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Gene expression in swine granulosa cells and ovarian tissue during the estrous cycle

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ABSTRACT. The components of the insulin-like growth factor (IGF) system appear to be involved in regulation of ovarian follicular growth and atresia in the pig. We investigated the expression pattern of mRNAs for IGF1 (*IGF1*), its binding proteins (*IGFBP1*, *IGFBP2*, *IGFBP3*, and *IGFBP5*), and epidermal growth factor in swine follicle cells and ovarian tissue throughout the estrous cycle using the real-time quantitative PCR technique. The results of gene expression were analyzed using linear regression with gene expression as a dependent variable and days of estrous cycle as an independent variable. Additionally, an analysis was made of the correlation of expression levels with plasma concentration of follicle-stimulating hormone, luteinizing hormone, estradiol-17β, progesterone, and prolactin. Expression of mRNA of all of these genes was detected in granulosa cells and ovarian tissue. *IGFBP3* mRNA showed a quadratic expression pattern ($P \leq 0.001$) and was significantly and positively correlated with progesterone ($r = 0.81$; $P \leq 0.01$) but negatively correlated with prolactin ($r = -0.596$; $P \leq 0.05$). Expression

of the other genes was unaffected by the stage of the estrous cycle. Real-time quantitative PCR effectively detected all transcripts, including the very low levels of *IGFBP1* transcripts, and could be used for studies of follicle dynamics.

Key words: Follicular development; Growth factors; RT-PCR

INTRODUCTION

During a regular estrous cycle, a complex regulatory network interacts to regulate ovarian follicular growth and function through autocrine and paracrine mechanisms. These events are marked by a high dynamism of gene expression and involve endocrine regulation by pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), but also include the action of steroids and peptides (Drummond, 2006) such as insulin-like growth factor 1 (*IGF1*), that acts as an amplifier of gonadotropin hormonal action (Adashi, 1998), and epidermal growth factor (EGF)-signaling network that potentializes LH action and modifies granulosa cell differentiation (Urban and Veldhuis, 1992; Hattori et al., 1995). Moreover, the IGF1 biological availability may be closely regulated by the presence of IGF-binding proteins (*IGFBP*) in follicular tissue (Mazerbourg et al., 2003).

In pigs, some experiments by *in situ* hybridization have allowed the detection of gene expression in granulosa cells and corpus luteum during the growth of antral follicles and luteal phase, respectively. These studies mainly describe genes involved in follicular development such as IGF system and growth factors (Samaras et al., 1993; Zhou et al., 1996; Gadsby et al., 1996) and the expression of genes involved in steroidogenesis (Guthrie et al., 1994; Conley et al., 1994; Balasubramanian et al., 1997). Despite numerous indications that the IGF system is important to ovarian follicular growth and development, the techniques used in previously reported studies were less accurate and an abundance of *IGFBP1* has not been detected in porcine granulosa cell. Also, few transcriptomic data are available concerning the pig ovarian function (Bonnet et al., 2008).

The quantitative real-time RT-PCR technique has proven to be a powerful method to quantify gene expression and would help to elucidate many of the specific mechanisms involved in follicular development, including the initiation of primordial follicle growth, antrum formation, follicular selection, and atresia. Thus, the aim of this study was to investigate the pattern of mRNA expression of most constituents of the IGF-system (*IGF1*, *IGFBP1*, *IGFBP2*, *IGFBP3*, and *IGFBP5*) and *EGF* in granulosa cells and ovarian tissue as a function of the day of estrous cycle and its correlation with FSH, LH, estradiol-17β (E_2), progesterone (P_4), and prolactin (PRL) plasma concentration.

MATERIAL AND METHODS

Animals and ovary collection

Sixteen prepubertal gilts (*Sus scrofa*, Landrace x Large White x Pietrain) used in this study were obtained from the pig farm at the Federal University of Viçosa (Viçosa, MG, Brazil). Animals were treated with P.G 600® (400 IU eCG and 200 hCG). The onset of estrus was

checked twice a day using a mature boar. The first day of estrus was designated as day 0 of the estrous cycle. The gilts (N = 4) were sacrificed by electrocution on days 0, 6, 12, and 18 of estrous cycle, corresponding to estrous, metaestrous, diestrous, and proestrous stages, respectively. Ovaries were collected immediately after death and transported on ice to the laboratory.

RNA isolation and cDNA synthesis

Granulosa cells from follicles were collected by vacuum aspiration, washed twice in phosphate-buffered saline by centrifugation at 5000 *g* for 6 min. The number of granulosa cells was determined using the hemocytometric method in order to adjust the volume of extraction buffer. After removal of granulosa cells by aspiration, the ovarian cortex was torn into small pieces and treated with RNAlater (Ambion, Austin, TX, USA) according to manufacturer instructions and stored at -80°C until RNA isolation. RNA isolation from granulosa cells and 30 mg ovarian tissue was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Genomic DNA was removed using the on-column DNase digestion with the RNase free DNase Set (Qiagen) according to manufacturer instructions. Quantification of total RNA was determined spectrophotometrically at 260 nm and its integrity verified by ethidium bromide staining and by optical density (OD) absorption ratio $OD_{260\ nm}/OD_{280\ nm}$ >1.9. For each animal at each stage, a pool of equivalent amounts of RNA from granulosa cells and ovary tissue was prepared in order to evaluate the total gene expression for the ovaries. One microgram total RNA pool from granulosa cells and ovarian tissue for each female was reverse transcribed with SuperScript III/RNaseOUT Enzyme Mix (Invitrogen Life Technologies, Carlsbad, CA, USA). The cDNA was then stored at -20°C until analysis by real-time RT-PCR.

Real-time RT-PCR

Quantitative real-time RT-PCR was performed using SYBR green fluorescent detection system during amplification on ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA), according to manufacturer recommendations. The primer oligonucleotides used for the reactions were designed using the "PrimerQuest" software available from Integrated DNA Technologies, Inc. (Coralville, IA, USA) from swine sequences available in GenBank (htpp://www.ncbi.nlm.nih.gov). Their sequences and expected PCR product length are shown in Table 1. Reactions were performed in duplicates using 12.5 µL 2X SYBR® Green PCR Master Mix (Applied Biosystems, São Paulo, SP, Brazil), 200 nM primer for *EGF* and *IGFBP5* and 400 nM primer for *IGF1*, *IGFBP1*, *IGFBP2*, *IGFBP3*, and 100 ng of the cDNA per reaction in a final volume of 25 µL. Prior to quantification by real-time RT-PCR, optimal primer concentration was determined for each transcript, and the linearity of amplification for each target (*IGF1*, *IGFBP1*, *IGFBP2*, *IGFBP3* or *IGFBP5*, and *EGF*) mRNA was similar to that of the endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The thermal cycling conditions for all genes allowed the use of the default program as follows: 40 cycles of 30 s of melting at 95°C followed by 30 s of annealing and extension at 60°C. After the 40 amplification cycles, all samples were subjected to a melt curve analysis in which they were heated at 1°C/30 s increments from 60° to 94°C to validate the absence of non-specific products. Normalized gene expression was presented using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). The final results were expressed as $2^{-\Delta Ct}$ x 1.000.

Table 1. Forward (F) and reverse (R) primer sequences, size of cDNA and GenBank number of the factors investigated.

Primer	Oligonucleotide sequence	Length (bp)	GenBank
*GAPDH**	F: CAAAGTGGACATTGTCGCCATCA	124	AF017079
	R: AGCTTCCCATTCTCAGCCTTGACT		
IGF1	F: TGCCCAAGGCTCAGAAGGAAGTA	146	NM_214256
	R: GGTAACTCGTGCAGAGCAAAGGA		
IGFBP1	F: CCCATCCTTTGGAACGCCATCAAT	121	AB053605
	R: TGGCTAGTCTGTCCAGCACTTTGT		
IGFBP2	F: AGCATGGCCTGTACAACCTCAAAC	156	AF120326
	R: CTGCTGCTCGTTGTAGAAGAGAT		
IGFBP3	F: GTCCACACCAAGATGGACGTGAT	188	J05228
	R: CATGTTCAGGAACTTGAGGTGGT		
IGFBP5	F: GCAAGCCAAGATCGAGAGAGACT	159	NM_214099
	R: TCAGCTTCTTTCTGCGGTCCTTCT		
EGF	F: TGTATTGGTGCGATGCCAAGCAG	114	NM_214020
	R: AACACAGCTACCGCAAATGGGTG		

*Endogenous control gene.

Hormone quantification

Prior to sacrifice, a single blood sample was collected into a 10-mL sterile vacutainer tube with anticoagulant EDTA (1.5 mg/mL) by venipuncture of the sinus orbitalis, centrifuged at 700 *g* for 15 min at room temperature and the plasma was then stored at -20°C until assayed for E_2, P_4, PRL, LH, and FSH. Hormone concentrations were quantified using radioimmunoassay kits (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

A linear regression analysis using gene expression ($2^{-\Delta Ct}$) after transformation ln (x + 1) (Voge et al., 2004) as dependent variable and days of estrous cycle as independent variables using the general linear model procedure (SAS procedure GLM, SAS Institute, Cary, NC, USA) was used. Outliers were detected using the Robustreg Procedure (SAS) as described by Huber (1973). Pearson correlations were calculated between transformed expression values and hormone concentrations.

RESULTS

In the current study, mRNA abundance for the members of the IGF system (*IGF1*, *IGFBP1*, *IGFBP2, IGFBP3*, *IGFBP5*) and a representative of the *EGF* system were investigated by real-time RT-PCR in the pig ovary. The specific expression of all genes analyzed was confirmed in a pool of ovarian tissue and granulosa cells for each animal in each stage. Evaluation of the changes in the IGF system components showed that mRNA abundance of *IGF1* and its binding proteins *IGFBP1*, *IGFBP2* and *IGFBP5* in the entire ovarian structure, considering granulosa cells and ovarian tissue, were not affected by the stages of the estrous cycle (P = 0.53), (P = 0.40) and (P = 0.68), respectively, with no significant correlation between expression values for these genes and the hormones analyzed (Table 2).

Table 2. Correlations between transformed values of gene expression and hormone concentration in plasma.

Target gene	Hormones	Correlation	P value	N
IGF1	E_2	0.213	0.5058	12
	P_4	-0.080	0.7947	14
	PRL	-0.056	0.8556	14
	FSH	0.158	0.5889	14
	LH	-0.041	0.8952	13
IGFBP1	E_2	0.068	0.8629	9
	P_4	0.127	0.7261	10
	PRL	-0.134	0.7130	10
	FSH	-0.407	0.2425	10
	LH	0.074	0.8507	9
IGFBP2	E_2	0.263	0.4345	11
	P_4	0.125	0.6984	12
	PRL	-0.051	0.8737	12
	FSH	0.079	0.7985	13
	LH	-0.123	0.7044	12
IGFBP3	E_2	-0.206	0.4991	13
	P_4	0.811	0.0004	14
	PRL	-0.569	0.0337	14
	FSH	-0.432	0.1081	15
	LH	-0.168	0.5649	14
IGFBP5	E_2	0.590	0.0560	11
	P_4	-0.105	0.7453	12
	PRL	0.335	0.2866	12
	FSH	-0.323	0.2815	13
	LH	-0.281	0.3755	12
EGF	E_2	0.100	0.7570	12
	P_4	0.049	0.8717	13
	PRL	0.228	0.4548	13
	FSH	0.213	0.4631	14
	LH	-0.109	0.7216	13

N = number of animals analyzed.

However, *IGFBP3* mRNA expression showed a quadratic pattern ($P \leq 0.01$) with the maximum expression occurring at approximately day 11 ($\delta y / \delta t = 10.76$ days). Figure 1 shows the regression of *IGFBP3* mRNA expression throughout the estrous cycle. Furthermore, a positive correlation was found between transformed expression values and progesterone concentrations ($r = 0.81$; $P \leq 0.01$) and a negative correlation between *IGFBP3* mRNA expression values and prolactin concentration ($r = -0.569$; $P \leq 0.05$) (Table 2). Real-time RT-PCR analysis also showed that the mRNA encoding for *EGF* was constantly expressed in the entire ovarian structure throughout the estrous cycle ($P = 0.58$).

DISCUSSION

The importance of the local growth factors for the modulation of follicular cell function, regarding cell proliferation and steroidogenesis (Spicer and Echternkamp, 1995; Mazerbourg et al., 2003; Paradis, 2009) is becoming clearly evident. However, there is still a lack of knowledge about some growth factors and their interactions in the follicle throughout follicle development. For a better understanding of the local regulation of porcine follicle development, the pattern of mRNA expression of many constituents of the IGF system (*IGF1*, *IGFBP1*, *IGFBP2*, *IGFBP3*, and *IGFBP5*) and *EGF* in granulosa cells and ovarian tissue as a function of day of the estrous cycle and its correlation with FSH, LH, E_2, P_4, and PRL plasma concentration were investigated in the current study.

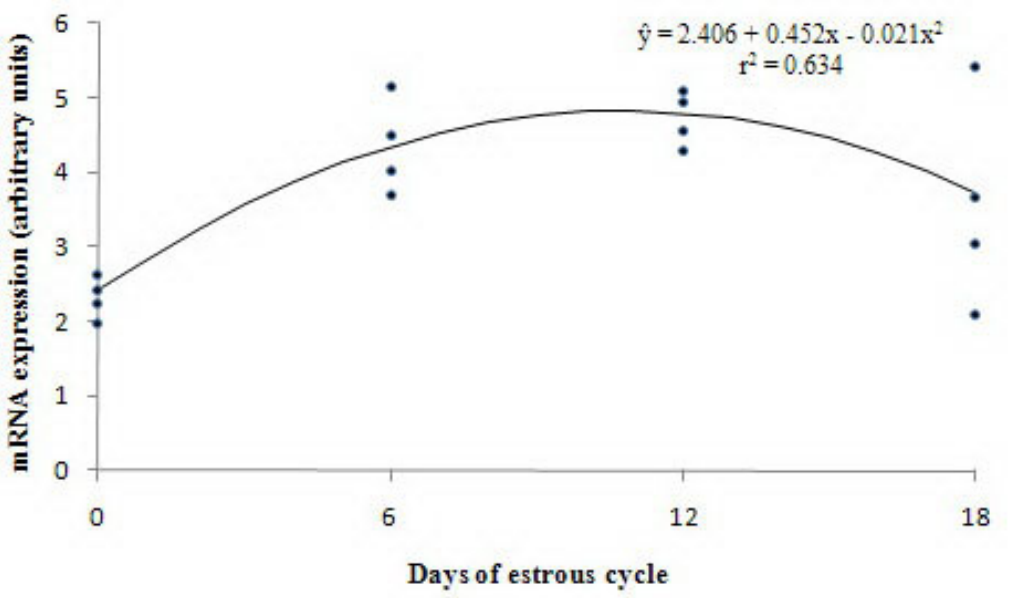

Figure 1. *IGFBP3* mRNA expression in granulosa cells and ovarian tissue.

The *IGF1* mRNA abundance was detected in all stages analyzed although it was not affected by days of the estrous cycle. The lack of significant correlation between *IGF1* mRNA expression and FSH levels (r = 0.158; P = 0.59) apparently imply that *IGF1* mRNA abundance is not dependent on FSH levels as suggested by Zhou et al. (1997). The detection of *IGF1* mRNA observed in the pool formed by granulosa cells and ovarian tissue is in agreement with previous studies in the pig (Zhou et al., 1996; Liu et al., 2000; Paradis, 2009). In contrast with the study of Samaras et al. (1993), the amount of *IGF1* mRNA in our study did not change during follicular phase and no significant correlation was found with estradiol-17β production.

However, since a pool of ovarian tissue and granulosa cells for each animal was used and the ovary section comprised steroidogenic as well as nonsteroidogenic cell types (endothelial cells, fibroblasts, white blood cells), it is not possible to know precisely which cell type was responsible for the synthesis of IGF system components. Moreover, it is important to emphasize that according to Mazerbourg et al. (2003), modulation of IGF1 bioavailability by the *IGFBPs*, and the IGFBP protease PAPP-A may be important for regulating follicle cell function.

Although *IGFBP1* expression was constantly expressed in the current study by real-time RT-PCR, its abundance was very low. To our knowledge, this is the first study to show *IGFBP1* mRNA expression in the gilt ovary, as previous studies by *in situ* hybridization did not detected it (Zhou et al., 1996). This shows that quantitative PCR is an effective technique for detection of low levels of transcripts. To date, recent studies in sheep (Hastie et al., 2004) and cattle (Schams et al., 2002) have localized *IGFBP1* mRNA expression in follicles throughout the estrous cycle, although expression tended to be extremely low. While El-Roeiy et al. (1994) demonstrated *IGFBP1* mRNA expression in normal, but not

polycystic ovary syndrome follicles, other studies in humans (Voutilainen et al., 1996), monkey (Arraztoa et al., 2002) and rats (Nakatani et al., 1991) have failed to demonstrate *IGFBP1* gene expression in follicles. Indeed, it has been postulated that any significant quantities of intra-follicular *IGFBP1* may represent transudation from serum rather than *de novo* synthesis, although given the equivocal data, local production cannot be discounted as a possibility (Hastie and Haresign, 2006).

IGFBP3 mRNA was produced at relatively high levels in the pool of ovarian tissue and granulosa cells in all stages of the estrous cycle, showing a quadratic pattern ($P \leq 0.01$). The high expression of *IGFBP3* mRNA in luteal phase, particularly between days 6 and 12, where granulosa cells were already luteinized, suggests that *IGFBP3* transcript is mainly produced by porcine luteal cells as previously described by Gadsby et al. (1996). A positive correlation found between transformed expression values and progesterone concentrations ($r = 0.81$; $P \leq 0.01$), suggests that this hormone regulates IGFBP3 mRNA expression, through differentiation and maintenance of luteal function or indirectly by another pathway. On the other hand, a negative correlation was found between *IGFBP3* mRNA expression values and prolactin concentration ($r = -0.569$; $P \leq 0.05$) suggesting their antagonistic roles on ovarian physiology. Prolactin plays a luteotropic role in early luteal phase (Dusza and Tilton, 1990) while the *IGFBP3* mRNA expression is related to luteolysis in rat ovary (Erickson et al., 1993). Thus, the negative correlation observed in the current study indicates that in the luteal phase the combination of increasing expression of *IGFBP3* mRNA and decreasing prolactin concentration would allow the corpus luteum regression in swine, in which *IGFBP3* would act as luteolytic factor.

In addition, the current results in a pool of granulosa cells and ovarian tissue showed that *IGFBP3* mRNA abundance was constant in the luteal phase (metaestrus and diestrus) in agreement with previous studies (Zhou et al., 1996; Gadsby et al., 1996), and low expression at the estrous stage while most studies have failed to detect it in developing follicles of the pig (Samaras et al., 1993; Zhou et al., 1996).

According to the previous findings regarding the inhibitory role of *IGFBP3* in *IGF1* modulation follicular cell function in corpus luteum (Ge et al., 2003) and based on these results it can be stated that in metaestrus and diestrus (luteal phase) *IGFBP3* expression was significant greater than in estrus (follicular phase) leading to an increase of *IGF1* bioavailability. The opposite occurred at proestrus and estrus leaving *IGF1* available to act in follicular tissue, particularly regarding cell proliferation and steroidogenesis (Mazerbourg et al., 2003).

IGFBP2 and *IGFBP5* mRNA abundance in the entire ovarian structure was not affected by the stages of estrous cycle with no significant correlation between expression values for these IGFBPs and the hormones analyzed (Table 2). In previous studies, Liu et al. (2000) reported that *IGFBP2* mRNA decreased during porcine follicular growth. However, this result was not verified in the current experiment corroborating the results reported by Paradis (2009) who found no effect of *IGBP2* mRNA level by the stage of follicle growth using real-time RT-PCR analysis. According to Paradis (2009) the utilization of a representative pool of follicles present on the ovary of each animal might be a confounding source of such effect, since it is possible that expression of the *IGFBPs* within the follicle population was different. In this context, it could be expected to find a decrease in *IGFBP2* mRNA expression during follicle growth since the *IGFBP2* would be responsible for maintaining

the bioavailability of *IGF1* in highest levels in the maturing ovulatory follicles (Samaras et al., 1993; Liu et al., 2000). Also it has been suggested that both transcriptional and post-transcriptional regulation are involved for the regulation of *IGFBP2* expression as well as proteolytic degradation found in growing ovulatory follicles (Mazerbourg et al., 2003) illustrating the complexity of the interactions between the components of IGF system and the importance of deciphering the regulation of the IGF systems toward a better understanding of ovarian function.

As well as in the present study, other experiments have verified the expression of *IGFBP5* mRNA on the surface or germinal epithelium, ovarian connective tissue and vasculature, but not in follicles (Zhou et al., 1996), and predominant expression in porcine luteal cells (Gadsby et al., 1996). Interestingly, Paradis (2009) using real-time RT-PCR technique reported decreased *IGFBP5* mRNA abundance after the exposure to LH surge in the pig granulosa cell, which was not verified between days 0 and 6 of estrous cycle in the current study.

It is important to remember that some discordance between mRNA abundance in granulosa and ovarian tissue, verified in the current study and among those consulted literature, could be explained by sensitivity of the technique used, physiological status of the follicles collected and utilization of the representative pool of follicles of each animal or breed differences. Given the role that the *IGFBPs* play in regulating IGF bioavailability, the observed differences in gene expression and potentially of the production of *IGFBPs* may well function as an important local regulatory mechanism controlling follicle growth and atresia (Hastie and Haresign, 2006).

The current results of real-time RT-PCR analysis showed that the mRNA encoding for *EGF* was stably expressed in the granulosa cells and ovarian tissue. This is consistent with observations in porcine follicles, in which *EGF* appears to be predominately expressed in the mural granulosa cells; however, in a lesser proportion in cumulus cells (Singh et al., 1995). In the current study, almost no significant correlation was found between expression values and the hormones analyzed (Table 2). According to Park et al. (2004) the LH stimulation of ovarian follicles involves activation of a local *EGF* network. Based on that, an alteration of its relative expression in estrous stage and a possible positive correlation between *EGF* mRNA abundance and LH hormone would be expected. Again, the utilization of a representative pool of follicles present on the ovary of each animal may have impaired the identification of a follicle size effect, since the ability of EGF to stimulate porcine cumulus cell expansion *in vitro* increases with follicular size (Prochazka et al., 2003). According to Fujinaga et al. (1992) *EGF* is one of the autocrine or paracrine factors regulating ovarian folliculogenesis in porcine, and may modulate not only the function of follicles, but also that of the corpus luteum. A point to be considered in future experiments not only for *EGF*, but also for the *EGF*-like ligants and *EGF* receptor, is the analysis of the gene expression in follicular cells of different sizes and in the oocyte, so that a more precise expression pattern could be obtained.

In conclusion, the results of the present study have demonstrated that all transcripts of the components of the IGF system analyzed, as well as *EGF*, can be detected at the porcine ovary (follicle and/or ovarian tissue), and for one of the components, its gene expression varied with the stage of the estrous cycle. This study further emphasizes the importance of IGF system in the autocrine and paracrine regulation of follicle growth and development in gilts. Further studies are currently being conducted to elucidate the precise patterns of

expression in relation to follicle size and health status at stages of the estrous cycle, including more than one breed of pigs. Furthermore, the involvement of the components of the IGF system in the process of follicular atresia is also being investigated, as well as the mechanisms by which IGF gene expression may be regulated and how the observed changes in gene expression are correlated with levels of protein production in porcine ovary. In the near future, analysis in gene expression in different follicle size and development stage will be presented by our group.

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