCR was performed using 300 ng total RNA with OligodT primer (Ambion) and master mix containing 5.5 μL of 5× Buffer (Invitrogen), 1.0 μL of dNTP (Promega, Madison, WI), 2.0 μL of DTT, and 0.5 μL of Superscript II (Invitrogen) for a total reaction volume of 20 μL. The samples and master mix underwent a standard RT protocol starting at 70°C for 10 min, then 4°C for 20 min, 37°C for 3 min, 42°C for 1 hr, 4°C for 3 min, and 90°C for 2.5 min.

Primers were designed using Primer3 and National Centers for Biotechnology Information BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), validated as previously described (Govoni et al., 2006), and synthesized by Integrated DNA Technologies (Table 1). Primers were validated by confirming product size in an agarose gel, efficiency of amplification, annealing temperature, and performing a BLAST search. For traditional PCR, products were amplified using GoTaq Green Master Mix (Promega) under standard thermal cycling conditions (Stage 1: 94°C for 3 min; stage 2: 94°C for 30 s; 60°C for 1 min; 72°C for 1 min for 40 cycles; stage 3: 72°C for 5 min and 10°C forever) and run on a 2% agarose gel. For real-time RT-PCR, ribosomal protein subunit 15 (RPS15) was used as the endogenous control gene (Bionaz and Loor, 2007). In addition to determining TBX2 and TBX3 expression, IGF-binding protein 3 and 5 (IGFBP3 and IGFBP5) expression were determined to confirm the effectiveness of IGF-I and GH treatment, respectively. Real-time RT-PCR was performed using Power SYBRgreen Master Mix (Invitrogen) and the ABI 7900 HT Fast Real-time PCR machine (Applied Biosystems, Foster City, CA).

The total volume of the reaction mixture was 25 μL, containing 5 μL of cDNA, 3 μL of nuclear-free water,
1 μL each of 10 nM forward and reverse primer, and 10 μL of SYBRgreen. For the TBX3 primer, 0.5 μL of each forward and reverse primer was used. Real-time RT-PCR was performed using standard cycling conditions (Stage 1: 50°C for 2 min and 95°C for 10 min; stage 2: 95°C for 15 s and 60°C for 1 min for 40 cycles; stage 3: 95°C for 15 s and 60°C for 15 s with a 2% ramp to 95°C for 5 min). The ΔCt values were obtained and were used to calculate the ΔΔCt values to determine relative gene expression (Livak and Schmittgen, 2001). Changes in gene expression are expressed relative to the control and these values are presented in the figures and text.

Statistical analysis of gene expression was performed using the ΔCt values for control and treatment groups. Controls were presented as relative to average of all controls to provide a mean and standard error for figures. Data were analyzed by using the PROC MIXED program in SAS (Version 2.9, SAS Institute Inc., Cary, NC). Treatment mean comparisons were performed using least squares means. All experiments for MAC-T cells were repeated 3 times. Similar findings were observed; therefore, 1 experiment was used for presentation in the figures. For primary MEC and fibroblasts, data were analyzed including animal as a variable. Statistical significance was considered at P ≤ 0.05.

T-box3 was expressed in whole tissue from the bovine mammary gland, as well as individual cell types from within the gland (primary MEC and fibroblast cells; Figure 1A). In addition, TBX3 was expressed in the MAC-T cell line (Figure 1A). Although TBX2 was detected in the bovine mammary gland tissue, it was not present in primary MEC or the MAC-T cell line (Figure 1A). However, TBX2 was detected in mammary gland fibroblasts (Figure 1A). T-box3 expression was detected in the bovine mammary gland, within all cell types, and TBX3 expression was 2.4-fold greater in the MAC-T cells than mammary gland tissue (P = 0.02; Figure 1B). T-box2 was expressed in the whole mammary gland tissue and fibroblast cells, but not observed in the primary MEC or MAC-T cells (Figure 1C). Based on these findings, MAC-T cells and primary MEC were used to determine the effects of GH and IGF-I on TBX3 expression and mammary gland fibroblasts were used to evaluate the effects of GH and IGF-I on TBX2 gene expression.

In MAC-T cells, GH treatment (100 and 500 ng/mL) did not alter TBX3 gene expression at 24 or 48 h (Figure 2A; P = 0.73). Insulin-like growth factor-I treatment (100 and 200 ng/mL) increased TBX3 gene expression 1.49 ± 0.12- and 1.45 ± 0.10-fold, respectively at 24 h (Figure 2B; P ≤ 0.05), but no change was observed at 48 h. To confirm the efficacy of our treatments, expression of IGFBP3 and IGFBP5, which are known to be responsive to IGF-I and GH, respectively, were determined (Mohan et al., 2003; Fleming et al., 2005). Expression of IGFBP3 increased 2.55 ± 0.21- and 2.65 ± 0.18-fold in response to IGF-I treatment at 100 and 200 ng/mL, respectively, at 24 h, and 3.24 ± 0.41- and 5.25 ± 0.42-fold, respectively, at 48 h (P ≤ 0.05). At 24 h, GH100 and GH500 did not alter IGFBP5 (P ≥ 0.10). At 48 h, GH100 reduced IGFBP5 expression 1.40 ± 0.06-fold (P = 0.02), but GH500 did not alter IGFBP5 expression (P = 0.23).

In primary MEC, GH treatment (GH100 and GH 500) did not alter TBX3 expression (Figure 3A; P = 0.68). Treatment with IGF-I (IGF100 and IGF200) did
not change \( TBX3 \) expression at 24 and 48 h (Figure 3B; \( P = 0.16 \)). To confirm the effectiveness of GH and IGF-I treatments, \( IGFBP \) expression was determined. Similar to MAC-T cells, IGF-I treatment (100 and 200 ng/mL) increased MEC \( IGFBP3 \) gene expression 6.10 ± 2.25 and 5.07 ± 1.97, respectively, at 24 h (\( P < 0.001 \)). However, treatment with GH100 or GH500 did not alter \( IGFBP5 \) gene expression at either time point (\( P = 0.38 \)).

In fibroblasts, GH (GH500) and IGF-I (IGF200) did not alter expression of \( TBX2 \) or \( TBX3 \) at 24 h (Figure 4; \( P \geq 0.46 \)). As expected, GH500 increased expression of \( IGFBP5 \) 1.85 ± 0.41-fold (\( P = 0.04 \)) and IGF200 increased \( IGFBP3 \) expression 11.53 ± 4.92-fold (\( P = 0.02 \)), demonstrating the effectiveness of our treatment using the fibroblast cells.

Although the role of GH and IGF-I in regulating the mammary gland is well known, downstream targets of these factors are not well elucidated. Our findings provide the first evidence that \( TBX2 \) and \( TBX3 \) are expressed in the bovine mammary gland. In addition, expression of \( TBX3 \) is present in MAC-T, primary MEC, and fibroblast cells, but \( TBX2 \) is only detected in fibroblasts. Lastly, \( TBX3 \) mRNA expression is increased in response to IGF-I, suggesting it may function as a mediator of IGF-I action in the bovine mammary gland.

T-box2 and 3 are expressed during several stages of embryonic development, including blastocyst formation and organogenesis (Law et al., 1995). At this point of development, these genes are expressed in a wide variety of rudimentary tissue types, including the central nervous system, peripheral nervous system, cartilage or skeleton, kidneys, lungs, muscle tissue, and the mammary gland (Law et al., 1995). T-box2 and 3 have been shown to have distinguished roles in the development of mammary glands in mice (Douglas and Papaioannou, 2013). However, the roles of these transcription factors in the adult mammary gland, including during pregnancy, lactation, and involution, are not well known. Current research in the adult mammary gland primarily focuses on the roles of \( TBX2 \) and \( TBX3 \) in stimulating cancer cell progression, as they are often upregulated in breast cancer cells (Abrahams et al., 2010; Peres et al., 2010). We provide the first evidence that \( TBX2 \) and \( TBX3 \) are expressed in the healthy adult bovine mammary gland. Interestingly, expression of \( TBX2 \) was only detected in the fibroblast cells, whereas \( TBX3 \) was detected in both MEC and fibroblasts. During embryonic development, \( TBX3 \), but not \( TBX2 \), is expressed within the mammary placodes, which will later become the parenchyma or the portion of the mammary gland that contains the MEC (Chapman et al., 1996). Alternatively, during embryonic development, \( TBX2 \), but not

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**Figure 1.** The gene T-box2 (\( TBX2 \)) is expressed in the mammary gland and mammary gland fibroblasts, but not in primary mammary epithelial cells (MEC). (A) Both PCR and gel electrophoresis were used to determine presence of \( TBX2 \) and T-box3 (\( TBX3 \)) mRNA expression in bovine mammary gland (MG) tissue, MAC-T, primary MEC, and fibroblasts. Ribosomal protein subunit 15 (\( RPS15 \)) served as the endogenous control gene. Real-time reverse transcription PCR was used to determine relative expression of \( TBX3 \) (B) and \( TBX2 \) (C) mRNA expression in mammary gland and various cell types. Data are expressed as fold change from the mammary gland tissue and presented as a mean ± SE. Data for primary MEC and fibroblasts are presented as an average of all animals (n = 3 animals). An asterisk (*) represents \( P = 0.02 \) versus MG.
TBX3, is expressed within the mammary mesenchyme, which will later become the stroma region of the mammary gland that contains various connective tissue elements, including the fibroblasts cells (Chapman et al., 1996). The cell-type-specific expression of TBX2 and TBX3 in the bovine mammary gland is consistent with expression patterns during embryonic development, suggesting that cell-type-specific expression of these transcription factors may be conserved during development.

Insulin-like growth factor-I is a critical factor in regulating growth, development, and function of the mammary gland. The GH-IGF axis and associated IGFBP, which modulate IGF-I action, have been extensively studied in the bovine mammary gland (Campbell et al., 1991; Cohick, 1998; Bauman, 1999; Berry et al., 2003; Akers, 2006; Fleming et al., 2007). However, additional downstream targets of GH and IGF-I likely exist, such as transcription factors that have not been identified. Increased TBX3 expression in response to IGF-I in the present study is consistent with the important roles of IGF-I and TBX3 in cell proliferation and the mammary gland function. For example, disruption of IGF-I (Ruan and Kleinberg, 1999; Lingbeek et al., 2002; Kleinberg and Ruan, 2008) or haploinsufficiency of TBX3 (Packham and Brook, 2003; Meneghini et al., 2006) both lead to little or no mammary gland development. Increased
This could be explained by the critical role of IGF-I expression in MAC-T, primary MEC, or fibroblasts. TBX3, however, we did not observe an effect of GH on TBX3, independent of IGF-I. How-specific to MAC-T cells or stage of lactation.

TBX3 expression is responsive to GH, independent of IGF-I. How-ever, we did not observe an effect of IGF-I on TBX3 expression in response to GH, but increase in TBX3 in response to IGF-I, is consistent with the critical role for IGF-I in stimulating mammary gland development and mediating GH action (Purup et al., 2000). Furthermore, the presence of functional GH receptors in MEC has not been verified (Cohick, 1998; Akers, 2002), even though the presence of GH receptor mRNA has been reported (Plath-Gabler et al., 2001). This could contribute to the lack of change in TBX3 expression in response to GH treatment alone and is supported by the lack of change in IGFBP5 expression in the primary MEC.

In conclusion, TBX2 and TBX3 are expressed in the bovine mammary gland, and expression patterns are similar to those found during embryonic development in mice. T-box3 expression increased in response to IGF-I in MAC-T cells, suggesting that it may be a down-stream target of this critical growth factor in cattle. Further work is needed to determine the function of TBX2 and TBX3 in adult mammary gland function, which will further our understanding of the complex changes that occur in the mammary gland during pregnancy, lactation, and involution.

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