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Increased abundance of aromatase and follicle stimulating hormone receptor mRNA and decreased insulin-like growth factor-2 receptor mRNA in small ovarian follicles of cattle selected for twin births1,2

S. E. Echternkamp,*,3 P. Y. Aad,† D. R. Eborn,* and L. J. Spicer†

*USDA4, ARS, U.S. Meat Animal Research Center, Clay Center, NE 68933; and †Department of Animal Science, Oklahoma State University, Stillwater 74078

ABSTRACT: Cattle genetically selected for twin ovulations and births (Twinner) exhibit increased ovarian follicular development, increased ovulation rate, and greater blood and follicular fluid IGF-1 concentrations compared with contemporary cattle not selected for twins (Control). Experimental objectives were to 1) assess relationships among aromatase (CYP19A1), IGF-1 (IGF1), IGF-2 receptor (IGF2R), and FSH receptor (FSHR) mRNA expression in small (≤5 mm) antral follicles and 2) determine their association with increased numbers of developing follicles in ovaries of Twinner females. Ovaries were collected from mature, cyclic (d 3 to 6) Twinner (n = 11), and Control (n = 12) cows at slaughter and pieces of cortical tissue were fixed and embedded in paraffin. Expression of mRNA was evaluated by in situ hybridization using 35S-UTP-labeled antisense and sense probes for CYP19A1, FSHR, IGF1, and IGF2R mRNA. Silver grain density was quantified within the granulosa and theca cells of individual follicles (2 to 7 follicles/cow) by Bioquant image analysis. Follicles of Twinners tended to be smaller in diameter than Controls (1.9 ± 0.1 vs. 2.3 ± 0.1 mm; P = 0.08), but thickness of granulosa layer did not differ (P > 0.1) by genotype. Relative abundance of CYP19A1 (P < 0.01) and FSHR (P < 0.05) mRNA was greater in granulosa cells of Twinners vs. Controls, respectively, whereas IGF2R mRNA expression was less in both granulosa (P < 0.01) and theca (P < 0.05) cells in follicles of Twinners vs. Controls, respectively. Abundance of CYP19A1 mRNA in granulosa cells was correlated negatively with IGF2R mRNA expression in both granulosa (r = −0.33; P < 0.01) and theca (r = −0.21; P = 0.05) cells. Expression of IGF1 mRNA was primarily in granulosa cells, including cumulus cells, and its expression did not differ between Twinners vs. Controls (P > 0.10). Detected increases in CYP19A1 and FSHR, but not IGF1, mRNA expression along with decreases in IGF2R mRNA expression in individual follicles of Twinners support the hypothesis that increased follicular development and steroidogenesis in Twinner females result from increased extra-ovarian IGF-1 production. Furthermore, a reduction in follicular IGF2R mRNA expression accompanied by a reduction in receptor numbers would increase availability of free IGF-2 and its stimulation of follicular development in Twinners.

Key words: aromatase, cattle, insulin-like growth factor, ovarian follicular development, twinning


INTRODUCTION

Long-term genetic selection of cattle (Twinner) for the production of twin ovulations and fraternal twin births, using an index of ovulation and twinning rate (Echternkamp et al., 1990a; Gregory et al., 1990; Van Vleck et al., 1991), has enhanced ovarian follicular development and increased the incidence of twin births. Ovaries of Twinner females had a 2-fold greater density of...
of secondary preantral follicles (Cushman et al., 2000), 50% more small (≤5 mm) and medium (6 to 12 mm) antral follicles (Echternkamp et al., 2004), and >70% frequency of twin or multiple ovulations (Echternkamp et al., 2007, 2009) compared with females not selected for twins (Control). Both IGF-1 and -2 are important promoters of FSH-mediated development of antral follicles, selection of pre-ovulatory follicles, and corpora lutea (CL) formation (Spicer and Echternkamp, 1995). Comparisons of IGF-1 concentrations in blood and follicular fluid between Twiner vs. Control females found greater IGF-1 concentrations in the Twinners (Echternkamp et al., 1990b, 2004). Actions of both IGF-1 and -2 are mediated through the IGF-1 receptor, whereas binding of IGF-2 to the IGF-2 receptor (IGF2R) results in degradation of the ligand-receptor complex (Hawkes and Kar, 2004). In addition, IGF-1 was reported to increase FSH receptor (FSHR) mRNA and decrease IGF2R mRNA in follicular cells. Thus, it is hypothesized that the enhanced ovarian follicular development and increased ovulation rate in cattle selected for twins are a consequence of an increase in extra- and intra-ovarian IGF-1 secretion, increasing numbers of FSHR, and decreasing IGF2R (i.e., increasing availability of free IGF-2) in ovarian follicular cells (Spicer and Aad, 2007). The experimental objectives were to 1) assess relationships among aromatase (CYP19A1), FSHR, IGF-1 (IGF1), and IGF2R mRNA expression in small (≤5 mm) antral follicles and 2) determine their association with increased follicular development in Twiner females.

MATERIALS AND METHODS

The experimental design and procedures employed in this study were approved by the USDA, ARS, U.S. Meat Animal Research Center (USMARC) Animal Care and Use Committee. Experimental procedures were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Animals and Experimental Design

Expression of mRNA for CYP19A1, FSHR, IGF1, and IGF2R was evaluated in small (1 to 5 mm) antral follicles of ovaries obtained from mature (5 to 8 yr of age), cyclic Twiner and Control cows. The USMARC Twiner population is a composite population of cattle selected for the production of twin ovulations and fraternal twin births since 1981 (Echternkamp et al., 1990a; Gregory et al., 1990; Van Vleck et al., 1991). Control females were from a contemporary crossbred population at USMARC that has not been selected for twinning.

Estrous cycles were synchronized among 12 Twiner and 12 Control cyclic cows by the administration of a single injection of PGF2α (PGF, 25 mg intramuscularly; Lutalyse, Pfizer Animal Health, New York, NY) during the luteal phase of the estrous cycle. Presence of a functional CL was determined transrectally by real-time ultrasonography. Cows were subsequently monitored for estrus, and ovulation was confirmed by ultrasonography; 1 Twiner cow ovulated asynchronously and was excluded from the study. Ovaries were collected at slaughter on d 3, 4, 5, or 6 (n = 3 cows d−1-genotype−1) after estrus (estrus = d 0), stored immediately on ice, and transported to the laboratory for processing. Pieces of ovarian cortical tissue containing multiple small antral follicles were dissected from the ovarian surface, fixed in neutral buffered formalin, dehydrated in ethanol and then xylene, and embedded in paraffin.

In situ Hybridization

Abundance of CYP19A1, FSHR, IGF1, and IGF2R mRNA within individual small antral follicles was determined by in situ hybridization using antisense and sense 35S-labeled-UTP cRNA probes transcribed from DNA sequences with primers described previously for CYP19A1 (Xu et al., 1995b), FSHR (Xu et al., 1995a), IGF1 (Radcliff et al., 2003), and IGF2R (Spicer and Aad, 2007). For IGF1 and IGF2R, reverse transcription-PCR was performed on DNase-treated RNA obtained from antral follicles and liver to produce a 113-bp amplicon of bovine IGF1 cDNA and a 188-bp amplicon of bovine IGF2R cDNA, respectively. Amplicons were ligated into the plasmid vector, pCRII (Invitrogen Life Technologies, Carlsbad, CA), and subsequently transformed into Top10 E. coli cells (Invitrogen Life Technologies). The identity and orientation of the clones were confirmed by sequencing. Plasmids containing the cDNA were linearized using restriction endonuclease Not1 for the sense probe and Spe1 for the antisense probe. Probes were generated by in vitro transcription in the presence of 35S-rUTP (PerkinElmer Health Sciences, Inc, Waltham, MA) using RNA polymerase SP6 (Maxiscript SP6/T7 kit; Ambion, Austin, TX) or T7 (Maxiscript T3/T7 kit; Ambion) for sense and antisense probes, respectively, according to the manufacturer’s directions. For CYP19A1 and FSHR, plasmids containing their respective cDNA sequences were obtained from Allen Garverick, University of Missouri, Columbia, and probes were synthesized as described previously (Xu et al., 1995a,b).

Multiple sections (8 μm) of the embedded tissue were cut, mounted on glass microscope slides, deparaffinized, and subsequently incubated with proteinase K for 10 min. Tissue sections were prehybridized at 42°C and then hybridized with the respective probes overnight at 55°C using 100 μL of hybridization solution/slide at a probe concentration of 5,000 dpm/μL for CYP19A1 or
FSHR or 20,000 dpm/µL for IGF1 or IGF2R. After hybridization, slides were washed, treated with RNasea for 30 min, washed, lightly counterstained with hematoxylin, and dehydrated. Slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) followed by exposure for 4 wk at 4°C in a desiccated dark box. Slides were developed in Dektol (Eastman Kodak), fixed with Kodak Fix (Eastman Kodak), dehydrated and cleared, and mounted for examination by brightfield and darkfield microscopy. For each animal, 2 sections were hybridized to the antisense probe, and one section was hybridized to the sense probe for each gene. Sections of cortical tissue from Twinner and Control cows collected from each day of the cycle were included within a hybridization run to minimize biases among runs.

Abundance of mRNA was measured within cross sections of intact small follicles exhibiting an antrum and well-defined granulosa and theca cell layers. Hybridization intensity was quantified in the granulosa and theca cells using the Bioquant Nova Prime image analysis system (BIOQUANT Image Analysis Corporation, Nashville, TN). Evaluated cross sections were perceived to be near the midpoint of the follicle. Within a marked area of interest within the granulosa or theca cell layer of an individual antral follicle, the program quantifies the total number of pixels and the number occupied by silver grains or graphic pixels. Hybridization intensity was defined as the proportion of total pixels occupied by graphic pixels. Specific hybridization intensity within an individual follicle was defined as the average of 4 fields within a follicle cell type for the antisense probe minus the sense probe and expressed as the proportion of the area occupied by specific grains. Measurements were collected on 2 to 7 follicles/cow, and similar numbers of small antral follicles were evaluated per population.

**Statistical Analysis**

Genetic effect on abundance of mRNA for the 4 genes within the small antral follicles was tested for each trait by PROC MIXED procedure (SAS Inst., Inc., Cary, NC) for repeated measurements for multiple follicles per cow. Independent fixed effects in the initial statistical model were genetic line and day of the estrous cycle. Day of cycle was not significant \((P > 0.10)\) and was subsequently removed from the model. Individual follicles were subsequently classified as estrogen-active or -inactive when either >5% or ≤5%, respectively, of the analyzed area was occupied by specific grains for CYP19A1. Expression data were reanalyzed by repeated measures analysis; the model included genetic line and estrogen status as independent fixed effects. Residual correlations among abundance of CYP19A1, FSH, IGF1, and IGF2R mRNA were calculated after accounting for the variation associated with the fixed effects in the model. Relationships among measured traits were assessed by PROC CORR (SAS Inst. Inc.).

**RESULTS**

Diameters of the small, intact antral follicles randomly selected for evaluation in the ovaries of the Twinner cows tended to be smaller than those of follicles selected for evaluation in ovaries of the Controls \((1.9 ± 0.1 \text{ vs. } 2.3 ± 0.1 \text{ mm}; P = 0.08)\), but the thickness of the granulosa layer did not differ between the 2 cattle populations \((76.8 ± 19.2 \text{ vs. } 75.0 ± 16.0 \mu\text{m}, \text{ respectively}; P > 0.10)\).

**Aromatase and Follicle-stimulating Hormone Receptor mRNA**

In situ hybridization analysis revealed that mRNA expression for both CYP19A1 and FSHR was localized specifically to the granulosa cells (Figure 1 B and C, respectively) of small antral follicles contained within the ovaries of cyclic Twinner and Control cows. Although expression of CYP19A1 and FSHR mRNA varied among follicles within and between animals, relative abundance of CYP19A1 or FSHR mRNA within the small antral follicles did not differ with follicle diameter (1 to 5 mm) or by day of the estrous cycle.

Collectively, the relative abundance of CYP19A1 mRNA (Table 1) was greater \((P < 0.01)\) in the small antral follicles of Twinner cows compared with Control cows. Furthermore, when individual follicles were classified as either estrogen-active or -inactive on the basis of the abundance of CYP19A1 mRNA, an increased abundance of CYP19A1 mRNA was found in estrogen-active, but not -inactive, small antral follicles (Figure 2) of...
Table 1. Comparisons of aromatase (CYP19A1), FSH receptor (FSHR), IGF-1 (IGF1), and IGF-2 receptor (IGF2R) mRNA expression in granulosa (GC) or theca interna (TC) cells of small antral follicles in ovaries of Twinner and Control females

<table>
<thead>
<tr>
<th>mRNA</th>
<th>n²</th>
<th>Twinner</th>
<th>n²</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19A1, GC</td>
<td>41</td>
<td>17.5 ± 1.4a</td>
<td>47</td>
<td>7.5 ± 1.3b</td>
</tr>
<tr>
<td>FSHR, GC</td>
<td>41</td>
<td>11.0 ± 1.2c</td>
<td>47</td>
<td>7.3 ± 1.1d</td>
</tr>
<tr>
<td>IGF1, GC</td>
<td>41</td>
<td>16.5 ± 2.5</td>
<td>47</td>
<td>13.2 ± 2.4</td>
</tr>
<tr>
<td>IGF2R, GC</td>
<td>41</td>
<td>2.9 ± 0.7a</td>
<td>47</td>
<td>6.2 ± 0.7b</td>
</tr>
<tr>
<td>IGF2R, TC</td>
<td>41</td>
<td>3.0 ± 0.6c</td>
<td>47</td>
<td>5.0 ± 0.6d</td>
</tr>
</tbody>
</table>

a,bMeans without a common superscript differ between genetic lines; P < 0.01.
c,dMeans without a common superscript differ between genetic lines; P < 0.05.

Table 2. Coefficients for residual correlations among relative abundance of aromatase (CYP19A1), FSH receptor (FSHR), IGF-1 (IGF1), and IGF-2 receptor (IGF2R) mRNA expression in ovarian granulosa (GC) and theca interna (TC) cells

<table>
<thead>
<tr>
<th>mRNA</th>
<th>CYP19A1</th>
<th>FSHR</th>
<th>IGF1</th>
<th>IGF2R, GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHR, GC</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF1, GC</td>
<td>0.05</td>
<td>0.33**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2R, GC</td>
<td>−0.33**</td>
<td>−0.16</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>IGF2R, TC</td>
<td>−0.21*</td>
<td>0.27**</td>
<td>0.28*</td>
<td>0.43**</td>
</tr>
</tbody>
</table>

1Number of observations, n = 88.

*Positive or negative coefficients are significant at P < 0.05.

**Positive or negative coefficients are significant at P < 0.01.

Twinner vs. Control cows (genetic line × estrogen status; P = 0.02). In addition, a greater proportion of the small follicles of Twiners were classified as estrogen-active compared with follicles of Controls (0.90 vs. 0.57 ± 0.08, respectively; P = 0.03); only 4 estrogen-inactive follicles were found among the ovarian tissue sections randomly selected and evaluated for Twinner cows compared with 20 estrogen-inactive follicles for Control cows. Abundance of FSHR mRNA was greater (P < 0.05) in the small antral follicles of Twinner cows compared with Control cows (Table 1), and FSHR mRNA tended to be greater (P = 0.11) in estrogen-active vs. -inactive follicles, but evidence of an interaction between estrogen status and genetic line was not detected (genetic line × estrogen status; P > 0.10). Coefficients (Table 2) for the positive correlations between abundance of CYP19A1 and FSHR mRNA or between CYP19A1 and IGF1 mRNA in granulosa cells were small (P > 0.10). In contrast, the positive coefficient (Table 2) for the correlation between abundance of FSHR and IGF1 mRNA in granulosa cells was statistically significant (r = 0.33; P < 0.01).

Insulin-like Growth Factor-1 and Insulin-like Growth Factor-2 Receptor mRNA

In contrast with CYP19A1 (P < 0.01) and FSHR mRNA, expression of IGF1 (Figures 3 and 4) and IGF2R mRNA (Figure 5) was detected within both granulosa and theca cells of the small antral follicles. Although IGF1 mRNA expression was detected within both the granulosa and theca cell layers (Figure 3), its expression was localized primarily within the granulosa layer, including cumulus cells surrounding the oocyte (Figure 4).

Abundance of IGF1 mRNA was low relative to the abundance of CYP19A1 mRNA within the same follicle and required a larger amount of 35S-labeled probes for detection. Abundance of IGF1 mRNA did not differ (P = 0.38) between small antral follicles in the ovaries of Twinner vs. Control cows (Table 1) or between estrogen-active vs. -inactive follicles (15.3 vs. 13.1 ± 2.1%, respectively; P = 0.63). Relative abundance of IGF1 mRNA in granulosa cells was correlated positively (Table 2) with abundance of FSHR mRNA (r = 0.33; P < 0.01) in granulosa cells and with IGF2R mRNA in theca cells (r = 0.28; P < 0.05), whereas the correlation coefficient with IGF2R mRNA in granulosa cells was small (r = 0.16; P > 0.10).

Relative abundance of IGF2R mRNA (Table 1) was found to be less within both the granulosa (P = 0.003) and theca (P = 0.02) cells of small antral follicles from the ovaries of Twinner cows compared with Control cows. However, the genotypic difference in IGF2R mRNA expression within the theca cells (Figure 6) was only detected within estrogen-active follicles, being less (P < 0.01) in estrogen-active follicles of Twinners...
vs. Controls, whereas $IGF2R$ mRNA did not differ ($P > 0.10$) by genotype for estrogen-inactive follicles (genetic line × estrogen status, $P = 0.01$).

Comparing estrogen-inactive vs. -active small follicles, relative abundance of $IGF2R$ mRNA was 2-fold greater within granulosa cells of estrogen-inactive vs. -active small follicles (10.6 ± 1.8 vs. 4.2 ± 0.9, respectively; $P = 0.01$). Again, $IGF2R$ mRNA was only greater in estrogen-inactive vs. -active small follicles of Twinners (genetic line × estrogen status, $P = 0.01$). Thus, abundance of $IGF2R$ mRNA in granulosa (r = −0.33; $P \leq 0.01$) or theca (r = −0.21; $P = 0.05$) cells was correlated negatively (Figure 7) with abundance of $CYP19A1$ mRNA expression in granulosa cells.

**DISCUSSION**

Cattle are primarily monovulatory, and thus, the frequency of dizygotic (fraternal) twin births is low (Rutledge, 1975). Because twin ovulations are the first prerequisite for dizygotic twins, the genetic selection criteria used for the USMARC Twinner herd included measurement of ovulation rate for consecutive multiple estrous cycles in all female progeny between 12 and 18 mo of age (Echternkamp et al., 1990a). Responses to this selection were an increase in numbers of preantral and antral ovarian follicles (Cushman et al., 2000; Echternkamp et al., 2004) and an increase in the frequency of twin or triplet ovulations to >70% (Echternkamp et al., 2007, 2009). The physiological mechanism for this increase in ovarian follicular development is not well described, but comparisons of IGF-1 con-
centrations in blood and ovarian follicular fluid between cattle selected (Twinner) vs. not selected for twinning (Control) indicated greater IGF-1, but not FSH, concentrations in Twinner females (Echternkamp et al., 1990b, 2004). Thus, the objective of the present study was to further explore the role of the IGF system in increasing follicle numbers and follicular development within the ovaries of Twinner females utilizing in situ hybridization analysis to compare mRNA expression within individual small antral follicles between Twinner and Control cows for genes linked with ovarian follicular development (i.e., CYP19A1, FSHR, IGF1, and IGF2R).

Comparison of CYP19A1 mRNA expression in small antral follicles between ovaries of Twinner and Control cows indicated that a greater proportion of the small follicles within the ovaries of Twinner cows had an increased CYP19A1 mRNA expression (i.e., estrogen-active) as well as a greater abundance of CYP19A1 mRNA within the granulosa cells of the individual estrogen-active, small antral follicles compared with small follicles from the ovaries of Control cows. As reported previously, CYP19A1 mRNA expression was localized specifically within granulosa cells and present in follicles ranging from 1 to 5 mm in diameter (Xu et al., 1995b). The expression of CYP19A1 mRNA is detectable in bovine fetal ovaries as early as ovarian tissue is detectable (Garverick et al., 2010) and is associated with detection of estradiol-17β production by bovine fetal ovaries early in gestation (Yang and Fortune, 2008). Steroid production within the bovine fetal ovary has been hypothesized to regulate germ cell meiosis and ovarian anatomical and vascular development (Yang and Fortune, 2008); thus, the increased abundance of CYP19A1 mRNA expression in the small Twinner follicles may indicate earlier recruitment of follicular development and maturation of ovarian follicles within the ovaries of Twinner females. In addition, CYP19A1 mRNA expression and aromatase activity are reported to be increased in granulosa cells from large bovine ovarian follicles, and to a lesser extent in granulosa cells from small bovine ovarian follicles, by either IGF-1 or -2 in vitro (Spicer et al., 1993; Spicer and Aad, 2007); thus, the observed increase in abundance of CYP19A1 mRNA in Twinner follicles is consistent with increased IGF-1 stimulation within the ovaries of Twinner females as a result of increased blood and follicular fluid IGF-1 concentrations (Echternkamp et al., 1990b, 2004).

Abundance of FSHR mRNA was also increased in the granulosa cells of Twinner small antral follicles compared with granulosa cells of Controls. Expression of FSHR mRNA in bovine granulosa cells begins shortly after the follicles leave the primordial pool (Xu et al., 1995a), but information on the regulation of FSHR mRNA transcription is limited and its regulation may differ among species. Evidence does exist for an involvement of IGF-1 in the regulation of the FSHR mRNA expression as FSH receptor numbers in bovine granulosa cells are reportedly increased by in vitro treatment with IGF-1 (Spicer and Aad, 2007), whereas female IGF1-null mice have reduced FSHR mRNA abundance in granulosa cells (Baker et al., 1996). Likewise, FSHR mRNA expression was correlated positively with IGF1 mRNA expression in granulosa cells in the present study. Thus, the greater abundance of FSHR mRNA in granulosa cells of Twinner cows may also be the consequence of greater IGF-1 concentrations in the blood or ovarian follicular fluid of Twinters.

Follicle stimulating hormone stimulation of steroidogenic activity within bovine granulosa cells is mediated via its binding to the transmembranal FSH receptor, which is consistent with the greater abundance of mRNA for both FSHR and CYP19A1 in follicles of Twinters compared with Controls. In contrast, the correlation coefficient for the overall positive correlation between abundance of FSHR and CYP19A1 mRNA in granulosa cells was small in the present study and in a previous comparison of CYP19A1 and FSHR mRNA expression in granulosa cells from healthy antral follicles ranging in diameter from 0.5 to 14 mm during the first follicular wave of the bovine estrous cycle.
cycle (Xu et al., 1995a). The exact role for FSH in the regulation of the FSH receptor and early follicular development is unclear. For instance, treatment of bovine granulosa cells with FSH in vitro did not affect FSHR mRNA abundance (Spicer and Aad, 2007), and ovarian follicles grew to the early antral stage in hypophysectomized ewes in the absence of gonadotropin support (Driancourt et al., 1987), whereas FSH was able to increase the number of small preantral follicles in hypophysectomized mice (Wang and Greenwald, 1993). Likewise, differences in plasma FSH concentration were not detected in blood between pseudopregnant Twinner and Control cows (Echternkamp et al., 2004).

Results from both in vitro and in vivo studies have documented stimulatory effects of IGF-1 on ovarian follicular cell proliferation, differentiation, steroidogenesis (Spicer et al., 1993; Spicer and Echternkamp, 1995), and inhibition of apoptosis (Chun et al., 1996; Guthrie et al., 1998), including bovine follicular cells. Furthermore, sustained treatment of cattle (Gong et al., 1993) or swine (Echternkamp et al., 1994) with exogenous recombinant bovine or porcine GH, respectively, increased blood IGF-1 concentrations and numbers of small antral follicles within the ovaries; however, an increase in ovulation rate only occurred if exogenous FSH was subsequently administered (Gong et al., 1993). In contrast with previously observed greater follicular fluid IGF-1 concentrations in Twinner ovaries, the numerically greater IGF1 mRNA expression in granulosa cells of small antral follicles from Twinner females did not differ significantly from expression in follicles of Controls. Unfortunately, it was not feasible to quantify IGF-1 in follicular fluid of the individual small ovarian follicles evaluated in the present study. Previous assessments of IGF-1 in bovine ovarian follicles found greater IGF1 mRNA expression, greater in vitro production by granulosa cells, and greater follicular fluid IGF-1 concentrations in large (especially dominant follicles) versus small antral follicles; thus, the small genetic difference in abundance between small follicles of Twinners and Controls may be the consequences of assessing IGF1 mRNA expression in follicular cells of small as opposed to large antral follicles. Alternatively, a major portion of the IGF-1 measured in follicular fluid of large follicles is of extra-ovarian origin and sequestered from the blood (Echternkamp et al., 1999b), which concurs with the decrease of IGF1 mRNA expression in follicular cells of mammalian ovaries. The IGF1 mRNA expression was also similar between granulosa and cumulus cells within the 3 follicles having cumulus cells adjacent to an oocyte in the sections evaluated. The small number of silver grains covering the oocyte in Figure 3 is likely the consequence of a thin layer of cumulus cell contamination on the surface of the oocyte, for IGF1 mRNA expression was not detected by RT-PCR analysis of bovine oocytes (Nuttinck et al., 2004). Information on the production of IGF 2 and the role of IGF2R in the regulation of bovine ovarian follicle development is limited. A recent comparison of biological activity for IGF-1 and -2 in bovine granulosa cells in vitro (Spicer and Aad, 2007) indicated that IGF-1 and -2 had similar biological potencies for the induction of granulosa cell steroidogenesis and mitosis. In addition, binding of IGF-2 to the IGF-1 receptor mimics the actions of IGF-1, whereas preferential binding of IGF-2 to the IGF2R sequesters its biological activity by endocytosis-mediated degradation of the non-glycosylated IGF-2 polypeptide hormone (Hawkes and Kar, 2004). In the present study, abundance of the IGF2R mRNA was less in both the granulosa and theca cells of small antral follicles of Twinners vs. Controls. As a consequence, IGF2R mRNA expression was correlated positively between the 2 follicular cell types, suggestive that IGF2R mRNA expression is commonly regulated in the 2 cell types. Because the IGF2R sequesters the biological activity of IGF-2, a reduction in IGF2R mRNA expression accompanied by a decrease in number of IGF2R in small ovarian follicles of Twinners could contribute to the increased numbers and growth of small antral follicles and their expression of CYP19A1 mRNA in the ovaries of Twinners compared with Controls. Treatment of granulosa cells from small bovine antral follicles with IGF-1 in vitro decreased abundance of IGF2R mRNA and increased abundance of FSHR mRNA (Spicer and Aad, 2007); thus, greater follicular fluid IGF-1 concentrations found in Twinners (Echternkamp et al., 1990b) may contribute to less IGF2R mRNA expression and greater FSHR mRNA expression noted in follicular cells of small ovarian follicles of Twinners vs. Control cows. Additional support for an inverse relationship between follicular cell function and IGF2R numbers was the negative correlation between CYP19A1 and IGF2R mRNA expression in granulosa and theca cells, implying an increase in steroidogenesis as a result of increased biological availability of IGF-2.

Additional evidence for a link between IGF-1 stimulation and increased follicular development and ovulation rate in cattle is the identification of a suggestive QTL for twinning rate on BTA 5 in the chromosomal region containing the IGF1 gene (Kappes et al., 2000). Subsequent refined mapping of this QTL region in Holstein cattle identified an SNP in intron 2 of IGF1 that was associated significantly with twinning rate (Kim et al., 2009).

In conclusion, analysis of mRNA expression by in situ hybridization allows the assessment of mRNA expression within individual follicles rather than a composite of follicles varying in physiological status, especially since major differences in physiological status appear to exist among ovarian follicles and between cattle populations. In addition to the ovaries of Twinner females containing more antral follicles (Echternkamp et al., 2004), detected increases in abundance of CYP19A1 and
FSHR mRNA in the present study indicate that a greater proportion of the follicles are FSH responsive and steroidogenically active. The increase in CYP19A1 and FSHR mRNA, but not IGF1, mRNA along with decreases in IGFB2 mRNA expression within individual small antral follicles of Twinner females compared with Control females support the hypothesis that increased numbers of FSH-responsive, steroidogenically active follicles in Twinner females result from increased extra-ovarian IGF-1 stimulation. Furthermore, a reduction in follicular IGFB2 mRNA expression and in accompanying receptor numbers would allow for increased availability of free IGF-2 and its stimulation of follicular development in Twinners.

LITERATURE CITED


