Possible role of IGF2 receptors in regulating selection of 2 dominant follicles in cattle selected for twin ovulations and births

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ABSTRACT

Abundance of IGF-2 receptor (IGF2R), FSH receptor (FSHR), and LH receptor (LHCGR) mRNA in granulosa cells (GCs) or theca cells (TCs) or both cells as well as estradiol (E2), progesterone (P4), and androstenedione concentrations in follicular fluid were compared in cows genetically selected (Twinner) or not selected (control) for multiple ovulations and twin births. Cows were slaughtered at day 3 to 4 (day 3) and day 5 to 6 (day 5) of an estrous cycle, and ovaries, follicular fluid, GCs, and TCs were collected. The two largest (F1 and F2) E2-active (EA) and E2-inactive (EI) follicles were selected according to their E2-to-P4 ratio and diameter. Androstenedione levels in EA F1 and F2 follicles were 5-fold greater (P < 0.05) in Twinner cows than in control cows on day 3 but did not differ on day 5. Twinner cows also had greater (P < 0.05) E2 and P4 concentrations, whereas steroid levels in EI follicles did not differ (P > 0.10) between genotypes. In EA F2 follicles, IGF2R levels in GCs were greater (P < 0.05) in control cows than in Twinner cows on day 3 and day 5, whereas IGF2R mRNA in TCs did not differ (P > 0.10). On day 3, FSHR mRNA levels were greater (P < 0.05) in GCs of EA F1 and EI F2 follicles of control cows than of Twinner cows. LH receptor mRNA expression was less in GCs and greater in TCs of EA F2 follicles in control cows than in Twinner cows (P < 0.05). We hypothesize that reduced GC IGF2R expression in F2 follicles of Twinner cows may play a role in the development of 2 or more dominant follicles.

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1. Introduction

In the ovary, follicular development is governed by a series of changes in endocrine and paracrine factors, among which IGFs play a major role. Both IGF1 and IGF2 stimulate steroidogenesis and mitogenesis within bovine follicles [1–3], and total (ie, free plus bound) follicular fluid concentrations of IGF2 are greater than those of IGF1 [4–6]. The biological actions of both IGF1 and IGF2 are mediated via the IGF type 1 receptor (IGF1R) [7], and IGFBP, which modulate IGF effects by sequestering IGF1 and IGF2, prevent degradation and signaling of these growth factors, and thus alter intrafollicular amounts of IGF1 or IGF2 to affect follicular growth [8]. Conversely, the mannose-6 phosphate/IGF type 2 receptor (IGF2R), which has been described as a membrane-bound IGF2 binding protein [9] that acts as a modulator of intrafollicular IGF2 activity [3] and is produced by a maternally inherited gene [10,11], is not well studied in the ovary. In addition, IGF2R mRNA abundance is significantly decreased in granulosa cells (GCs) collected from women with a reduced ovarian reserve [12] and is lower in small (1–5 mm) antral follicles of cattle selected for twin births [13].

Over the past 25 y, researchers at the US Meat Animal Research Center have genetically selected cattle for a high propensity to ovulate 2 dominant follicles and to produce
fraternal twin calves [13–17], providing an excellent model for the study of the regulation of ovarian follicle growth and selection. Previously, Echternkamp et al [18] identified IGF1 to be greater in follicular fluid and plasma of Twinner cattle and later found that Twinner cattle also have greater intrafollicular IGFBP5 and lower IGFBP4 binding [19]. The IGF2R inactivates IGF2 [3], but whether IGF2R mRNA abundance in GCs or theca cells (TCs) differs between Twinner and control cattle or changes during follicular recruitment and selection is unknown. The objective of the present study was to determine and compare changes in IGF2R mRNA abundance in GCs and TCs during follicular growth and selection of one vs multiple dominant follicles in cattle.

2. Materials and methods

2.1. Experimental design

The experimental design and procedures used were approved by the US Meat Animal Research Center Animal Care and Use Committee. Ovaries of 15 cows with the propensity for twin births (ie, Twinner) [16,20,21] and 15 MARC I, II, and III (control) cows were evaluated transectally by real-time ultrasonography with the use of a 7.5-MHz linear-array probe, Aloka 500 instrument (Corometrics Medical Systems, Wallingford, CT) to determine the presence of corpora lutea (CL) and to record the follicular population. Cows with CL were injected with prostaglandin F-2 alpha (30 mg), and ovaries were scanned by real-time ultrasonography 2 d before slaughter to confirm ovulation and to record follicular populations. Cows were slaughtered (no more than 6 cows per d) at follicle recruitment (day 3–4; day 3) or deviation (day 5–6; day 5) of the estrous cycle to evaluate if IGF2R gene expression changes during the time the dominant follicle is being selected and initiates its dominance over subordinate follicles [22]. Ovaries were immediately recovered and transported on ice to the laboratory. Up to 10 of the largest antral follicles (>4 mm in diameter) per pair of ovaries were excised from the ovaries and individually snap-frozen and stored in liquid nitrogen.

Frozen follicles (range, 5–17 mm) were processed as described previously [21,23] with modifications. Follicle diameters were measured on dissected follicles before collection of the GCs and TCs. Briefly, frozen follicles were bisected, and the outer follicle wall was thawed slightly for the TCs to be peeled out of the follicle shell as described previously [21,23]. Follicular fluid that contained GCs was thawed by incubation at 37 °C for 5 min and centrifuged at 1000 × g at 4 °C for 5 min. The GC pellet was lysed in 0.5 mL of TRIzol reagent (Invitrogen Corp, Carlsbad, CA), vortexed, incubated for 5 min at 37 °C, and stored at −80 °C until RNA extraction. The follicular fluid supernatant was transferred into clean expendor tubes and stored at −20 °C until hormone assays. Theca cells were suspended in 0.5 mL of RNAlater (Ambion Inc, Austin, TX) at 4 °C overnight, homogenized in TRIzol Reagent (Invitrogen Corp), and stored at −80 °C until RNA extraction as described below.

2.2. Hormone assays

Concentrations of estradiol (E2), progesterone (P4), and androstenedione in follicular fluid were determined by RIA as described previously [24,25]. The intra-assay CV was 12% for the E2 RIA, 10% for the P4 RIA, and 14% for the androstenedione RIA. Data for 2 control cows without estrogen-active (EA; E2-to-P4 ratio >1) follicles, and 1 control cow without a CL were removed from all subsequent data analyses.

2.3. RNA extraction and quantification

Ribonucleic acid from GC samples was extracted in 13 batches with an average of 13 samples per extraction batch, and RNA from TCs was extracted in 8 batches with an average of 20 samples per extraction batch as previously described [21]. Each batch contained an equal number of samples from each treatment group and a similar number of different size follicles. Granulosa cells, stored in 0.5 mL of TRIzol at −80 °C, had RNA extracted with the TRIzol protocol as described previously [3,26,27]. Theca cells, stored in 0.5 mL of RNAlater (Ambion Inc), were transferred into 0.75 mL of TRIzol reagent (Ambion Inc), were transferred into 0.75 mL of TRIzol reagent and homogenized for 2 to 3 min on ice with the use of the Omni TH tissue homogenizer (Omni International Inc, Marietta, GA) with Omni Tip disposable generator probes to prevent contamination between treatments [21]. After extraction, RNA was quantified by ultrasensitive fluorescent nucleic acid staining by using the RiboGreen RNA Quantitation Reagent Kit (Molecular Probes, Eugene, OR) with modifications as described previously [28] and using a fluorescent plate reader (Wallac 1420; PerkinElmer, Boston, MA). The intra-assay CV was <10%.

2.4. Multiplex real-time RT-PCR for mRNA quantitation

Differential expression of target genes in GCs or TCs was quantified with the multiplex 1–step real-time RT-PCR Taqman Gold RT-PCR Kit (Applied Biosystems Inc, Foster City, CA) and 18S ribosomal RNA (18S rRNA) control kit (Applied Biosystems Inc) as the internal control to normalize for variation in loaded RNA. Combinations of primers for each target gene and 18S rRNA were tested to identify the concentrations that would achieve optimal amplifications in the RT-PCR reaction. Primers and probes for quantitative RT-PCR were designed with Primer Express software as previously described for FSHR and IGF2R [3] and for LHCGR [29,30]. A “short, nearly exact matches” BLAST query search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted to ensure specificity of the designed primers and probes and that they were not designed from any homologous regions coding for other genes. All designed probes were synthesized with a 5′ FAM reporter dye and a 3′ TAMRA quencher dye (TaqMan TAMRA; Applied Biosystems Inc); the internal control 18S rRNA probe (TaqMan Ribosomal RNA Control Reagents; Applied Biosystems Inc) was supplied as a VIC Probe. For all RT-PCR runs, a no-template control and a no-reverse transcriptase control were included to ensure the lack of contaminants in the master mix and the absence of any genomic DNA contamination, respectively. In addition, the RT-PCR products were run on agarose gels to verify the length and size of the expected target genes, and the same RT-PCR cDNA samples were used to verify the amplified sequences.
For experimental samples, 50 or 100 ng of total RNA was amplified in a total reaction volume of 25 μL that consisted of 200 nM forward primer, 200 nM reverse primer, 100 nM FAM/TAMRA fluorescent-labeled probe for the target gene, 20 nM of the supplied 18S forward and reverse primers, 100 nM of the 18S probe labeled with VIC/TAMRA dyes, 12.5 μL of TaqMaster Master Mix without uracil N-glycosylase, and 1 U Multiscribe with RNase inhibitor (Applied Biosystems Inc). Thermal cycling conditions that used an ABI 7500 PCR machine (Applied Biosystems Inc) were set to 30 min at 48.8°C for reverse transcription, 10 min at 95°C for AmpliTaq Gold activation, and finished with >45 cycles at 95°C for 15 s for denaturing and at 60°C for 1 min for annealing and extension.

Because the total number of samples was greater than the 96-well plate capacity, cows were sorted by genotype and cycle. Cows within genotype and cycle were assigned randomly to each plate, with all follicles from 1 individual cow on the same plate; all individual samples were run in duplicate. Quantification of gene expression was calculated by setting an arbitrary threshold (Ct) on the FAM or VIC curves in the geometric portion of the RT-PCR amplification plot after examining the log view. The 18S rRNA values were used as internal controls to normalize samples for any variation in amounts of RNA loaded, and relative quantity of target gene mRNAs was expressed as 2−ΔΔCt by using the relative comparative threshold cycle method as described previously [3,31–33].

2.5. Statistical analyses

Data were analyzed as a completely randomized design with a 2 × 2 × 2 factorial treatment structure for EA and EI follicles separately: main effects were genotype (control vs Twinner), day of cycle (recruitment day 3 or deviation day 5), and follicle rank (F1 or F2), with cow as a random effect. The model included all 2- and 3-way interactions. To assign follicle rank within EA or EI follicles on the basis of their theoretical dominance. Estrogen active follicles were sorted first by E2 concentration, followed by follicular diameter, and then by E2-to-P4 ratio (F1 having the greatest E2-to-P4 ratio). Estrogen inactive follicles were sorted first by size and followed by their E2-to-P4 ratio. In both cattle populations, EA F1 always had an E2-to-P4 ratio >1; EA F2 in control cows had E2-to-P4 ratio >0.25 and E2 >10 ng/mL.

Treatment effects on dependent variables (eg, E2 concentration, target gene mRNA levels) were determined with the mixed model procedure of SAS for Windows version 8.02 (SAS Institute Inc, Cary, NC). Outliers were detected according to the procedure described by Grubbs [34] as provided by GraphPad Software (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Data were tested for homogeneity of variance by using Hartley’s F max test. To correct for heterogeneity of variance, steroids and target gene mRNA were analyzed after transformation to natural log (x + 1). Differences in main effects were considered only when the 2- and 3-way interactions were not significant (P > 0.05). Mean differences were determined by Fisher’s protected least significant differences test, if significant treatment effects in ANOVA were detected. Results are presented as the least square means ± SEM.

3. Results

3.1. Diameter, steroids, and abundance of FSHR, LHCGR, and IGF2R mRNA in EA F1 and F2 follicles

3.1.1. Diameter of EA follicles

Diameter of the 2 largest EA follicles (F1 and F2) within each control and Twinner cow, selected on size and E2-to-P4 ratio, was not affected (P > 0.10) by genotype or any of the 2- and 3-way interactions, but diameter did differ (P < 0.05) between days and follicle rank (Table 1). Day 5 EA follicles were 2 mm larger (P < 0.05) than day 3 EA follicles. Diameter of EA F1 and F2 in Twinner cows averaged 12.3 ± 0.6 mm and 10.7 ± 0.6 mm and in control cows averaged 11.9 ± 0.8 mm and 9.6 ± 0.8 mm, respectively.

3.1.2. Steroids in EA follicles

Concentrations of E2 in follicular fluid in EA follicles were affected by genotype (P < 0.05) and follicle rank (P < 0.01), whereas effect of day of cycle and 2- and 3-way interactions were not significant. Concentrations of E2 in EA F1 and F2 were 47% greater (P < 0.05) in Twinner cows than in control cows and were 2-fold greater (P < 0.05) in EA F1 than in EA F2 (Fig. 1A). Concentrations of P4 in the follicular fluid of the 2 largest EA follicles were affected only by genotype (P < 0.05; Table 1). Averaged between days and follicle rank, EA follicles of Twinner cows had 48% greater (P < 0.05) P4 concentrations than of control cows (Table 1). The E2-to-P4 ratio was only affected by follicle rank (P < 0.01; Table 1) with E2-to-P4 ratio averaging

Table 1

<table>
<thead>
<tr>
<th>Main Effect</th>
<th>Group</th>
<th>No.</th>
<th>Diameter (mm)</th>
<th>P4 (ng/mL)</th>
<th>E2-to-P4 ratio</th>
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<tr>
<td>EA follicles</td>
<td>Genotype</td>
<td>Control</td>
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<td>10.5</td>
<td>21.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>Twinner</td>
<td>30</td>
<td>11.4</td>
<td>32.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Day of cycle</td>
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<td>27</td>
<td>9.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6.13</td>
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<td>5</td>
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<td>26.2</td>
<td>7.22</td>
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<tr>
<td>Follicle rank</td>
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<td>27.9</td>
<td>8.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>26</td>
<td>9.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.7</td>
<td>4.73&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>SEM</td>
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<td>3.1</td>
<td>0.91</td>
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<tr>
<td>EI follicles</td>
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<td>105.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>125.7</td>
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<tr>
<td>Day of cycle</td>
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<td>SEM</td>
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<td>37.2</td>
<td>0.036</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: E2, estradiol; EA, estrogen-active; EI, estrogen-inactive; P4, progesterone.

<sup>a</sup> No 2- or 3-way interactions were significant (P > 0.10).

<sup>b</sup>c Within main effect, means without a common superscript differ (P < 0.05).
1.8-fold greater in F1 than F2 EA follicles. Concentration of androstenedione in follicular fluid was affected \((P < 0.05)\) by genotype and day of cycle (Fig. 1B) and tended to be affected by their interaction (genotype by day, \(P < 0.08\)). Concentrations of androstenedione in EA F1 and F2 were 5-fold greater \((P < 0.05)\) in Twinner cows than in control cows on day 3. Because androstenedione levels decreased \((P < 0.05)\) between day 3 and day 5 in control cows, FSHR mRNA abundance did not differ between genotypes on day 5 (Fig. 3). Abundance of GC and TC LHCGR mRNA in EA follicles (Fig. 4) was affected by genotype by follicle rank \((P < 0.05)\) but not \((P > 0.10)\) day of estrous cycle. Abundance of GC LHCGR mRNA (Fig. 4A) was similar \((P > 0.10)\) for EA F1 of control and Twinner cows, whereas abundance of GC LHCGR mRNA was 5-fold less \((P < 0.05)\) in EA F2 follicles of control cows than of Twinner cows. In contrast, abundance of TC LHCGR mRNA tended to be affected \((P < 0.10)\) by genotype and genotype by day by follicle rank interaction (Fig. 3). In EA F1 follicles, abundance of FSHR mRNA was 7-fold greater \((P < 0.01)\) in GCs of control than of Twinner cows at day 3 (Fig. 3). Because FSHR mRNA decreased \((P < 0.05)\) 65% between day 3 and day 5 in control cows, FSHR mRNA abundance did not differ between genotypes on day 5 (Fig. 3).

### 3.1.3. Receptor mRNA in EA follicles

Among the 2 largest EA follicles, abundance of GC IGF2R mRNA in F2 of control cows was 4-fold greater \((P < 0.05)\) than F2 of Twinner cows on day 3 and day 5 (Fig. 2A). Abundance of IGF2R mRNA in TCs of EA follicles did not differ between control and Twinner cows on day 3 or day 5 (Fig. 2B) but averaged 5.8-fold greater \((P < 0.05)\) abundance of IGF2R mRNA in TCs than in GCs of EA follicles.

Overall, GC abundance of FSHR mRNA in EA follicles tended to be affected \((P < 0.10)\) by genotype and genotype by day by follicle rank interaction (Fig. 3). In EA F1 follicles, abundance of FSHR mRNA was 7-fold greater \((P < 0.01)\) in GCs of control than of Twinner cows at day 3 (Fig. 3). Because FSHR mRNA decreased \((P < 0.05)\) 65% between day 3 and day 5 in control cows, FSHR mRNA abundance did not differ between genotypes on day 5 (Fig. 3). Abundance of GC and TC LHCGR mRNA in EA follicles (Fig. 4) was affected by genotype by follicle rank \((P < 0.05)\) but not \((P > 0.10)\) day of estrous cycle. Abundance of GC LHCGR mRNA (Fig. 4A) was similar \((P > 0.10)\) for EA F1 of control and Twinner cows, whereas abundance of GC LHCGR mRNA was 5-fold less \((P < 0.05)\) in EA F2 follicles of control cows than of Twinner cows. In contrast, abundance of TC LHCGR mRNA...
was 9-fold greater in EA F2 follicles of control cows than of Twinner cows but similar \( (P > 0.10) \) between EA F1 of control and Twinner cows (Fig. 4B).

### 3.2. Diameter, steroids, and abundance of FSHR, LHCGR, and IGF2R mRNA in EI F1 and F2 follicles

#### 3.2.1. Diameter of EI follicles

The largest 2 EI follicles (F1 and F2) within the control and Twinner cows were identified on the basis of their E2-to-P4 ratio and diameter. Follicle rank and genotype by day affected \( (P < 0.05) \) follicle diameter, whereas main effects of genotype, day, or other interactions were not significant. Twinner cows had smaller \( (P < 0.05) \) EI F2 follicles than F1 follicles on day 3 and day 5, but control EI F2 and F1 follicles did not differ in diameter between days (Table 1).

#### 3.2.2. Steroids in EI follicles

Concentrations of E2 in follicular fluid were not affected by genotype, follicle rank, day of cycle, or any of the 2- and 3-way interactions \( (P > 0.10) \). Concentration of E2 in the 2 largest EI follicles of Twinner and control cows averaged 4.58 ± 1.09 ng/mL and 3.41 ± 1.58 ng/mL, respectively \( (P > 0.20) \). Concentrations of P4 in the follicular fluid of the 2 largest EI follicles did not differ \( (P > 0.20) \) between control and Twinner cows or between day 3 and day 5 of the estrous cycle (Table 1); however, follicle rank influenced \( (P < 0.001) \) P4 such that P4 concentrations were 6-fold greater in EI F1 than in EI F2 follicles (Table 1). The E2-to-P4 ratio was not affected by genotype, follicle rank, day of cycle, or their interactions \( (P > 0.10); \) Table 1 \) and averaged 0.11 in EI F1 and 0.10 in EI F2.

#### 3.2.3. Receptor mRNA in EI follicles

Abundance of GC IGF2R mRNA in EI follicles tended to be affected \( (P < 0.07) \) by day and genotype by day by follicle rank interaction (Table 2). In Twinner cows, abundance of IGF2R mRNA in GCs of EI follicles tended to be less \( (P < 0.07) \) in F1 on day 5 than in F2 on day 3 (Table 2). Abundance of TC IGF2R mRNA in EI follicles was not affected \( (P > 0.10) \) by any main effect or interaction and averaged 630 and 863 ± 568 relative abundance in control and Twinner cows, respectively \( (P > 0.20) \).

Abundance of FSHR mRNA in EI follicles tended to be affected \( (P < 0.10) \) by day and genotype by day by follicle rank interaction (Table 2); other effects and interactions were not significant. On day 3, abundance of FSHR mRNA in GCs of EI F1 was greater \( (P < 0.05) \) in Twinner than in control cows but did not differ at day 5 (Table 2).
contrast, abundance of FSHR mRNA in EI F2 was greater ($P < 0.05$) in GCs of control cows than of Twinner cows on day 3 (Table 2). Abundance of FSHR mRNA in GCs of control cows (2130 ± 515 relative abundance) was 3-fold greater than in EI follicles (722 ± 255 relative abundance). Abundance of GC LHCGR mRNA in EI follicles was affected by day ($P < 0.05$) but not by genotype, follicle rank, or any 2- or 3-way interactions ($P > 0.10$). Abundance of GC LHCGR mRNA was 2.5-fold greater ($P < 0.05$) in EI follicles on day 5 (1299 ± 506 relative abundance) than on day 3 (515 ± 372 relative abundance). In EI follicles, genotype by day by follicle rank tended to affect ($P < 0.10$) abundance of TC LHCGR mRNA such that in TCs of EI F2 on day 5, abundance of LHCGR mRNA was greater ($P < 0.07$) in control cows than in Twinner cows (Table 2).

### 4. Discussion

Expression of IGF2R, LHCGR, and FSHR mRNA in GCs and TCs of follicles from cattle genetically selected (Twinner) or not selected (control) for multiple ovulations and twin births were characterized with sensitive quantitative RT-PCR analyses. Results indicate that a) abundance of IGF2R mRNA was less in GCs of EA F2 follicles of Twinner cows than of control cows and similar to EA F1 follicles; b) on day 3, abundance of FSHR mRNA was greater in GCs of EI F1 follicles of control cows than of Twinner cows; c) averaged over day 3 and day 5, LHCGR mRNA in GCs and TCs was less and greater, respectively, in EA F2 follicles of control cows compared with Twinner cows; and d) E2, P4, and androstenedione concentrations were greater in EA F1 and F2 follicles of Twinner cows than of control cows.

The decreased abundance of IGF2R mRNA in GCs of EA F2 follicles on day 3 and day 5 of Twinner cows compared with control cows, and the decreased FSHR mRNA abundance in EA F1 follicles on day 3 in Twinner cows compared with control cows coincide with the increased frequency for selection of twin or multiple dominant follicles within a follicular wave in Twinner cattle [19,35]. Perhaps less FSHR expression may prevent any single follicle to respond better to declining FSH concentrations than its competitor follicles, enabling >1 follicle to become dominant. In addition, the decreased abundance of GC IGF2R mRNA expression in EA F2 follicles of Twinner cows compared with control cows concurs with the reported negative relationship between abundance of CYP19A1 mRNA in GCs and IGF2R mRNA in GCs of small (1–5 mm) antral follicles [13].

Thus, it is possible that increased bioavailability of IGF2 (via decreased IGF2R) alters sensitivity of the second largest follicle (i.e., EA F2) to FSH in Twinner cows so that 2 follicles, rather than 1 follicle, are selected within a follicular wave of an estrous cycle. In a previous study, Spicer and Aad [3] characterized IGF2R mRNA in GCs and hypothesized that IGF2R may act as a type of decoy receptor, binding to and inactivating IGF2. This latter suggestion was also made for bovine TCs [6] and is further supported by studies of others [7], indicating that the GC IGF2R does not participate in transmembrane IGF signaling. Greater plasma and intrafollicular IGF1 concentrations in Twinner cows than in control cows [18,19] may be a possible cause for the reduced IGF2R mRNA abundance in Twinner cows because in vitro studies indicate that IGF1 decreases IGF2R binding and IGF2R mRNA abundance in GCs [3].

Recently, Echternkamp et al [13] reported a reduction in abundance of IGF2R mRNA in GCs and TCs of small (1–5 mm) antral follicles of Twinner cows compared with control cows, whereas abundance of CYP19A1 and FSHR mRNA was increased in GCs of these same-sized follicles, and a greater proportion of these follicles were EA in Twinner cows, indicating increased IGF1 stimulation. Therefore, lower abundance of FSHR mRNA in EA F1 on day 3 in Twinner cows than in control cows together with decreased IGF2R mRNA in EA F2 may allow the second largest follicle of Twinner cows to compete equally with the largest follicle and thus gain codominance. It should be noted that mRNA levels do not always correlate with protein levels and are an indirect measure of protein amount and activity. Additional studies should be conducted to verify that changes occur at the protein level.

Earlier findings by Echternkamp et al [18,19] and Echternkamp [36] pointed to the importance of the IGF system and its involvement in increased ovulation rate. Inheritance of components of the IGF system in mammals include imprinted genes such as IGF2 and IGF2R as well as non-imprinted genes such as IGF1 and IGF1R [10,37–39]. Maternal imprinting of IGF2R is regulated by epigenetics [10,11,40,41], and its loss has been linked to various
does not acquire an enhanced ability to respond to FSH as in control EA follicles or is more developed by day 3 than in control cows. Previous studies that evaluated only 3 or 4 animals per day found that in bovine follicles with the use of semiquantitative in situ hybridization techniques FSHR mRNA did not differ significantly between day 0, 2, 4, and 8 of the first follicular wave (ie, day 2–10 of the estrous cycle) [57], and GC FSHR binding did not change significantly between day 2 and 10 of an estrous cycle [58]. In addition, FSHR mRNA abundance did not change after E2-induced atresia of follicles [59] but decreased after the preovulatory LH surge [60] and was less in GCs of large (>8 mm) vs small (1–5 mm) follicles [27]. Reasons for why a decrease in FSHR mRNA was observed between day 3 and day 5 in control cows of the present study may be due to the small sample size used in previous studies because both reported numerical decreases in FSHR of 44% to 54% during the same time period [57,58].

The switch in follicle dependency from FSH to LH after selection may be due to an increased expression of LHCGR and a decreased expression of FSHR in growing follicles [45,53,54]. In the present study, abundance of LHCGR mRNA in GCs and TCs of EA F1 follicles did not differ between control and Twinner cows, whereas GC LHCGR mRNA abundance in EA F2 follicles was less in control cows than in Twinner cows, suggesting that EA F2 in Twinner cows gained their ability to respond to LH. This idea is supported by greater androstenedione levels in F2 follicles of Twinner cows than of control cows on day 3. Previous studies indicated that GC LHCGR binding was greater in dominant than in subordinate follicles on day 5 and 10 of an estrous cycle [25]; did not change significantly in the largest follicle between day 2, 4, 6, and 10 of an estrous cycle [58]; and did not change after E2-induced atresia of follicles [59]. Whether selection of the dominant follicle or expression of LHCGR occurs first is not clear, but LHCGR mRNA is detectable in 1 healthy follicle per cow with a diameter of ≥8 mm [57,61]. Echternkamp [36] suggested that more follicles in Twinner cows were at a more advanced stage of development than in control cows, and the present study supports this suggestion. Because IGF1 induces LHCGR protein in bovine TCs [5] and amplifies FSH-induced LHCGR mRNA in rat GCs [62,63], perhaps elevated IGF1 in Twinner cows plays a role in the increased GC LHCGR mRNA in F2 follicles of Twinner cows. Further research is needed to clarify the sequence of events and changes in GC LHCGR during follicle development in Twinner cattle.

The 2 largest EI follicles were compared to help determine whether the atresia process was different between control and Twinner cattle. The E2-to-P4 ratio of <1 in follicular fluid is commonly used as an index of follicular atresia [47]. In bovine follicles, E2 concentrations decrease and P4 concentrations increase during atresia [64]. Follicles categorized as EI in the present study also suggest that, as atresia progressed from day 3 to day 5, GC abundance of LHCGR mRNA increased and FSHR mRNA decreased regardless of genotype. Moreover, concentrations of E2 or P4 in EI follicles did not differ among genotypes or days, but P4 concentrations in EI F1 were 6-fold greater than in EI F2. In addition, abundance of GC IGF2R mRNA decreased
between day 3 and day 5 in EI F1 of Twinner cows and in EI F2 of control cows. Because changes in follicular variables between day 3 and day 5 in EI follicles were qualitatively similar between control and Twinner cows, it is likely that the atresia process is similar in these 2 genotypes of cattle.

In summary, GC abundance of $IGF2R$ in EA F2 and $FSHR$ in EA F1 was less in Twinner cows than in control cows during growth of follicles early in the follicular wave. Because $IGF2R$ sequesters free IGF2 and, thus, reduces its binding to the IGF1R, we hypothesize that reduced GC $IGF2R$ levels in follicles of Twinner cows may increase the amount of free or bioavailable IGF2. The increased IGF2 may act in an autocrine or paracrine fashion to regulate follicular development and selection of 2 or more dominant follicles.

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