Differential abundance of IGF1, bile acids and the genes involved in their signaling in the dominant follicle microenvironment of lactating cows and nulliparous heifers

Ricardo Sanchez, Yasmin Schuermann, Laurianne Gagnon-Duval, Hernan Baldassarre, Vilceu Bordignon, Bruce D. Murphy, Nicolas Gevry, Luis B. Agellon, Raj Duggavathi

PII: S0093-691X(14)00025-9
DOI: 10.1016/j.theriogenology.2014.01.005
Reference: THE 12682

To appear in: Theriogenology

Received Date: 1 November 2013
Revised Date: 29 December 2013
Accepted Date: 1 January 2014

Please cite this article as: Sanchez R, Schuermann Y, Gagnon-Duval L, Baldassarre H, Bordignon V, Murphy BD, Gevry N, Agellon LB, Duggavathi R, Differential abundance of IGF1, bile acids and the genes involved in their signaling in the dominant follicle microenvironment of lactating cows and nulliparous heifers, Theriogenology (2014), doi: 10.1016/j.theriogenology.2014.01.005.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Differential abundance of IGF1, bile acids and the genes involved in their signaling in the dominant follicle microenvironment of lactating cows and nulliparous heifers

Ricardo Sanchez¹, Yasmin Schuermann¹, Laurianne Gagnon-Duval², Hernan Baldassarre¹, Vilceu Bordignon¹, Bruce D. Murphy³, Nicolas Gevry³, Luis B. Agellon⁴ and Raj Duggavathi¹*

1 Department of Animal Science, McGill University, Ste-Anne-de-Bellevue
2 Centre de recherche en reproduction animale, Université de Montréal, St-Hyacinthe
3 Département de Biologie, Université de Sherbrooke, Sherbrooke
4 School of Dietetics and Human Nutrition, McGill University, Ste-Anne-de-Bellevue

Short title: Metabolic stress in dominant follicle

* Correspondence: raj.duggavathi@mcgill.ca, Ph: +1 514 398-7803
ABSTRACT

It is well documented that incidence of fertility problems is high in lactating cows but not in heifers of the same genetic merit. Understanding the metabolic and molecular differences between fertile heifers and relatively infertile lactating cows will help us understand the pathogenesis of infertility in dairy cows. Follicular waves in lactating cows (30-50 days in milk; N = 12) and heifers (N = 10) were synchronized by ultrasound-guided follicle ablation. Follicular fluid and granulosa cells of the dominant follicle were collected by ultrasound-guided aspiration along with blood sampling on Day 6 after synchronization. Dominant and subordinate follicles were larger in lactating cows than in heifers. Metabolic stress in lactating cows was evidenced by lower glucose and higher β-hydroxy butyric acid (BHBA) compared to heifers. Insulin-like growth factor 1 (IGF1) signaling was reduced in the dominant follicle in lactating cows through reduced IGF1 concentrations in plasma and follicular fluid of the dominant follicle, and reduced expression of pregnancy associated plasma protein A (PAPPA) in their granulosa cells. We also found increased levels of total bile acids in the follicular fluid of the dominant follicle of lactating cows compared to heifers. Granulosa cells of the dominant follicle had higher expression of SLC10A2 and GPBAR1 (bile acid transporter and receptor, respectively) in lactating cows. These novel data are indicative of increased bile acid signaling within the dominant follicles of lactating cows compared to heifers. Overall, we demonstrate in the present study the metabolic, endocrine and molecular differences within the microenvironment of the dominant follicles in lactating cows and heifers. These differences in follicular microenvironment may contribute toward abnormal ovarian function in lactating dairy cows.
1. INTRODUCTION

Fertility during the early postpartum period in high-producing dairy cows has declined over the past few decades [1] requiring premature culling of infertile cows and causing economic burden on dairy enterprises. There is consensus that the altered metabolic milieu in lactating cows, characterized by low plasma levels of glucose and high non-esterified fatty acids (NEFA) and BHBA, is the major cause of the ovarian dysfunction that results in infertility [2-6]. At this time, the mechanisms responsible for impaired ovarian function in lactating cows remain to be completely elucidated.

Intriguingly, fertility in nulliparous heifers with the genetic potential for milk production has been consistently high [7-10]. Some differences between the two populations have been identified; lactating cows have higher incidence of double ovulation [11], larger dominant follicles [12], lower progesterone concentrations [11, 12] and lower estradiol concentration at estrus [9] than do heifers. Thus, understanding the metabolic and molecular differences between heifers and lactating cows may enable us to develop novel therapeutic/management strategies to improve reproductive success in lactating dairy cows.

Very few studies have compared metabolic parameters of lactating cows with those of heifers. Among these is an elegant study from Bender et al. [13] reporting that the microenvironment of the dominant follicle in cows was different from that in heifers. However, whether such altered follicular fluid composition is associated with an altered gene expression pattern in follicular granulosa cells has not been tested. Therefore, our goal was to study metabolic differences in plasma and follicular fluid, and gene expression in granulosa cells of lactating cows as compared to those of heifers.

IGF1 is important for ovarian function. It is the major metabolic hormone that is reduced in lactating cows, potentially due to changes in liver function during early lactation [4, 6]. However, there is little direct evidence for variant IGF1 concentrations
between heifers and lactating cows at the level of follicular microenvironment. Further, overall IGF1 signaling is a result of interactions among signaling molecules of the IGF system, composed of IGF-1 and IGF-2; six IGF-binding proteins (IGFBP-1 to -6) and proteases of IGFBPs [14, 15]. Pregnancy associated plasma protein A (PAPPA) has been shown to be the main IGFBP protease regulating the levels of free IGF1 in bovine dominant follicle [14, 16]. However, specific molecular features underlying the IGF system of the dominant follicle in lactating cows relative to that in heifers have not been reported.

Recent studies in dairy cows have revealed that the liver undergoes major enhancement of its bile acid synthetic machinery during the onset of lactation [17, 18]. An increase in plasma levels of total bile acids during early lactation has been demonstrated in several species including rats [19] and sows [20]. This increase has been attributed to the increased demand for cholesterol and triglycerides for milk production [21]. Interestingly, recent studies demonstrate that bile acids can act as endocrine signals through their membrane receptor, G protein-coupled bile acid receptor 1 (GPBAR1) [22]. Due to the widespread expression of GPBAR1, bile acids can regulate cellular functions in multiple tissues [23]. Bile acids have been linked to gonadal steroid synthesis through their nuclear receptor, NR1H4, which inhibits the transcriptional activity of another nuclear receptor, NR5A2 via NR0B2 [24]. However, links between bile acids and follicular microenvironment in dairy cows have never been examined.

The objectives of our study were to determine: 1. the levels of IGF1 and total bile acids in the plasma and follicular fluid of the dominant follicle of a synchronized follicular wave in lactating cows and nulliparous heifers; 2. the mRNA abundance of the genes for IGF and other proteins known to be involved in bile acid metabolism in granulosa cells of the dominant follicle of a synchronized follicular wave in lactating cows and nulliparous heifers.
2. MATERIALS AND METHODS

2.1. Animal model

All experimental procedures using cattle were approved by the Animal Care and Use Committee of McGill University. Heifers (n = 10; age 12.95 ± 0.99 months) used in this study were nulliparous with a history of normal health and estrous cyclicity. Lactating cows (n = 12; 3-5 years old; 44.69 ± 1.51 days in milk) in their second or third lactation and free of disease (e.g. milk fever, mastitis, etc) were recruited during the period of 30-50 days into their lactation.

2.2. Follicular wave synchronization and sample collection

The emergence of a new follicular wave was induced by ultrasound-guided aspiration of all follicles ≥5 mm in diameter [25]. Following follicular aspiration, animals were treated with a luteolytic dose of prostaglandin F2α (Lutalyse®, 2 mL, im) and an intra-vaginal progesterone releasing devise (Eazi-Breed™ CIDR®) to maintain a similar progesterone milieu in both groups of animals. With this treatment regimen, a new follicular wave was expected to emerge between 36 to 48h after follicular aspiration (Day 0). On Day 6, the implant was removed and transrectal ultrasonography was performed to map the position and size of all follicles ≥3 mm in diameter. The total number and position of all follicles were mapped, and the diameter of the dominant follicle was noted. The follicular fluid of the dominant follicle was collected by ultrasound-guided aspiration [26]. The aspirated fluid was centrifuged at 3000 rpm for 5 min to collect granulosa cells, which were then incubated with red-blood-cell lysis buffer (2.075g NH4Cl, 0.25g KHC3 and 5% EDTA in distilled water) for 5 min. Both follicular fluid and granulosa cells of each dominant follicle were snap frozen in liquid nitrogen and stored at -80 °C until molecular analyses. Blood samples were also collected from the coccygeal vein in heparinized
vacutainers (366450, BD Glass tubes with K3EDTA) on Day 6. Plasma samples were
stored at –80 °C until endocrine and biochemical analyses.

2.3. Endocrine and biochemical analyses
Glucose, BHBA and total bile acids in plasma and follicular fluid were analyzed on a RX
Monza Analyzer (RX2901, Randox Laboratories) using kits: Glucose assay (GL2623,
Randox Laboratories), Ranbut (RB1007, Randox Laboratories) and Total Bile Acid Kit
(Diazyme Laboratories). Cholesterol and triglycerides were analyzed by enzymatic
colorimetric method using Genzyme cholesterol-SL and Triglycerides SL kits,
respectively. Total IGF-1 was measured using an IGF-1 ELISA kit (Immunodiagnostic
Systems Ltd).

2.4. Measurement of mRNA abundance by quantitative PCR (qPCR)
Total RNA was extracted from granulosa cells using the Directzol RNA miniprep isolation
kit (Zymo Research) and then cDNA was synthesized from 100 ng total RNA using the
iScript cDNA Synthesis kit (Biorad). Primers were designed using transcript sequences
retrieved by the UCSC genome browser (assembly: Oct. 2011 (Baylor
Btau_4.6.1/bosTau7) and by Primer-BLAST (Table 1). All qPCR assays were performed
on CFX384 thermal cycler (BioRad) using iQ™ SYBR® Green Supermix using the
following profile: an initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C
for 15 s and 58°C for 30s. The mRNA abundance data were analyzed by standard
curve method. Ribosomal protein L19 (RPL19) was used as the reference gene to
determine relative mRNA abundance.

2.5. Statistical Analyses
Analyses were performed using SAS 9.3 PROC IML. Normality of data was confirmed by Shapiro-Wilk test. Means (± SEM) were compared by unpaired Student’s t-test. A significance level of $P < 0.05$ was employed.

### 3. RESULTS

#### 3.1. Ovarian status at the time of the dominant follicle aspiration

On the day of follicular sample collection, the diameter of the largest follicle was greater than the second largest follicle ($P < 0.001$) in both heifers and lactating cows confirming that the largest follicle was the dominant follicle (Fig. 1A). Both the dominant and subordinate follicles were larger in lactating cows than the dominant and subordinate follicles from heifers ($P < 0.001$; Fig. 1A), respectively. There was no difference in the number of subordinate follicles between cows and heifers ($P > 0.05$, Fig. 1B).

#### 3.2. Glucose, triglycerides and BHBA in plasma and follicular fluid of the dominant follicle

Lactating cows had lower glucose concentration than heifers (Fig. 2A) both in plasma ($P = 0.05$) and follicular fluid ($P < 0.01$) of the dominant follicle. Plasma concentrations of triglycerides were lower in lactating cows ($P < 0.03$) than heifers, but the triglyceride levels in follicular fluid of the dominant follicle did not differ between groups ($P > 0.05$, Fig. 2B). While plasma concentrations of BHBA tended to be higher in lactating cows than heifers ($P = 0.07$, Fig. 2E), their follicular fluid levels were significantly higher in cows than heifers ($P < 0.03$, Fig. 2F).
3.3. Plasma and follicular fluid concentrations of IGF1, bile acids and cholesterol

Concentrations of IGF1, bile acids and cholesterol in plasma and follicular fluid of the dominant follicle in lactating cows and heifers are shown in Figure 3. Concentrations of IGF1 were lower in lactating cows than heifers in both plasma (P<0.001) and follicular fluid (P<0.001). The plasma concentrations of total bile acids were higher in lactating cows than heifers (P < 0.01). Follicular fluid of lactating cows contained higher levels of total bile acids than heifers (P < 0.05). Similarly, concentrations of total cholesterol were higher in lactating cows than heifers in both plasma (P < 0.003) and follicular fluid of the dominant follicle (P < 0.004).

3.4. Genes of IGF network in granulosa cells of the dominant follicle

We assessed the mRNA abundance of nine genes involved in IGF signaling in granulosa cells: IGF1, IGF2 and their receptors, IGF1R and IGF2R, IGF binding proteins (IGFBP2, 3, 4 and 5) and the major IGFBP protease in bovine follicular fluid, PAPPA [14, 16, 27, 28]. IGFBP1 and 6 were not included in our analyses as they are not highly expressed nor regulated in granulosa cell of bovine follicles [29].

Relative mRNA abundance of IGF1, IGF1R, IGF2, IGF2R, IGFBP2, 3, 4 and 5 did not differ between heifers and lactating cows (P > 0.05, Fig. 4). However, the relative mRNA abundance of PAPPA was markedly lower in granulosa cells of the dominant follicle in lactating cows as compared to heifers (P < 0.02, Fig. 4).

3.5. Genes involved in bile acid signaling in granulosa cells of the dominant follicle

We assessed the mRNA abundance of six genes involved in bile acid signaling: membrane-bound bile acid transporter, SLC10A2; transmembrane receptors of bile
acids, GPBAR1 [30] and S1PR2 [31, 32]; and nuclear receptors of bile acid signaling,
NR1H4, NR0B2 and NR5A2 [33]. Relative mRNA levels of SLC10A2 were higher in
granulosa cells of lactating cows than those of heifers (P < 0.02, Fig. 5). Transcript levels
of GPBAR1 (P < 0.05), but not S1PR2 (P > 0.05) were higher in granulosa cells of
lactating cows than heifers (Fig. 5). The expression of NR1H4, NR0B2 and NR5A2
genes was similar in granulosa cells of the dominant follicles in heifers and lactating
cows (P > 0.05, Fig. 5).

4. DISCUSSION

It has been well documented that dairy cows experience negative energy
balance as a consequence of increase in nutrient demand for lactation and inability of
cows to meet these demands by feed intake [34, 35]. Negative energy balance is
characterized by changes in hormones and metabolites in blood circulation. These
alterations appear to underlie infertility in lactating cows, as heifers of the same genetic
background do not suffer from reproductive failure [11]. Thus, establishing differences
between heifers with high fertility and lactating cows with low fertility, and determining
how many of these are represented at the ovarian follicular level is important for
understanding the pathogenesis of infertility during early lactation.

In the present study, we found that both the dominant and subordinate follicles in
lactating cows were substantially larger than their respective counterparts in nulliparous
heifers. Previous studies have shown larger diameters of the dominant follicle in cows
than heifers [9, 12], but the diameter difference observed in these studies was less
compared to our study. This discrepancy appears to be due to differences in the time at
which dominant follicle diameters were measured. We measured on Day 6 of the
follicular wave when the dominant follicle would still be in the growing phase, in contrast
to other studies [9, 12] that reported diameters on the day before ovulation. Nonetheless,
the dominant follicle in cows appears to grow faster to larger diameters than in heifers. In agreement with our data, others studies have reported similar numbers of smaller follicles between cows and heifers [9, 12]. Therefore, it appears that the follicular growth, but not the number of follicles, is affected in lactating cows. This increased follicular growth has been attributed to lower estradiol and progesterone levels as a result of increased catabolism of these steroids [36, 37], thereby leading to higher concentrations of follicle stimulating hormone (FSH) in cows as compared to heifers [9, 12]. In another study, decreased steroid concentrations in lactating cows were attributed to the reduced steroidogenic gene expression in follicles of cows as compared to heifers [38].

During peak lactation in the cow, over 80% of the available glucose in the body is partitioned to the mammary gland for milk production and the majority of fats mobilized from adipose tissue contribute to milk fat synthesis [34]. Lower glucose in plasma of cows compared to heifers is indicative of increase glucose mobilization toward milk production [35]. Higher BHBA and lower triglycerides in plasma of lactating cows are indicative of increased lipid mobilization and fatty acid oxidation [2, 39]. The altered metabolite imbalance of lower glucose and higher BHBA found in plasma were reflected in the microenvironment of the dominant follicle in cows. While lower glucose has been shown to negatively affect oocyte maturation in vitro [40], direct effects of BHBA on oocyte maturation or granulosa cell function have not been reported. Nonetheless, high BHBA levels in follicular fluids of lactating cows have been shown to be associated with inferior quality of oocytes [41]. Overall, metabolic profiles of plasma and the dominant follicles clearly demonstrate that the lactating cows used in the present study were in negative energy balance and subjected to metabolic stress as compared to nulliparous heifers.

As expected, lactating cows in the present study had lower plasma IGF1 concentrations compared to heifers. While numerous studies have shown reduced IGF1
levels in circulation of lactating cows, very few studies have reported IGF1 concentrations in the follicular fluid [42]. Our data of reduced IGF1 levels in the follicular fluid of the dominant follicle of cows compared to heifers are in agreement with a previous study [38]. While the main source of IGF1 is the liver, levels of IGF1 and IGF2 are locally regulated in many tissues including ovaries. Granulosa cells express IGF1 and IGF2 genes, which are regulated during dominant follicle selection [38, 43]. Our qPCR analyses showed that there was no difference in mRNA abundance of both genes in granulosa cells of the dominant follicles in lactating cows and heifers. Thus, granulosa cells may not regulate IGF1 concentrations within the microenvironment of ovarian follicles in lactating cows. Also, lower IGF1 concentrations in the dominant follicular microenvironment of lactating cows are probably due to reduced IGF1 production in the liver, which is the main source of IGF1 in circulation.

The ultimate bioavailability of IGF1 is the result of an orchestrated interplay among IGFBPs and their proteases such as PAPPA [14, 16, 44]. Similar mRNA abundance of IGF1R in granulosa cells of the dominant follicle of both lactating cows and heifers indicates that metabolic differences between these two groups do not affect the receptor expression. Major IGFBPs regulated during follicular selection are IGFBP2, 3, 4 and 5 [45]; specifically mRNA abundance of IGFBP2 and 4 has been shown to decrease in granulosa cells of the dominant follicle in multiple species including the cattle, sheep and humans [16]. None of these major IGFBPs were differentially expressed in granulosa cells of cows and heifers in the present study.

It is well established that higher expression of PAPPA in granulosa cells [10, 14, 16, 29, 43, 44] leading to proteolytic degradation of both IGFBP4 and 5, is the basis for the selection of the dominant follicle in cattle [16, 27]. Higher levels of PAPPA enhance intra-follicular free IGF in the dominant follicle, thereby increasing steroidogenesis [46] [47]. Granulosa cells of the dominant follicle in lactating cows had lower levels of PAPPA
mRNA as compared to those of heifers. Taken together, reduced IGF1 along with reduced IGF1 expression are indicative of compromised IGF1 signaling in the dominant follicle of lactating cows. Reduced PAPPA mRNA, without change in the expression of its substrates, the IGFBPs, in granulosa cells of cows may result in decreased availability of free IGF1, further exacerbating compromised IGF signaling. This perturbed IGF system in the dominant follicle of lactating cows may also contribute toward their reduced steroidogenic ability [38].

It is well established in rats that the liver undergoes a remarkable increase in its bile acid synthesizing capability during lactation [48]. Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme of the classical pathway of bile acid synthesis from cholesterol [49]. Recent studies have demonstrated increases in the hepatic expression of CYP7A1, during early lactation in rats [50] and dairy cows [17]. Accordingly, we observed that lactating cows had three-fold higher total bile acids in plasma than heifers. Three-fold higher levels of total bile acids were also seen in the follicular fluid of the dominant follicle in lactating cows relative to that in heifers. In line with this, total cholesterol concentrations were also significantly higher in plasma and dominant follicle follicular fluid in cows than heifers. To our knowledge, this is the first time that detection of bile acids in bovine follicular fluid has been reported. These data suggest that bile acids of hepatic provenance reach the microenvironment of ovarian follicles. It is therefore hypothesized that bile acids affect follicular cells, granulosa and theca cells and the oocyte. In support of this hypothesis, mRNA abundance of SLC10A2, the bile acid transporter, and GPBAR1, the membrane receptor of bile acids, was higher in granulosa cells of the dominant follicle of lactating cows than heifers. We speculate that bile acid signaling within ovarian follicles may be enhanced in lactating cows suffering from fertility problems.
Although nothing is known about the potential effects of bile acids on follicular granulosa cells and ovarian functions, one can speculate a possible bile acid signaling network based on our data. Discovery of GPBAR1 as a membrane receptors and its widespread expression in multiple organs suggests that bile acids can function as signaling molecules [23] [30]. Indeed, bile acids have been shown to enhance energy expenditure in brown adipose tissue in a GPBAR1-dependent manner via protein kinase A (PKA) in mice [51]. Differential expression of GPBAR1 in lactating cows and heifers hints toward potential actions of bile acids on granulosa cells. It is known that bile acids impinge upon the transcription of their target genes (e.g. CYP7A1 in hepatocytes) through NR1H4 mediated induction of NR0B2, which in turn represses another nuclear receptor, NR5A2 [33]. We previously demonstrated in mice that Nr0b2 represses the expression of the steroidogenic acute regulatory protein (Star) through its inhibitory actions on Nr5a2, and that Nr5a2 is essential for ovarian functions [53, 54]. Taken together, the expression of the genes associated with bile acid signaling in granulosa cells is indicative of the potential effects of bile acids on granulosa cells. Further experiments are warranted to demonstrate a direct effect of bile acids on ovarian granulosa cells.

5. CONCLUSION
In the present study we compared biochemical, hormonal and granulosa cell gene expression patterns in the dominant follicle of lactating cows with those of heifers. There was clear evidence for increased metabolic stress in lactating cows in terms of follicular growth and metabolite alterations. IGF1 signaling was reduced in the dominant follicle microenvironment in lactating cows through reduced IGF1 concentrations and reduced expression of PAPPA in their granulosa cells. We also demonstrate, for the first time, that bile acids are present in bovine follicular fluid and are at increased levels in
dominant follicles of lactating cows. Higher abundance of \textit{SLC10A2} and \textit{GPBAR1} transcripts is supportive of the hypothesis that increased bile acids within follicular microenvironment may contribute toward abnormal ovarian function in lactating dairy cows.

\textbf{ACKNOWLEDGEMENTS:}

This work was supported by Programme Projet de Recherche en Equipe from Fonds de Recherche du Quebec – Nature et Technologies (FRQNT) to NG, VB, BDM and RD; a Discovery Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada (371850-09 to RD). Authors would like to thank Ms. Susan Cook and Ms. Gloria Patry (Prairie Diagnostic Services Inc., Saskatoon) for excellent technical service. Authors are also thankful to Dr. Roger Sauvé (Clinique Vétérinaire St-Louis-Embryobec, Quebec), Mr. Paul Meldrum and Dr. Dana Praslickova for help with experiments. RS was supported by the Graduate Excellence Fellowship of the Department of Animal Science. YS was supported by the RQR-CREATE scholarship and Graduate Excellence Fellowship of the Department of Animal Science. LGD was supported by an NSERC undergraduate student research award.

\textbf{REFERENCES}


et al. Differential gene expression in liver and small intestine from lactating rats 
compared to age-matched virgin controls detects increased mRNA of cholesterol 

[20] Hedemann MS, Flummer C, Kristensen NB, Theil PK. Metabolic profiling of plasma 
from sows before parturition and during lactation using a liquid chromatography-mass 

and lactation on lipoprotein and cholesterol metabolism in the rat. Journal of lipid 

[22] Smith Z, Ryerson D, Kemper JK. Epigenomic regulation of bile acid metabolism: 
emerging role of transcriptional cofactors. Molecular and cellular endocrinology. 
2013;368:59-70.

[23] Zwicker BL, Agellon LB. Transport and biological activities of bile acids. The 

receptor alpha: a molecular link between bile acids and steroid signaling? Cellular and 

[25] Berfelt DR, Lightfoot KC, Adams GP. Ovarian synchronization following ultrasound-

al. In vivo collection of follicular fluid and granulosa cells from individual follicles of 
different diameters in cattle by an adapted ovum pick-up system. Reproductive biology 

[27] Rivera GM, Fortune JE. Selection of the dominant follicle and insulin-like growth 
factor (IGF)-binding proteins: evidence that pregnancy-associated plasma protein A
contributes to proteolysis of IGF-binding protein 5 in bovine follicular fluid.


Table 1. List of primer sets used in real-time PCR analyses

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene name</th>
<th>Primer sequence (5' to 3')</th>
<th>Annealing Temp (C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_175049.3</td>
<td>GPBAR1</td>
<td>AGGGTGGACCTTGACTTGAA</td>
<td>57</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGTGGGTCATACAGGGGAC</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>NM_001077828.1</td>
<td>IGF1</td>
<td>TGTCACTGCTAAAATCCAGAGCA</td>
<td>59</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCTGAAGTGAAGAATGGGAA</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_001244612.1</td>
<td>IGF1R</td>
<td>CACATCCGTCAATTTCCA</td>
<td>60</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATGACACGGGGCGTAGTTG</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_174087.3</td>
<td>IGF2</td>
<td>GTGCTTTGCTCTTCCGGGC</td>
<td>60</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCAACACTCTTTACAGATGC</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_174352.2</td>
<td>IGF2R</td>
<td>CGATGAGAGTGAGAGAGAGG</td>
<td>60</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGGATTAGGGATCGAGGT</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_174555.1</td>
<td>IGFBP2</td>
<td>CGGGAACGTAACTGATGG</td>
<td>59</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGTGATGTTTGGCACCCTTG</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_174556.1</td>
<td>IGFBP3</td>
<td>AGCAGCTATTCAAGCTGAGT</td>
<td>59</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AACGTCAGTGCGGTCAAGG</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>NM_174557.4</td>
<td>IGFBP4</td>
<td>AAGATGAGGTCATCAGGGGC</td>
<td>60</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCAGTTGGAGATGGGAATGA</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_001105327.2</td>
<td>IGFBP5</td>
<td>GCAAGGCAAAGATCGAAAGAG</td>
<td>60</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTGGGTCTAGCTTCTTTCTGC</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_001040516.1</td>
<td>L19</td>
<td>GCCAATCCCTCCGTACAGA</td>
<td>60</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGCTGTACCTTTCCGCTT</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_001034708.2</td>
<td>NR1H4</td>
<td>TGTGTTGTCTGGAAAGAGCCG</td>
<td>60</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCGACACTCTTTGGCACTTCC</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>XM_592675.2</td>
<td>NR0B2</td>
<td>GTCTCTAGCCCTTCATCCAC</td>
<td>60</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCTCAATGACAGGGCGAGAG</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_001206816</td>
<td>NR5A2</td>
<td>CTACAGAATGACGCAAGGC</td>
<td>60</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCCACGTAGGAGTAGGCCAT</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NM_175049.3</td>
<td>S1PR2</td>
<td>ATGGAGGGAGGAGATATTGC</td>
<td>54</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGCAGCTCTACCTACAGC</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>XM_002689953.2</td>
<td>PAPPA</td>
<td>TGCATGGAGACAGAGCCCT</td>
<td>61</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGTCGTCATCTGCATAGCTC</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>XM_604179.7</td>
<td>SLC10A2</td>
<td>AGCAACCCAGGGTTGACCAT</td>
<td>60</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGCTAACCACCTTGAGGAG</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Ovarian status at the time of the dominant follicle aspiration. Mean (±SEM) diameter of the dominant (DF) and subordinate (SF) follicles (top panel), and total number of follicles in heifers and lactating cows on Day 6 of the synchronized follicular wave.

Figure 2. Glucose, triglycerides and β-hydroxy butyric acid (BHBA). Mean (±SEM) concentrations of glucose, triglycerides and BHBA in plasma and follicular fluid of the dominant follicle in heifers and lactating cows. Follicular fluid samples were collected by ultrasound guided follicular aspiration of the dominant follicle on Day 6 of the synchronized follicular wave. NS, non-significant (P > 0.05).

Figure 3. Insulin-like growth factor-1 (IGF1), bile acids and cholesterol. Mean (±SEM) concentrations of IGF1, bile acids and cholesterol in plasma and follicular fluid of the dominant follicle in heifers and lactating cows. Follicular fluid samples were collected by ultrasound guided follicular aspiration of the dominant follicle on Day 6 of the synchronized follicular wave.

Figure 4. Genes of IGF network in granulosa cells of the dominant follicle. Relative mRNA abundance (Mean±SEM) of insulin-like growth factors, and their receptors and binding proteins (IGF1, IGF1R, IGF2, IGF2R, IGFBP2, 3, 4 and 5), and pregnancy associated plasma protein A (PAPPA) in granulosa cells of the dominant follicle in heifers and lactating cows. Granulosa cells were collected by ultrasound guided follicular aspiration of the dominant follicle on Day 6 of the synchronized follicular wave. NS, non-significant (P > 0.05).
Figure 5. Genes involved in bile acid signaling in granulosa cells of the dominant follicle. Mean (± SEM) relative mRNA abundance of membrane-bound bile acid transporter, SLC10A2; transmembrane receptors of bile acids, GPBAR1 and S1PR2, and the nuclear receptors NR1H4, NR0B2 and NR5A2 in granulosa cells of the dominant follicle in lactating cows and heifers. Granulosa cells were collected by ultrasound-guided follicular aspiration of the dominant follicle on Day 6 of the synchronized follicular wave. NS, non-significant (P > 0.05).
Table 1. List of primer sets used in real-time PCR analyses

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Annealing Temp (C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_175049.3</td>
<td>GPBAR1</td>
<td>AGGGTGGACCTTGACTTGAA AGTGGGGTCTACAGGGACAG</td>
<td>57</td>
<td>131</td>
</tr>
<tr>
<td>NM_001077828.1</td>
<td>IGF1</td>
<td>TGTCAGTGGTAAATTCAGGCA TCTGAAGTGCAAAAGTCTGAA</td>
<td>59</td>
<td>158</td>
</tr>
<tr>
<td>NM_001244612.1</td>
<td>IGF1R</td>
<td>CACATCTGTCTATTCCAA GATGACGGGCGAGTTGT</td>
<td>60</td>
<td>180</td>
</tr>
<tr>
<td>NM_174087.3</td>
<td>IGF2</td>
<td>GTGCTTCTTGCCTCCCTG       GCAACCTCTCCACGATGC</td>
<td>60</td>
<td>183</td>
</tr>
<tr>
<td>NM_174352.2</td>
<td>IGF2R</td>
<td>CGATGAGAGTGAGGACAGCG TGGATTGAGGATCGGAGG</td>
<td>60</td>
<td>179</td>
</tr>
<tr>
<td>NM_174555.1</td>
<td>IGFBP2</td>
<td>CGGGAGCTGAATTCTGAGG      GGTGATGTTGGGCCACCTTG</td>
<td>60</td>
<td>137</td>
</tr>
<tr>
<td>NM_174556.1</td>
<td>IGFBP3</td>
<td>AGCAGCTATTTCAAAGCTGATG AACGTCAGTGCGTTCAAGAG</td>
<td>59</td>
<td>177</td>
</tr>
<tr>
<td>NM_174557.4</td>
<td>IGFBP4</td>
<td>AAGATGAGGTCATCGGGGC GCAGTGGGAGGATGGGAATGA</td>
<td>60</td>
<td>150</td>
</tr>
<tr>
<td>NM_001105327.2</td>
<td>IGFBP5</td>
<td>GCAAGCCAAGATCGAAAGAG CTGGTGACCTTTCTGTC</td>
<td>60</td>
<td>163</td>
</tr>
<tr>
<td>NM_001040516.1</td>
<td>L19</td>
<td>GCCAACTCGGTCAGACAGT     TGGCTGTACCCTCCGCTT</td>
<td>60</td>
<td>154</td>
</tr>
<tr>
<td>NM_001034708.2</td>
<td>NR1H4</td>
<td>TGTGTGCTCTTGCGGACGCG   TCGACACTCTTGCGGACTTT</td>
<td>60</td>
<td>171</td>
</tr>
<tr>
<td>XM_592675.2</td>
<td>NR0B2</td>
<td>GTCTCTACGCCTCATCCCCAC TCTCAATGCAGGAGGGAAGG</td>
<td>60</td>
<td>187</td>
</tr>
<tr>
<td>NM_001206816</td>
<td>N5A2</td>
<td>CTACAGACTACGACCGACGC   TCCACGAGAGTACACCAT</td>
<td>60</td>
<td>178</td>
</tr>
<tr>
<td>NM_175049.3</td>
<td>S1PR2</td>
<td>ATGGAGGAGAGAGGATATTGC TGGCAGCTCTACAGACAG</td>
<td>54</td>
<td>136</td>
</tr>
<tr>
<td>XM_002699953.2</td>
<td>PAPPA</td>
<td>TGACATGGAGACGAGGGCGCT   CAGTCATCGCTAGCTGGA</td>
<td>61</td>
<td>170</td>
</tr>
<tr>
<td>XM_604179.7</td>
<td>SLC10A2</td>
<td>AGCAACCCAGTGGTACCAT GGGCTAACCACCTTTGAGG</td>
<td>60</td>
<td>151</td>
</tr>
</tbody>
</table>
Figure 1 Sanchez et al

Diameter (mm)

Follicle number

Heifers
Lactating cows

P < 0.001

DF
SF

0 4 8 12 16 18

0 4 8
Figure 2 Sanchez et al

Plasma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Heifers</th>
<th>Lactating cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.0</td>
<td>0.00</td>
</tr>
<tr>
<td>P</td>
<td>0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>Triglycerides (umol/l)</td>
<td>0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>BHBA (mmol/l)</td>
<td>1.0</td>
<td>0.00</td>
</tr>
<tr>
<td>P</td>
<td>0.07</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

Follicular fluid

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Heifers</th>
<th>Lactating cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.0</td>
<td>0.00</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Triglycerides (umol/l)</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>0.06</td>
</tr>
<tr>
<td>BHBA (mmol/l)</td>
<td>0.8</td>
<td>0.00</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.03</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Heifers
Lactating cows

P < 0.03 NS
P = 0.07 P < 0.03
Figure 3 Sanchez et al

Plasma

IGF1 (ng/ml)

0

70

140

P < 0.001

Heifers

Lactating cows

Follicular fluid

Bile Acids (umol/l)

0

20

40

P < 0.01

Plasma Follicular fluid

P < 0.001

P < 0.003

P < 0.004

P < 0.01

P < 0.05

Cholesterol (mmol/l)

0

0.8

1.6

P < 0.001

Heifers

Lactating cows
Figure 4 Sanchez et al

Heifers  
Lactating cows
Figure 5

Sanchez et al

- **SLC10A2**
  - *P* < 0.02
  - NS

- **GPBAR1**
  - *P* < 0.05
  - NS

- **S1PR2**
  - NS

- **NR1H4**
  - NS

- **NR0B2**
  - NS

- **NR5A2**
  - NS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Heifers</th>
<th>Lactating cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC10A2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPBAR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1PR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1H4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR0B2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR5A2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heifers vs. Lactating cows