Possible Intracellular Regulators of Female Sexual Maturation

A. KOLESAROVA[1], A. V. SIROTKIN[2], M. MELLEN[3], S. ROYCHOUDHURY[4]

[1]Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, 949 76 Nitra, Slovak Republic

[2]Institutute for Farm Animal Genetics and Reproduction, National Agricultural and Food Centre, Nitra, 95141 Lužianky, Slovak Republic

[3]Poultry Slovakia, LTD., 962 21 Zvolen-Lieskovec, Slovak Republic

[4]Department of Life Science and Bioinformatics, Assam University, 788 011 Silchar, India

Summary

Protein kinases, transcription factors and other apoptosis- and proliferation-related proteins can regulate reproduction, but their involvement in sexual maturation remains to be elucidated. The general aim of the *in-vivo* and *in-vitro* experiments with porcine ovarian granulosa cells was to identify possible intracellular regulators of female sexual maturation. For this purpose, proliferation (expression of proliferating cell nuclear antigen - PCNA, mitogen-activated protein kinases - ERK 1,2 related MAPK and cyclin B1), apoptosis (expression of the apoptotic protein Bax and apoptosis regulator Bcl-2 protein), expression of some protein kinases (cAMP dependent protein kinase - PKA, cGMP-dependent protein kinase - PKG, tyrosine kinase - TK) and cAMP responsive element binding protein 1 (CREB-1) was examined in granulosa cells isolated from ovaries of immature and mature

gilts. Expression of PCNA, ERK1,2 related MAPK, cyclin B1, Bcl-2, Bax, PKA, CREB-1, TK and PKG in porcine granulosa cells were detected by immunocytochemistry. Sexual maturation was associated with significant increase in the expression of Bcl-2, Bax, PKA, CREB-1 and TK and with decrease in the expression of ERK1,2 related MAPK, cyclin B1 and PKG in granulosa cells. No significant difference in PCNA expression was noted. The present data obtained from *in vitro* study indicate that sexual maturation in females is influenced by puberty-related changes in porcine ovarian signalling substances: increase in Bcl-2, Bax, PKA, CREB-1, TK and decrease in ERK1,2 related MAPK, cyclin B1 and PKG. It suggests that these signalling molecules could be potential regulators of porcine sexual maturation.

Key words

Ovary • Kinases • Proliferation • Apoptosis • Transcription Factor

Corresponding author

A. Kolesarova, Department of Animal Physiology, Faculty of Biotechnology & Food Sciences, Slovak University of Agriculture, 949 76 Nitra, Slovak Republic. E-mail: Adriana.Kolesarova@uniag.sk

Introduction

Sexual maturation is associated with ovarian follicular growth and differentiation (Onagbesan et al., 2009; Palma et al., 2012). These processes are governed by hormones,

47 growth factors (Kolesarova et al., 2009a,b; 2010a,b; Roychoudhury et al., 2009, 2014),
48 which through protein kinases and transcription factors, affect ovarian cell proliferation,
49 apoptosis and secretion activity (Onagbesan et al., 2009; Sirotkin, 2013). There is indirect
50 evidence for involvement of several candidate signalling substances in control of sexual
51 maturation and/or related ovarian follicle development.
52 Action of hormones and growth factors on ovarian follicullogenesis and functions is
53 mediated via protein kinases and related proliferation- and apoptosis-related peptides
54 (Sirotkin et al., 2000, 2008). The involvement of cAMP/protein kinase A (PKA)-dependent
55 intracellular mechanisms (Makarevich et al., 2000; Sirotkin and Grossmann, 2003, 2006) in
56 the regulation of proliferation- and apoptosis-related substances (Sirotkin and Grossmann,
57 2003, 2006) has already been reported. Furthermore, cAMP/PKA can regulate the secretion
58 activity of mammalian ovarian cells as noted in cases of porcine (Sirotkin et al., 2004),
59 chicken (Sirotkin and Grossmann, 2006) and human (Sirotkin et al., 2008) ovarian cells and
60 also mediate the action of hormones and growth factors on ovarian functions (Makarevich
61 et al., 2004a,b). Apoptosis-related substances are crucial in follicular selection, atresia and
62 *corpus luteum* regression (Greenfeld et al., 2007; Maeda et al., 2007; Parborell et al., 2001,
63 2008). Mitochondrial apoptotic protein Bax is considered as the key pro-apoptotic
64 substance (Elmore, 2007), whilst apoptosis regulator Bcl-2 protein, which binds and
65 inactivates Bax, has an opposite, anti-apoptotic action (Greenfeld et al., 2007; Lomonosova
66 and Chinnadurai, 2008). The mitogen-activated protein kinases (MAPK) signalling cascade
67 including intracellular regulated kinases (ERK) also act as promoters of cell cycle
68 progression as well as mediators of mitogenic action of hormones and growth factors
69 (Cameron et al., 1996; Lapthorn et al., 1994; Sirotkin and Grossmann, 2003), stimulators of

ovarian cell proliferation, differentiation and secretion activity (Sirotkin and Grossmann, 2003) and suppressors of apoptosis (Dent et al., 1998; Gunter et al., 2013; Kyriakis, 1999; Xia et al., 1995). Tyrosine kinase (TK) localized in growth factor receptors and cytoplasm plays an important role in promoting cell proliferation, differentiation and mediation effects of some hormones and growth factors in signal transduction (Arora and Scholar, 2005; Okamura et al., 2001; Sirotkin and Grossmann, 2003). TK may be involved in activation of ovarian porcine follicle growth and maturation (Okamura et al., 2001) and in control of chicken ovarian cell proliferation and hormone release (Sirotkin and Grossmann, 2003).

The involvement of cGMP dependent protein kinase (PKG) along with cGMP in control of the production of steroid, nonapeptide hormone, growth factor, cAMP and cAMP-dependent PKA, as well as the induction of apoptosis in porcine ovarian cells has been reported, too (Sirotkin et al., 2000). Protein kinases (PKA, MAPK) can also target cAMP responsive element binding protein 1 (CREB-1). It is required for mediating stimulatory influence of FSH on granulosa and luteal cells differentiation and steroidogenesis during the follicular recruitment estrous cycle and pregnancy of mouse (Mendelson and Kamat, 2007). There exist indirect evidences for involvement of CREB in control of sexual maturation (He et al., 2006; Sirotkin et al., 2004). Cell cycle peptides especially proliferating cell nuclear antigen PCNA (Naryzhny and Lee, 2001) and cyclin B1 (Wyllie et al., 1998) are also involved in ovarian cell proliferation, growth and development (Tomanek and Chronowska, 2006). Proliferation-related peptide PCNA is a known promoter of the cell cycle transition through G1 and G2 phases. Furthermore, it activates the cyclin/cyclin dependent kinase complex (McHugh and Sarkar, 2006; Moldovan et al., 2007), which promotes the G_2-M transition of the cell cycle (Hassan et al., 2001).

Expression of PCNA and cyclin B1 in ovarian cells has been reported from different mammalian species (Hutt et al., 2006; Sirotkin et al., 2008).

The general aim of the *in-vivo* and *in-vitro* experiments with porcine ovarian granulosa cells was to identify possible intracellular regulators of female sexual maturation. For this purpose, expression of these signalling molecules were evaluated in granulosa cells collected from sexually mature and immature gilts of the same age.

Materials and methods

Animals

Healthy gilts of Slovakian White breed (100-120 days of age) were reared under standard conditions at the Experimental Station of the Slovak University of Agriculture in Nitra, Slovakia. Conditions of their care and handling corresponded to the instructions of the European Commission (EC) no. 178/2002 and related EC documents and as approved by local ethics committee. Animals (n=35) were assigned at slaughter into two groups: sexually immature (n=18) and animals of the same age having reached sexual maturity (n=17) according to visual characteristics of ovaries (presence of follicles larger than 5 mm).

Preparation, culture and processing of granulosa cells

Ovaries were transported to the laboratory at 4ºC and washed in sterile physiological solution. Ovaries from immature and mature gilts were processed separately. Follicular fluid was aspirated from 3-5 mm follicles, granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker™) and 1% antibotic-antimycotic solution (Sigma, St. Louis, Mo, USA) at a final concentration of 10^6 cells/mL of medium. Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1 ml/well; for RIA) or Lab-Tek 16-welled chamber slides (Nunc Inc., International, Naperville, USA, 100 µl/well; for immunocytochemistry). Both the plate wells and chamber slides were incubated at 37.5ºC and 5% CO_2 in humidified air until a 75 % confluent monolayer was formed (5-7 days), at which point the medium was replaced with fresh medium. Further culture was performed in 300 µl medium in 16-welled chamber slide cells or 1 ml of culture plate. After 2 days of culture the media from wells were removed, wells from chamber slides were washed in ice-cold PBS (pH 7.5). Cells were fixed for 1 h at room temperature in 4% paraformaldehyde, dehydrated in alcohols (70, 80, 96%; 10 min each) and stored in 96% alcohol at -4ºC to await immunocytochemical analysis. Media from plate wells were aspirated and kept at -70 ºC to await RIA.

Immunocytochemistry

Immunocytochemistry was used to detect PKA, PKG, TK, ERK1,2 related MAP kinase, CREB-1, PCNA, cyclin B1, Bax, Bcl-2 in granulosa cells plated on chamber slides

(Osborn and Isenberg, 1994). Primary mouse monoclonal antibodies to each petide IGFBP-3, IGFBP-4, PKA, PKG, TK, CREB-1, PCNA, cyclin B1, Bax, Bcl-2 (cross-reacting with corresponding rat, human, porcine and chicken substances; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as directed by the manufacturer at a dilution of 1:100 and ERK1,2 at a dilution of 1:50. Visualisation of the primary antibody binding sites was done with a secondary rabbit polyclonal antibody against mouse IGs, labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:500) and diaminobenzidine (DAB) reagent (Roche Diagnostics Corporation, IN, USA, 10%). The presence of each peptide was determined by light microscopy. To verify these data, in some selected cases primary antibodies were visualised by secondary rabbit or goat monoclonal antibodies against mouse IGs labelled with FITC (Sevac, Prague, Czech Republic) and fluorescent microscopy. Negative control was presented by stained cells omitting primary antibody. During microscopic inspection, the percentage of cells containing visible antigen was determined.

Statistics

Each experimental group was represented by four culture wells with granulosa cells. Assays of hormonal substances in incubation medium were performed in duplicate. The data presented concerning the effects of each substance are means of values obtained in three separate experiments performed on separate days using separate ovaries, and blood samples obtained from 10-12 animals. The values of blank controls were subtracted from the values determined by RIA in cell-conditioned medium to exclude any non-specific

161 background (less than 13% of total values). The rates of hormone secretion were calculated
162 per 10^6 cells per day. The proportion of cells containing each analysed substance was
163 calculated following immunocytochemical analysis by counting at least 1000 cells per
164 chamber slide well. Firstly, the data obtained in each experiment were processed by
165 ANOVA. Thereafter, significant differences between the immature groups and mature gilts
166 were evaluated by paired t-test or chi-square (χ^2) test by using statistical software Sigma
167 Plot 9.0 (Jandel, Corte Madera, USA). Differences from controls ($P<0.05$) were considered
168 as significant.
169
170 **Results**
171 Percentage of ovarian granulosa cells containing PCNA did not differ between sexually
172 mature (22.4±4.1%) and immature gilts (17.5±1.2%) (Fig. 1). On the contrary, the
173 expression of ERK1,2 related MAPK and cyclin B1 was significantly ($p<0.05$) lower in
174 granulosa cells of sexually mature gilts (ERK1,2 35.3±1.6%, cyclin B1 21.8±0.6%) in
175 comparison to their immature counterparts (ERK1,2 46.2±1.8%, cyclin B1 38.2±1.6%)
176 (Fig. 1). The expression of Bcl-2 and Bax by ovarian granulosa cells was significantly
177 ($p<0.05$) higher in sexually mature gilts in comparison to immature animals (37.7±1.8% vs.
178 28.7±1.0% for Bcl-2 and 48.7±2.6% vs. 31.9±2.3% for Bax, respectively) (Fig. 2).
179 Proportion of cells containing PKA was also significantly ($p<0.05$) higher in sexually
180 mature gilts than in sexually immature animals (PKA 54.4±1.2% vs. 32.7±1.7%) (Fig. 3).
181 Similarly, expression of TK was significantly ($p<0.05$) higher in sexually mature gilts in
182 comparison to sexually immature ones (TK 41.4±1.1% vs. 32.4±3.3%) (Fig. 3). Also,

proportion of cells containing CREB-1 was significantly ($p<0.05$) higher in sexually mature gilts than the immature animals (CREB 46.3±1.0% vs. CREB 38.6±0.7%). However, the expression of PKG was significantly lower ($p<0.05$) in granulosa cells of sexually mature gilts than the immature animals (PKG 16.7±2.2% vs. PKG 61.3±2.1%) (Fig. 3).

Discussion and conclusions

Do peptides of cell proliferation (PCNA, ERK1,2 related MAPK and cyclin B1) relate to sexual maturation?

Follicular growth and development in porcine ovary was associated with increased expression of PCNA in granulosa cell (Tomanek and Chronowska, 2006). Peng et al. (1998) reported decreased expression of PCNA in granulosa cells during apoptosis. Our investigation did not reveal any differences in PCNA in granulosa cells between immature and mature gilts (Peng et al., 1998). In contrast to PCNA, the expression of cyclin B1 was associated with sexual maturation. Since cyclin B1 is a promoter and marker of G-phase of cell cycle (Wyllie et al., 1998), it might be suggested that sexual maturation is associated with suppression of cell cycle at this phase. Since ERK1,2 related MAPK is an important marker and promoter of cell cycle (Grossmann, 2009), its role in stimulation of ovarian cell proliferation and related follicle growth during puberty may be suggested. During establishment of ovarian cyclicity the expression and probably the importance of ERK1,2 related MAPK declines. Besides cell proliferation ERK1,2 related MAPK can control apoptosis. In mammalian cells, the MAPK pathway can prevent (Allen et al., 1999;

Anderson and Tolkovsky, 1999; Nishio et al., 1999) or induce (Bhat and Zhang, 1999; Goillot et al., 1997) apoptosis depending on the type of cell and the extracellular stimuli that initiate the pathway. Therefore, the changes in ERK1,2 related MAPK as observed in the present study could be due to its involvement in control of apoptosis during sexual maturation. Evidence also persists (Sirotkin and Grossmann, 2003) that MAPK could be involved not only in control of apoptosis, but also in control of ovarian secretion activity and in mediating the effect of hormonal regulators of reproduction.

Results of the present study indicate that sexual maturation is associated with a reduction in the expression of ERK1,2 related MAPK and cyclin B1, but not of PCNA, which could result in reduction of ovarian cell proliferation, increase in their apoptosis and might even change their secretion activity and response to hormonal regulators during establishment of ovarian cycle.

Do anti-apoptotic peptide Bcl-2 and pro-apoptotic peptide Bax relate to sexual maturation?

In this study, sexual maturation in gilts was found to be associated with increased expression of both Bcl-2 and Bax by ovarian granulosa cells. Through their effect on apoptosis, these peptides could be involved in control of ovarian follicular growth, development and fertility. This is probably the first indication of involvement of ovarian Bax and Bcl-2 in control of porcine sexual maturation. The puberty-related increase in expression of both Bax and its antagonist Bcl-2 as observed in the present study suggest the involvement of these apoptosis-related peptides in regulation of porcine sexual maturation,

229 although understanding their exact role in control of porcine reproduction requires further
230 investigation.
231
232 **Do PKA, CREB-1, TK and PKG relate to sexual maturation?**
233
234 Stimulatory influence of PKA on ovarian secretory activity and in mediating the action of
235 hormones and growth factors has been demonstrated previously (Makarevich et al.,
236 2004a,b). The present study for the first time reports involvement of PKA in regulation of
237 not only basal ovarian functions, but also of sexual maturation. It is possible that sexual
238 maturity-related increases in the expression of PKA as observed in this study are important
239 for sexual maturity-associated increases in hormone and growth factor release and action.
240 It was previously mentioned that TK can be involved in activation of porcine ovarian
241 follicle growth and maturation (Okamura et al., 2001) and in control of chicken ovarian cell
242 proliferation and hormone release (Sirotkin and Grossmann, 2003). Our observations
243 present further involvement of TK in control of sexual maturity-related changes in ovarian
244 functions. Our observations provide the first indications of involvement of PKG in
245 regulation of porcine sexual maturation. Involvement of cGMP/PKG system in control of
246 release of porcine ovarian hormones has been reported previously (Sirotkin et al., 2000).
247 Also, our data provide the first indications of the role of CREB-1 in sexual maturation and
248 related processes in gilts. Although involvement of CREB-1 in control of sexual maturation
249 (He et al., 2006; Sirotkin et al., 2004) and in mediating the effect of growth factor on these
250 processes (Sirotkin and Grossmann, 2003) in non-porcine species has been documented,
251 details of CREB-1 targets and action remain to be studied.

Possible interrelationships between studied substances

Effect of hormones and growth factors on the ovary can be mediated by protein kinases and protein kinases-dependent transcription factors. PKA can mediate the action of hormones and growth factors (Makarevich et al., 2004a,b). Furthermore, hormones and growth factors can affect ovarian function during and after puberty through MAPK-dependent intracellular mechanisms. At least, growth factors can activate MAPK in a variety of cell types (Lapthorn et al., 1994), and inhibitors of MAPK cascade can block the mitogenic action of the growth factors (Alessi et al., 1995). Furthermore, ability of PKA and TK to affect MAPK and MAPK-activated CREB-1 in non-ovarian cells has been reported (Gao et al., 2009; McAlees and Sanders, 2009; Sun et al., 2009; Zu et al., 2009). Therefore, the functional interrelationships between these substances within the ovary in regulating porcine sexual maturation can't be excluded. Fine interrelationships between analysed processes occurring in porcine ovary during sexual maturation require further elucidation. Nevertheless, the present observations expand the existing knowledge concerning changes during sexual maturation in porcine ovarian hormones, growth factors and growth factors binding proteins. Furthermore, this is the first indication of involvement of some intracellular signalling substances in control of this process. Obtained results suggest that sexual maturation is associated with increase in expression of apoptosis-related substances (Bcl-2, Bax), PKA, TK, PKG, CREB-1, with decreases in the expression proliferation-related substances of ERK1,2 related MAPK and cyclin B1, but not PCNA. Analyzed data indicate puberty-related changes in porcine ovarian signalling substances: Bcl-2, Bax,

PKA, CREB-1, TK, ERK1,2 related MAPK, cyclin B1 and PKG. Results obtained from both *in vivo* and *in vitro* studies indicate the involvement of some apoptosis- and proliferation-related substances, protein kinases and transcription factor CREB-1 in porcine sexual maturation. The results of present study indicate that sexual maturation is associated with decrease in ovarian cells proliferation and increase in their secretory activity, apoptosis and expression of some protein kinases and transcription factor. Although the puberty-related changes don't provide direct evidence of the involvement and physiological role of these signalling molecules in control of sexual maturation, our study enables to identify several extra-and intracellular signalling substances, which could be potential candidates for induction of porcine puberty and sexual maturation.

Acknowledgements

This work was supported by the Ministry of Education, Science, Research and Sports of the Slovak Republic (1/0022/13) Slovak Research and Development Agency of the Slovak Republic (APVV-0304-12) and the Department of Biotechnology, Government of India (BT/265/NE/TBP/2011).

Reference

1. ALESSI DR, CUENDA A, COHEN P, DUDLEY DT, SALTIEL AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem* **270:** 27489-27494, 1995.
2. ALLEN MP, ZENG C, SCHNEIDER K, XIONG X, MEINTZER MK, BELLOSTA P, BASILICO C, VARNUM B, HEIDENREICH KA, WEIRMAN ME. Growth

arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt. *Mol Endocrinol* **13:** 191-201, 1999.

3. ANDERSON CNG, TOLKOVSKY AM. A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNKindependent induction of apoptosis by cytosine arabinoside. *J Neurosci* **19**: 664–673, 1999.
4. ARORA A, SCHOLAR EM. Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* **315:** 971-979, 2005.
5. BHAT NR, ZHANG P. Hydrogen peroxide activation of multiple mitogen-activated protein kinases in an oligodendrocyte cell line: role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death. *J Neurochem* **72:** 112-119, 1999.
6. CAMERON MR, FOSTER JA, BUKOVSKY A, WIMALASENA J. Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5'-monophosphates in porcine granulosa cells. *Biol Reprod* **55:** 111-119, 1996.
7. DENT P, JARVIS WD, BIRRER MJ, FISHER PB, SCHMIDT-ULLRICH RK, GRANT S. The roles of signaling by the p42/p44 mitogen-activated protein (MAP) kinase pathway; a potential route to radio- and chemo-sensitization of tumor cells resulting in the induction of apoptosis and loss of clonogenicity. *Leukemia* **12**: 1843–1850, 1998.
8. ELMORE S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35:** 495–516, 2007.

9. GAO J, SIDDOWAY B, HUANG Q, XIA H. Inactivation of CREB mediated gene transcription by HDAC8 bound protein phosphatase. *Bioch Biophys Res Comm* **379**: 1-5, 2009.
10. GOILLOT E, RAINGEAUD J, RANGER A, TEPPER RI, DAVIS RJ, HARLOW E, SANCHEZ I. Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. *Proc Natl Acad Sci* **94:** 3302-3307, 1997.
11. GREENFELD CR, BABUS JK, FURTH PA, MARION S, HOYER PB, FLAWS JA. BAX is involved in regulating follicular growth, but is dispensable for follicle atresia in adult mouse ovaries. *Reproduction* **133:** 107-116, 2007.
12. GROSSMANN AB. The molecular biology of pituitary tumors: a personal perspective. *Pituitary* **12:** 265-270, 2009.
13. GUNTER JH, SARKAR PL, LUBIK AA, NELSON CC. New players for advanced prostate cancer and the rationalisation of insulin-sensitising medication. *Int J Cell Biol* Article ID 834684, 13 pages, 2013, http://dx.doi.org/10.1155/2013/834684
14. HASSAN KA, EL-NAGGAR AK, SORIA JC, LIU D, HONG WK, MAO L. Clinical significance of cyclin B1 protein expression in squamous cell carcinoma of the tongue. *Clin Cancer Res* **7:** 2458-2462, 2001.
15. HE PJ, FUJIMOTO Y, YAMAUCHI N, HATTORI MA. Real-time monitoring of AMP response element binding protein signaling in porcine granulosa cells modulated by ovarian factors. *Mol Cell Biochem* **290:** 177-184, 2006.
16. HUTT KJ, McLAUGHLIN EA, HOLLAND MK. Primordial follicle activation and follicular development in the juvenile rabbit ovary. *Cell Tissue Res* **326:** 809-822, 2006.

17. KOLESAROVA A, CAPCAROVA M, SIROTKIN A, MASSANYI P. Insulin–like growth factor–I and progesterone release by ovarian granulosa cells of hens after experimental lead and molybdenum administrations *in vitro. Int J Poultry Sci* **8:** 890-895, 2009a.
18. KOLESAROVA A, SLIVKOVA J, SIROTKIN A, MASSANYI P, CAPCAROVA M. The release of insulin–like growth factor – I by ovarian granulosa cells of pregnant sows after lead and mercury administration *in vitro*. *Slovak J Anim Sci* **42:** 35–41, 2009b.
19. KOLESAROVA A, ROYCHOUDHURY S, SLIVKOVA J, SIROTKIN AV, CAPCAROVA M, MASSANYI P. *In vitro* study on the effect of lead and mercury on porcine ovarian granulosa cells. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 45: 320-331, 2010a.
20. KOLESAROVA, A.; SIROTKIN, A.V.; ROYCHOUDHURY, S.; CAPCAROVA, M. Puberty related changes in hormonal levels, productive performance, carcass traits, and their interactions in Slovakian White gilts. *Asian Australas J Anim Sci* **23:** 182-187, 2010b.
21. KYRIAKIS JM. Making the connection: coupling of stress-activated ERK/MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) core signalling modules to extracellular stimuli and biological responses. *Biochem Soc Symp* **64:** 29-48, 1999.
22. LAPTHORN AJ, HARRIS DC, LITTLEJOHN A, LUSTBADER JW, CANFIELD RE, MACHIN KJ, MORGAN FJ, ISAACS NW. Crystal structure of human chorionic gonadotropin. *Nature* **369:** 455-461, 1994.

23. LOMONOSOVA E, CHINNADURAI G. BH3-only proteins in apoptosis and beyond: an overview. *Oncogene* **27:** S2-S9, 2008.

24. MAEDA A, INOUE N, MATSUDA-MINEHATA, F, GOTO, Y, CHENG Y, MANABE N. The role of interleukin-6 in the regulation of granulosa cell apoptosis during follicular atresia in pig ovaries. *J Reprod Dev* **53:** 481-490, 2007.

25. MAKAREVICH AV, SIROTKIN AV, CHRENEK P, BULLA J, HETENYI L. The role of IGF-I, cAMP/protein kinase A and MAP-kinase in the control of steroid secretion, cyclic nucleotide production, granulosa cell proliferation and preimplantation embryo development in rabbits. *J Steroid Biochem Mol Biol* **73:** 123-133, 2000.

26. MAKAREVICH AV, SIROTKIN AV, FRANEK J, KWON HB, BULLA J. The role of oxytocin, protein kinase A, and ERK-related MAP-kinase in the control of porcine ovarian follicle functions. *Exp Clin Endocrinol Diab* **112:** 108-114, 2004a.

27. MAKAREVICH AV, SIROTKIN AV, GENIESER HG. Action of protein kinases A regulators on secretory activity of porcine granulosa cells *in vitro*. *Anim Reprod Sci* **81:** 125-136, 2004b.

28. McALEES JW, SANDERS V.M. Hematopoietic protein tyrosine phosphatase Mediates beta2-adrenergic receptor-induced regulation of p38 mitogen- activated protein kinase in B lymphocytes. *Mol Cell Biol* **29:** 675-686, 2009.

29. McHUGH PJ, SARKAR S. DNA interstrand cross-link repair in the cell cycle: a critical role for polymerase zeta in G1 phase. *Cell Cycle* **5:** 1044-1047, 2006.

30. MENDELSON CR, KAMAT A. Mechanisms in the regulation of aromatase in developing ovary and placenta. *J Steroid Biochem Mol Biol* **106:** 62-70, 2007.

31. MOLDOVAN GL, PFANDER B, JENTSCH S. PCNA, the maestro of the replication fork. *Cell* **129:** 665-679, 2007.
32. NARYZHNY SN, LEE H. Protein profiles of the Chinese hamster ovary cells in the resting and proliferating stages. *Electrophoresis* **22:** 1764-1775, 2001.
33. NISHIO K, FUKUOKA K, FUKUMOTO H, SUNAMI T, IWAMOTO Y, SUZUKI T, USUDA J, SAIJO N. Mitogen-activated protein kinase antisense oligonucleotide inhibits the growth of human lung cancer cells. *Int J Oncol* **14:** 461-469, 1999.
34. OKAMURA Y, MYOUMOTO A, MANABE N, TANAKA N, OKAMURA H, FUKUMOTO M. Protein tyrosine kinase expression in the porcine ovary. *Mol Hum Reprod* **7:** 723-729, 2001.
35. ONAGBESAN O, BRUGGEMAN V, DECUYPERE E. Intra-ovarian growth factors regulating ovarian function in avian species: a review. *Anim Reprod Sc*i **111:** 121-140, 2009.
36. OSBORN M, ISENBERG S. Immunocytochemistry of frozen and of paraffin tissue sections. In *Cell Biology: A Laboratory Hanbook*, pp 361-367, 1994. New York/London: Academic Press.
37. PALMA A, ARGANARAZ ME, BARRERA AD, RODLER D, MUTTO AA, SINOWATZ F. Biology and biotechnology of follicle development. *Scient World J*, Article ID 938138, 14 pages, doi:10.1100/2012/93813, 2012.
38. PARBORELL F, ABRAMOVICH D, TESONE M. Intrabursal administration of the antiangiopoietin 1 antibody produces a delay in rat follicular development associated with an increase in ovarian apoptosis mediated by changes in the expression of BCL2 related genes. *Biol Reprod* **78:** 506-513, 2008.

411 39. PARBORELL F, DAIN L, TESONE M. Gonadotropin-releasing hormone agonist
412 affects rat ovarian follicle development by interfering with FSH and growth factors
413 on the prevention of apoptosis. *Mol Reprod Dev* **60:** 241-247, 2001.
414 40. PENG X, MARUO T, MATSUO H, TAKEKIDA S, DEGUCHI J. Serum
415 deprivation-induced apoptosis in cultured porcine granulosa cells is characterized
416 by increased expression of p53 protein, Fas antigen and Fas ligand and by decreased
417 expression of PCNA. *Endocrinol J* **45:** 247-253, 1998.
418 41. ROYCHOUDHURY S, BULLA J, SIROTKIN AV, KOLESAROVA A. *In*
419 *vitro*changes in porcine ovarian granulose cells induced by copper. *J Environ Sci*
420 *Health A Tox Hazard Subst Environ Eng* **49:** 625-633, 2014.
421 42. ROYCHOUDHURY S, KOLESAROVA A, SLIVKOVA J, MASSANYI P,
422 SIROTKIN AV, BULLA J. Release of IGF-I by porcine ovarian granulosa cells
423 after *in vitro* copper administration. *Acta Fytotech et Zootechn* **Special issue:** 587-
424 593, 2009.
425 43. SIROTKIN AV. New endocrine and intracellular regulators of ovarian functions.
426 *Endocrine Abstracts* **32:** P583, 2013. DOI:10.1530/endoabs.32.P583
427 44. SIROTKIN AV, GROSSMANN R. Role of tyrosine kinase- and MAP kinase-
428 dependent intracellular mechanisms in control of ovarian functions in the domestic
429 fowl (*Gallus domesticus*) and in mediating effects of IGF-II. *J Reprod Dev* **49:** 99-
430 106, 2003.
431 45. SIROTKIN AV, GROSSMANN R. The role of protein kinase A and cyclin-
432 dependent (CDC2) kinase in the control of basal and IGF-II-induced proliferation
433 and secretory activity of chicken ovarian cells. *Anim Reprod Sci* **92:** 169-181, 2006.

46. SIROTKIN AV, MAKAREVICH AV, PIVKO J, KOTWICA J, GENIESER H, BULLA J. Effect of cGMP analogues and protein kinase G blocker on secretory activity, apoptosis and the cAMP/protein kinase A system in porcine ovarian granulosa cells in vitro. *J Steroid Biochem Mol Biol* **74:** 1-9, 2000.
47. SIROTKIN AV, SANISLO P, SCHAEFFER HJ, FLORKOVICOVA I, KOTWICA J, BULLA J, HETENYI L. Thrombopoietin regulates proliferation, apoptosis, secretory activity and intracellular messengers in porcine ovarian follicular cells: involvement of protein kinase A. *J Endocrinol* **183:** 595-604, 2004.
48. SIROTKIN AV, MLYNCEK M, MAKAREVICH AV, FLORKOVICOVA I, HETENYI L. Leptin affects proliferation-, apoptosis- and protein kinase A-related peptides in human ovarian granulosa cells. *Physiol Res* **57:** 437-442, 2008.
49. SUN XX, BOSTROM SL, GRIFFITH LC. Alternative splicing of the eag potassium channel gene in drosophila generates a novel signal transduction scaffolding protein. *Mol Cell Neurosci* **40:** 338-343, 2009.
50. TOMANEK M, CHRONOWSKA E. Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the pig ovary. *Folia Histochema Cytobiol* **44:** 269-274, 2006.
51. WYLLIE A, DONAHUE MS, FISCHER B, HILL D, KEESEY J, MANZOW S. In *Apoptosis and Cell Proliferation*, edn 2, pp 1-5, 64-66, 1998. Mannheim: Boehringer.
52. XIA Z, DICKENS M, RAINGEAUD J, DAVIS RJ, GREENBERG ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270:** 1326-1331, 1995.

53. ZU L, HE J, JIANG H, XU C, PU S, XU G. Bacterial endotoxin stimulates adipose lipolysis via toll-like receptor 4 and extracellular signal-regulated kinase pathway. *J Biol Chem* **284:** 5915-5926, 2009.

Figure Descriptions

Fig. 1 Distribution of PCNA, ERK1,2 and cyclin B1 in ovarian granulosa cells of sexually immature and mature gilts. * significant difference ($P<0.05$) between corresponding groups of sexually immature (n=18, black column) and mature (n=17, grey column) gilts evaluated by t-test and chi-square (χ^2) test. Immunocytochemistry.

Fig. 2 Distribution of Bcl-2 and Bax in ovarian granulosa cells of sexually immature and mature gilts. * significant difference ($P<0.05$) between corresponding groups of sexually immature (n=18, black column) and mature (n=17, grey column) gilts evaluated by t-test and chi-square (χ^2) test. Immunocytochemistry.

Fig. 3 Distribution of PKA, CREB, TK and PKG in ovarian granulosa cells of sexually immature and mature gilts. * significant difference ($P<0.05$) between corresponding groups of sexually immature (n=18, black column) and mature (n=17, grey column) gilts evaluated by t-test and chi-square (χ^2) test. Immunocytochemistry.

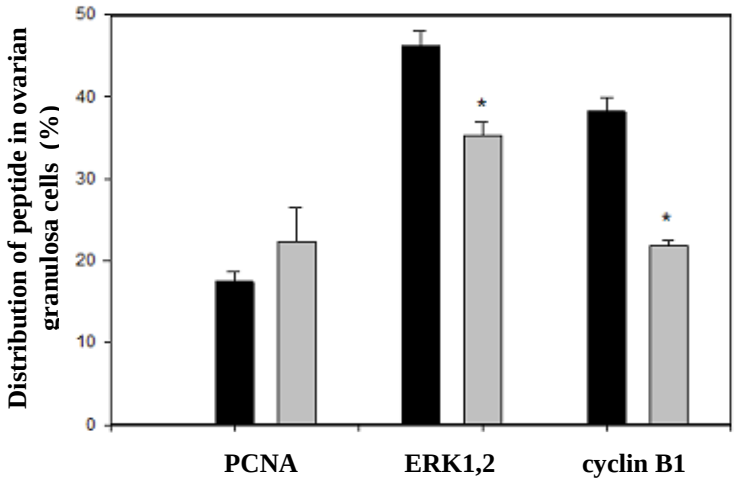
Distribution of peptide in ovarian granulosa cells (%)
50
40
30
20
10
0
*
*
PCNA
ERK1,2
cyclin B1

Fig. 1

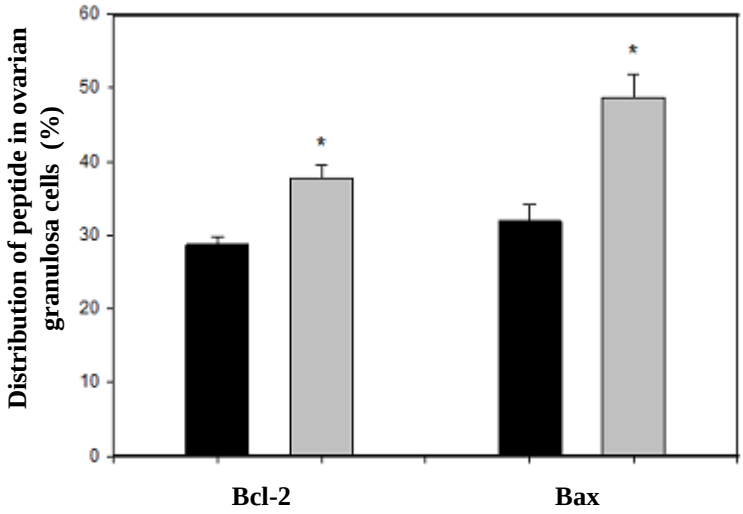
Distribution of peptide in ovarian granulosa cells (%)
60
50
40
30
20
10
0
*
*
Bcl-2
Bax

Fig. 2

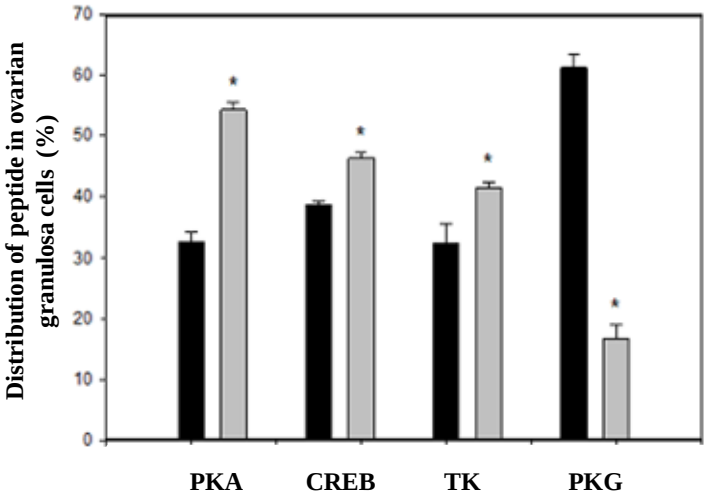
Distribution of peptide in ovarian granulosa cells (%)
70
60
50
40
30
20
10
0
*
*
*
*
PKA
CREB
TK
PKG

Fig. 3