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

P.Y. Aad [a,†], S.E. Echternkamp [b], L.J. Spicer [a,*]

[a] Department of Animal Science, Oklahoma State University, 114 Animal Science Bldg., Stillwater, OK 74078, USA
[b] USDA, US Meat Animal Research Center, Clay Center, NE 68933, USA

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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 ($E_2$), progesterone ($P_4$), 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) $E_2$-active (EA) and $E_2$-inactive (EI) follicles were selected according to their $E_2$-to-$P_4$ 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 ($P < 0.05$) $E_2$ and $P_4$ 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.



## 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

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* Corresponding author. Tel.: +1 405 744 6077; fax: +1 405 744 7390.
*E-mail address:* leon.spicer@okstate.edu (L.J. Spicer).
† Present address: Department of Natural and Applied Sciences, Notre Dame University, Louaizeh, Lebanon.

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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 transrectally 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-2α (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 eppendorf tubes and stored at −20°C until hormone assays. Theca cells were suspended in 0.5 mL of RNA*later* (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 ($E_2$), progesterone ($P_4$), and androstenedione in follicular fluid were determined by RIA as described previously [24,25]. The intra-assay CV was 12% for the $E_2$ RIA, 10% for the $P_4$ RIA, and 14% for the androstenedione RIA. Data for 2 control cows without estrogen-active (EA; $E_2$-to-$P_4$ 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 RNA*later* (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.