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Teze disertace
k získání vědeckého titulu "doktor věd"
ve skupině věd
biologicko-ekologické

Cellular and molecular events associated with oocyte maturation in preovulatory follicles.

název disertace

Komise pro obhajoby doktorských disertací v oboru
“Zoologie a fyziologie živočichů“

Jméno uchazeče **MVDr. Nagyová Eva, CSc.**

Pracoviště uchazeče
Ústav živočišné fyziologie a genetiky AVČR, v.v.i., Liběchov

Místo a datum ……

Content

1/Introduction

Morphological and biochemical events triggered by LH, including cumulus expansion and oocyte maturation

The ovary life cycle begins with the formation of a cell, the primordial germ cell that becomes an egg, is fertilized and then becomes a new female organism in which the events of the ovary life cycle become progressively expressed in the next generation. The life cycle of the ovary has four major developmental phases;

1. The phase of embryogenesis whereby populations of primordial germ cells and somatic cells become an integrated ovary mass containing oocytes and granulosa cells located within primordial follicles.
2. The phase of folliculogenesis in which oogenesis, granulosogenesis, and thecogenesis occur as a recruited primordial follicle grows and develops to the preovulatory stage or it dies by atresia.
3. The phase of ovulation whereby the oocyte transforms into a mature egg which is secreted into oviduct to await fertilization.
4. The phase luteogenesis whereby the follicle, now lacking an oocyte, luteinizes into an endocrine structure, the corpus luteum, which if implantation does not occur, dies be a process termed luteolysis (Suh et al.2002).

In healthy Graafian follicles of mammalian ovaries, oocytes are maintained at immature germinal vesicle stage and form a gap junction -mediated syncytium -like structure with surrounding layers of compact cumulus cells, which is termed the " oocyte-cumulus complex"(OCC). OCC persists at the immature stage until the preovulatory surge of LH induces it to mature (Eppig, 2001). Expression of LH/choriongonadotropin receptors is restricted to theca and mural granulosa cells that line the follicular wall, and murine cumulus cells or oocytes express no detectable LH/choriongonadotropin receptors (Eppig et al. 1997; Lawrence et al. 1980; Peng et al.1991). Therefore, the mechanism by which the LH induces maturation of OCC in intact follicles was a longstanding puzzle. Only recently, compelling evidence from studies in mice showed that LH stimulation induces the transient and sequential expression of the epidermal growth factor (EGF) family members: amphiregulin, epiregulin and betacelulin (Park et al. 2004). These authors showed that incubation of follicles with these growth factors recapitulates the morphological and biochemical events triggered by LH, including cumulus expansion and oocyte

maturation. They concluded that EGF-related growth factors are paracrine mediators that propagate the LH signal throughout the follicle. In addition, Conti et al. (2006) summarizes published data and creates model describing LH signaling. According to this model, LH activation of mural granulosa cells stimulates cAMP signaling, which, in turn, induces the expression of the EGF-like growth factors epiregulin, amphiregulin and betacelulin. These growth factors function by activating EGF receptors in either an autocrine /juxtacrine fashion within the mural layer, or they diffuse to act on cumulus cells. Activation of EGF receptor signaling in cumulus cells, together with cAMP priming, triggers oocyte nuclear maturation and acquisition of developmental competence as well as cumulus expansion.

Importantly, the cumulus expansion is PROCESS that involves synthesis of a backbone of long hyaluronan oligosaccharide chains that are cross-linked by a complex of HA binding cell surface and ECM proteins and proteoglycans. Active components of the cumulus matrix are synthesized directly by cumulus cells under the control of endocrine and oocyte-derived factors; secreted by mural granulosa cells, or enter the follicle in blood plasma (Russell and Salustri, 2006). Hyaluronan (HA) is the major molecule in expanded cumulus ECM. Its synthesis is rapidly induced in vivo after the LH surge (Eppig, 1980; Salustri et al.1992) and increases up to 20-30-fold with FSH stimulation in mouse OCC (Salustri et al.1989) and porcine OCC (Nagyova et al. 1999). Another ECM associated components necessary for organization of cumulus ECM in mouse and porcine are proteins both derived from the serum and locally synthesized. These include the heavy chains of IαI, TNFAIP6, PTX3 and the proteoglycan versican (Russel and Salustri, 2006; Nagyova, 2015). Thus, the expansion, involves cumulus cell production of a sticky mucinous extracellular matrix essential for ovulation, fertilization and the embryonic development. Failure to undergo these maturational processes causes female infertility (Eppig, 2001; Russell and Salustri, 2006).

In this thesis I have summarized our results concerning the transformation oocyte into a mature egg in porcine preovulatory follicle. In particular, I put together our results concerning cellular and molecular events that are associated with oocyte maturation such as gonadotropin-induced resumption of meiosis, role of cAMP as second messenger, cumulus expansion, oocyte-secreted factors, expression of extracellular matrix related components, EGFR and TGFβ signaling pathways, proteasomal protelysis and steroidogenesis.

2/ Short characterization of our results and methods involved in the thesis.

Stručné vyjádření základních myšlenek, metod a závěrů disertace.

I

Is cAMP decrease essential for resumption of meiosis in mouse oocytes?

E Nagyova, J Kalous, P Sutovsky, J Motlik

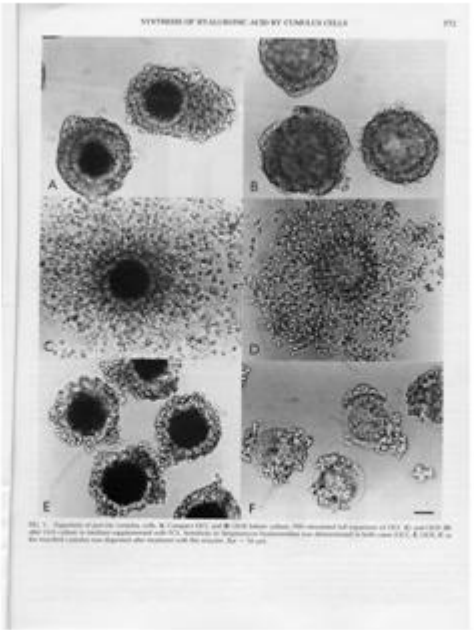
Our experiments were undertaken to answer 2 questions: 1/ Does porcine membrane granulosa (PMG) prevent resumption of meiosis in mouse oocytes with rapid time sequence of GVBD? 2/ Does, co-culture mouse oocytes with PMG influence cAMP levels in the cytoplasm of the mouse oocytes? We found that when freshly isolated mouse oocyte-cumulus complexes (OCC) were cultured on PMG, the OCC did not resume meiosis during the 3-h and 6-h co-culture period. We concluded that the direct contact of the mouse denuded or cumulus -enclosed oocytes with PMG was essential to prevent resumption of meiosis and that the inhibitory activity of PMG was not species-specific. Our experiments also documented the drop of cAMP levels in oocytes blocked at the GV stage by PMG. Importantly, the RIA- assay for measurement of cAMP in oocytes was established.

II

Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: Retention of hyaluronic acid after insulin-like growth factor 1 treatment in serum free medium.

Eva Nagyová, Radek Prochazka and Barbara C. Vanderhyden

The aim of our study was to assess FSH-induced synthesis of hyaluronan (also called hyaluronic acid; HA) by porcine oocyte cumulus complexes (OCC) before and after oocytectomy. The experiments provide evidence that *in vitro* synthesis of HA by porcine OCC in serum supplemented medium is induced by FSH and that oocytectomy does not change the HA synthetic capacity of the cumulus cells or the ability to retain HA within the complex (Fig.1). The experiments performed in serum-free medium suggest that porcine cumulus cells are still capable of responding to FSH and synthesizing HA-but that the levels generated are reduced, both in total and with respect to retention within the complexes. The addition of IGF1 to the chemically defined medium enabled cumulus cells to expand in response to FSH in a manner similar to that observed when serum was present in the culture medium. We have demonstrated that the ability of IGF1 to enhance cumulus expansion in chemically defined media is associated with normal capabilities of the cumulus cells to both synthesize HA and retain HA within the complex (Nagyova et al, 2002; Nemcova et al.2007). Importantly; 1/the ability of chemically defined media to support normal HA synthesis and cumulus expansion will enable to characterize the cumulus-expansion enabling factor (CEEF), 2/ the assay for measurement of HA in porcine OCC was established.



III

Secretion of paracrine factors enabling expansion of cumulus cells is developmentally regulated in pig oocytes.

Eva Nagyova, Barbara C. Vanderhyden and Radek Prochazka

We used the interspecies testing system, to demonstrate secretion of "cumulus expansion-enabling factor" (CEEF) by porcine oocytes. FSH-stimulated expansion and synthesis of HA by mouse oocytectomized complexes (OOX) were assessed after 18 h of culture in media conditioned by porcine oocytes: 1/at different stages of maturation and 2/ in which maturation was inhibited with a specific inhibitor of cdk-kinases, butyrolactone I. We demonstrated that porcine oocytes produce at least two factors that contribute to the production and stability of the preovulatory matrix. We have shown that secretion of both factors occurred only in GV stage oocytes and during the GV to MI transition. Oocytes that progressed to and beyond MI appeared to produce a factor or factors that enabled production of HA after stimulation of cumulus cells with FSH but not its retention within complexes. Our recent data suggest that growth differentiation factor-9 (GDF9) might be a candidate for the CEEF (Vanderhyden et al., 2003; Prochazka et al, 2004). We have demonstrated for the first time that both factors produced by porcine oocytes could be stored frozen, while maintaining both their expansion-promoting activity and ability to retain HA within the expanded cumulus extracellular matrix.

Table 1 Effect of freezing CEEF on the expansion of intact mouse OCC and OOX complexes

Culture Conditions	Number of Complexes *	Type of Complex	Degree of Expansion **
Fresh medium	40	OCC	+3 to +4
Fresh medium	40	OOX	0
Frozen medium	40	OCC	+3 to +4
Frozen medium	40	OOX	0
Frozen medium, GV oocyte-conditioned	60	OOX	+3 to +4
Frozen medium, LD oocyte-conditioned	60	OOX	+3 to +4

* Total number of complexes examined in at least three replicates.

** Subjective evaluation of the degree of expansion (see Materials and Methods).

For evaluation of expansion of mouse OOX complexes cultured in frozen-thawed oocyte- conditioned media, there were 6 replicates (n=60).

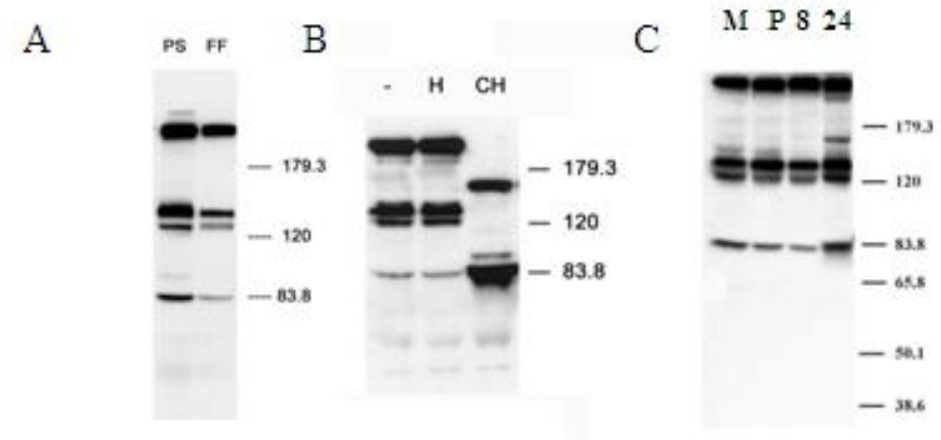
IV

Covalent transfer of heavy chains of inter-alpha-trypsin inhibitor family proteins to hyaluronan in *in vivo* and *in vitro* expanded porcine oocyte-cumulus complexes

Eva Nagyova, Antonella Camaioni, Radek Prochazka and Antonietta Salustri

We provide for the first time the evidence that heavy chains of inter-alpha-trypsin inhibitor family proteins (IαI) are covalently linked to HA in porcine OCC expanded *in vivo* and *in vitro*, thereby directly participating to the formation of cumulus extracellular matrix (ECM). We identified IαI molecules in porcine serum and follicular fluid (Fig.1). For the first time we show that IαI molecules can freely cross the blood-follicle barrier and that follicular fluid collected at any stage of folliculogenesis can be successfully used instead of serum for stabilizing *in vitro* -expanded oocyte cumulus ECM. Finally, we demonstrate that porcine cumulus cells show an autonomous ability to promote the incorporation of the heavy chains of IαI in the HA rich-cumulus ECM.

Western blot analysis of IαI molecules in pig serum and follicular fluid at different stages of folliculogenesis.



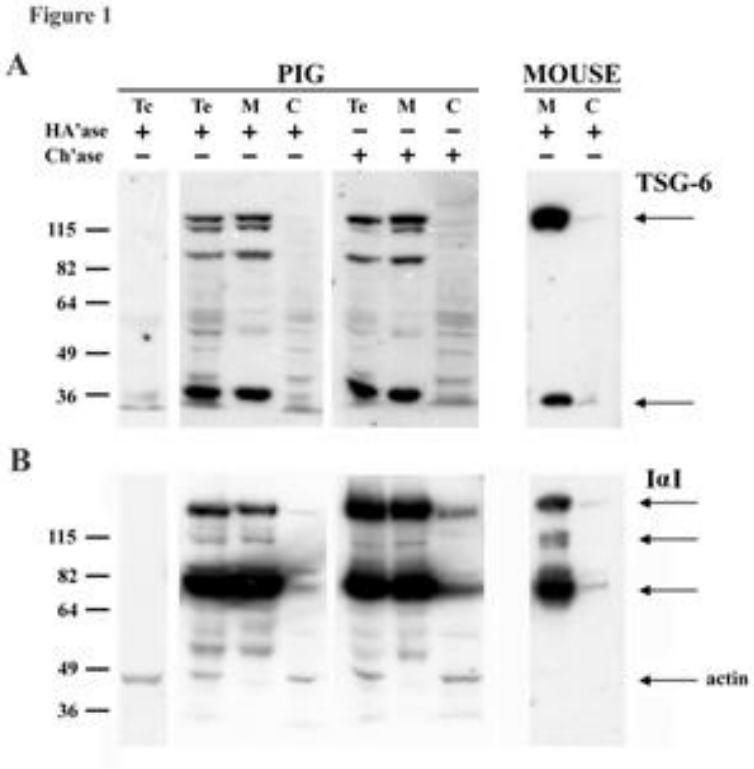
V

Synthesis of tumor-necrosis factor alpha-induced protein 6 in porcine preovulatory follicles: A study with A38 antibody.

Eva Nagyova, Antonella Camaioni, Radek Prochazka, Anthony J Day, and Antonietta Salustri

Our study documents for the first time the presence of the tumor-necrosis factor alpha-induced protein 6 (TNFAIP6 also called TSG6) in porcine expanding OCC and provides several lines of evidence that TNFAIP6 catalyzes the formation of heavy chains (IαI) - HA complexes. Western blot analysis with anti-TNFAIP6 antibody of matrix extracts from 24 h cultured OCC showed intense positive signals at 120 kDa and 35 kDa, corresponding to the heavy chains (IαI)-TNFAIP6 complex and the free TNFAIP6, respectively (Fig.1). Moreover, we found by using cell free assay that porcine follicular fluid collected from follicles 24 h after hCG stimulation contains heavy chains (IαI)-HA coupling activity.

Figure 1



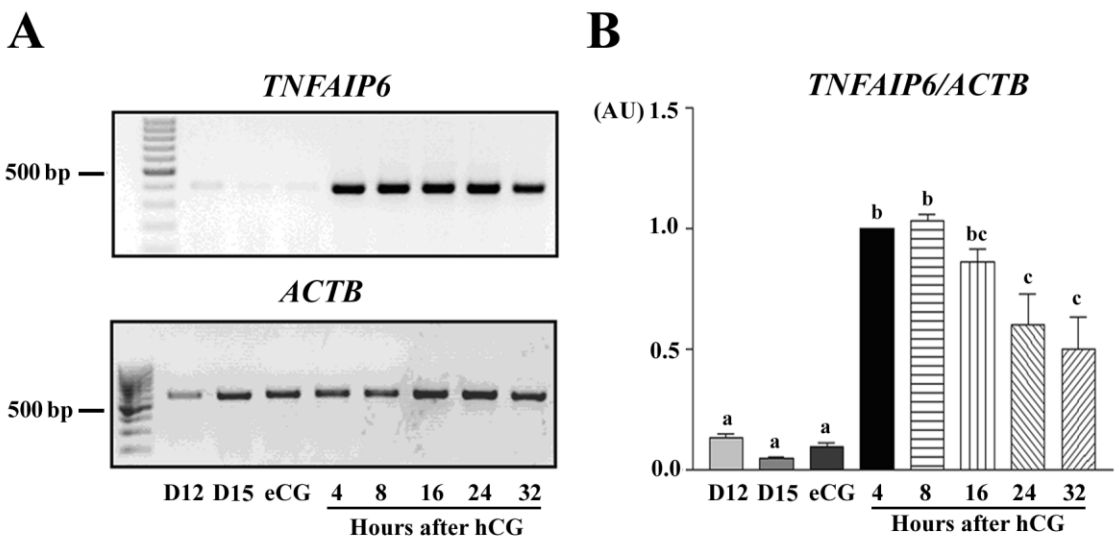
VI

Expression of tumor necrosis factor alpha-induced protein 6 messenger RNA in porcine preovulatory ovarian follicles

Eva Nagyova, Lucie Nemcova and Radek Prochazka

We determined the temporal pattern of expression and relative abundance (RA) of *TNFAIP6* mRNA in porcine growing and preovulatory follicles. We assessed the changes in the RA of *TNFAIP6* mRNA in mural granulosa cells (MGC) during the preovulatory period and in the gonadotropin-stimulated OCC and MGC cultured in vitro. The levels of TNFAIP6 mRNA were low in the follicles on Day 12 and Day 15 of the estrous cycle and at 66 h after PMSG stimulation but were significantly increased at 4 h after hCG. The high level of TNFAIP6 expression was maintained until 16 h after hCG stimulation and gradually decreased at 24 h and 32 h after hCG (Fig.1). During *in vitro* culture, FSH/LH -induced TNFAIP6 mRNA was expressed in both OCC and MGC in a similar temporal pattern as seen *in vivo*. We conclude that TNFAIP6 expression in the pig, like other species, increases in preovulatory follicles soon after the LH (hCG) surge. The OCC and MGC display similar patterns of TNFAIP6 expression under both *in vivo* and *in vitro* conditions. Our finding strengthens the hypothesis that TNFAIP6-catalyzes the formation of heavy chains (of IαI)-HA complexes and that this mechanism is common to mammalian OCC.

Figure 1



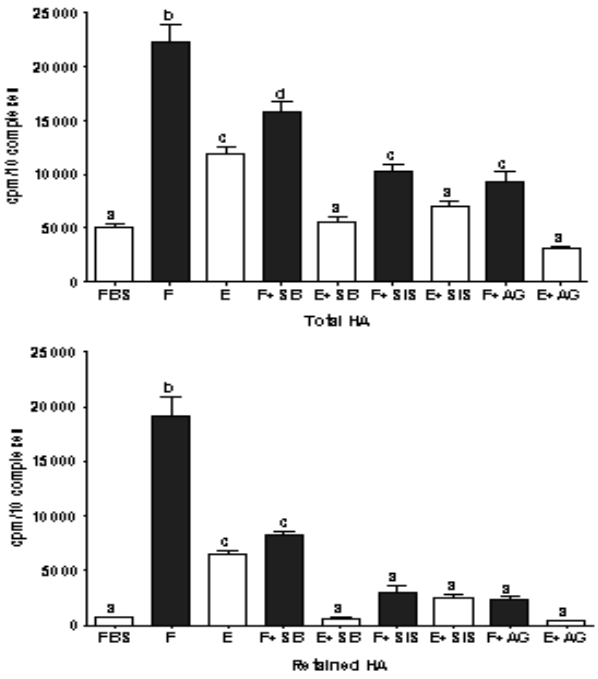
VII

Activation of cumulus cell SMAD2/3 and epidermal growth factor receptor pathways are involved in porcine oocyte-cumulus cell expansion and steroidogenesis

Eva Nagyova, Antonella Camaioni, Sona Scsukova, Alzbeta Mlynarcikova, Radek Prochazka, Lucie Nemcova, Antonietta Salustri.

The aim of the present study was to determine if together with EGFR activation, the SMAD2/3 signaling pathway is involved in regulating cumulus expansion and steroidogenic activity of porcine OCC stimulated in vitro with either FSH or EGF. The individual signaling pathways were disrupted by specific small molecule inhibitors, that is SB431542 (specific inhibitor of SMAD2 and SMAD3 activation), SIS3 (specific inhibitor of SMAD3 activation) and AG1478 (EGFR tyrosine kinase inhibitor).We provide the first evidence that the SMAD2/3 signaling pathway is involved in regulating expansion and HA synthesis in porcine OCC. We also show that SMAD2/3 activation by GDF9/TGFβ growth factors produced by the oocyte and/or cumulus cells contribute to limiting gonadotropin induction of progesterone synthesis by porcine cumulus cells. Moreover, we demonstrate that in FSH-stimulated porcine OCC inhibition of EGFR kinase activity by AG1478 strongly decreases HA synthesis and its retention in the matrix (Fig.1), as well as progesterone synthesis. Our findings show that cross talk between FSH/EGFR and TGFβ/GDF9 signaling pathways is essential for functional activities of porcine OCC.

Figure 1



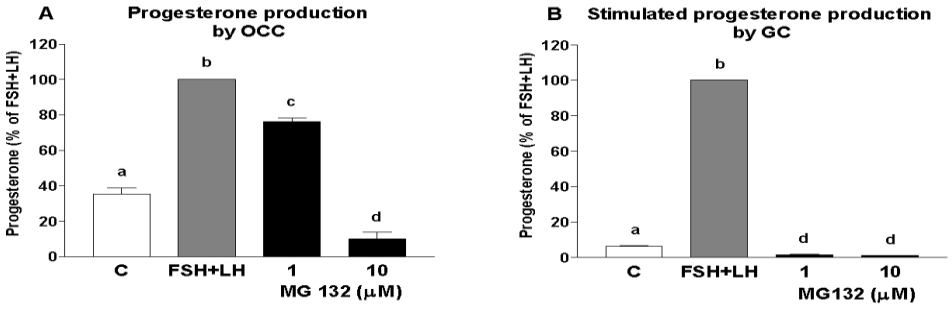
VIII

Inhibition of proteasomal proteolysis affects expression of extracellular matrix components and steroidogenesis in porcine oocyte-cumulus complexes.

Eva Nagyova, Sona Scsukova, Lucie Nemcova, Alzbeta Mlynarcikova, Young-Joo Yi, Miriam Sutovsky, Peter Sutovsky

We determined the targets of proteasomal inhibition that prevent cumulus expansion and formation of cumulus ECM. We have found that treatment with MG132 (a specific proteasomal inhibitor) reduced the expression of cumulus matrix-related transcripts: *TNFAIP6* and *HAS2*. Concomitantly HA was detected with biotinylated HABP within FSH/LH-stimulated OCC, but not in those treated with MG132. The FSH/LH-stimulated progesterone production was significantly suppressed by MG132 in OCC and granulosa cells (Fig.1). Importantly, our results show that the ability of gonadotropin-stimulated porcine cumulus cells to produce progesterone to a level comparable with control OCC was not restored when MG132 was present 20 h in the culture, but it was restored when MG132 was present only 3 h.

Figure 1

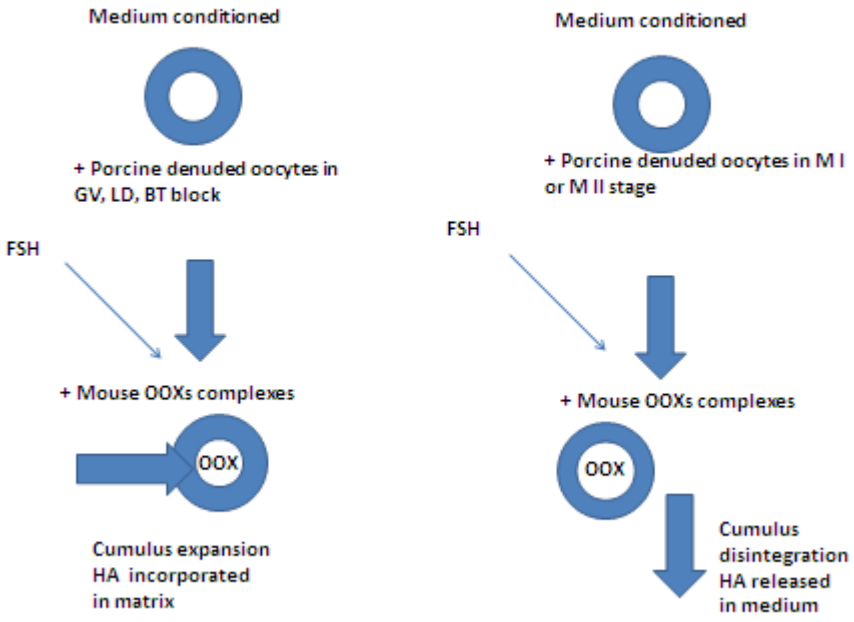


IX

Regulation of cumulus expansion and hyaluronan"synthesis in porcine oocyte-cumulus complexes during in vitro maturation

Nagyova Eva

This review deals with molecular mechanisms controlling three important ovarian follictlar processes: 1/ expansion of the porcine OCC, 2/ synthesis of HA by cumulus cell, and 3/ production of the progesterone by OCC. We show that the net synthesis of HA, during the FSH-stimulated expansion of the OCC in the presence of serum, correlates directly with accumulation of glycosaminoglycans in the cumulus ECM; that porcine oocytes produce at least two factors that have influence on the formation and stability of the preovulatory cumulus ECM (Fig.1) and that the IGF1 is the component of the serum that promotes the FSH-stimulated synthesis and retention of HA within the expanded cumulus ECM by PI3K/AKT and MAPK3/1 -dependent mechanisms. Moreover, we have found that cross talk between FSH/EGFR and TGFβ/GDF9 signaling pathways is essential for functional activities of the porcine OCC; since, FSH-induced synthesis of both HA and progesterone is reduced but not abolished by EGFR tyrosine kinase inhibitor, AG1478, indicating that other signaling pathways elicited by FSH are operating in parallel. Furthermore, SMAD2/3 signaling pathway is involved in the control of both cumulus expansion and steroidogenesis in porcine OCC; since SMAD2/3 activation by TGFβ/GDF9 produced by oocyte and /or cumulus cells, significantly affects gonadotropin-induced HA and progesterone synthesis by porcine cumulus cells.



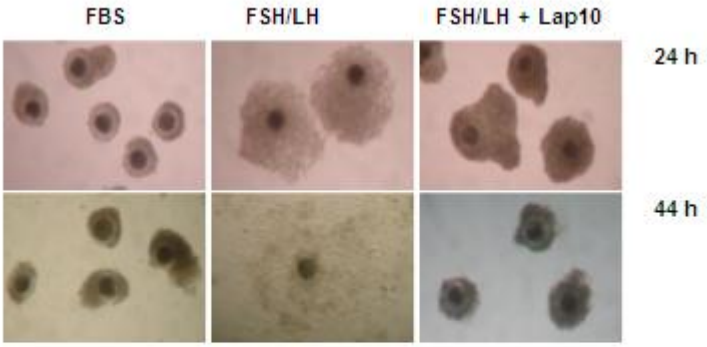
X

Lapatinib inhibits meiotic maturation of porcine oocyte-cumulus complexes cultured in vitro in gonadotropin-supplemented medium.

Eva Nagyova, Lucie Nemcova, Alzbeta Mlynarcikova, Sona Scsukova, Jaroslav Kalous.

It has been suggested that the use of biological agents in female cancers increases the probability that some women will conceive while taking a growth factor signaling pathway inhibitor. The aim of this study was to determine whether inhibition of EGF receptor (EGFR) tyrosine kinase activity with lapatinib affects oocyte maturation. We found that lapatinib, through the EGF receptor signaling pathway inhibits oocyte maturation. Importantly, lapatinib reduced the expression of cumulus expansion-related transcripts (*TNFAIP6, PTGS2*), synthesis of HA, cumulus expansion (Fig.1), and progesterone secretion by OCC that were cultured in FSH/LH-supplemented medium. Taken together, the present data indicate that porcine OCC are sensitive to lapatinib -mediated inhibition of the EGFR pathway, since lapatinib completely blocked nuclear maturation of porcine OCC cultured in vitro.

Figure 1



Cumulus expansion stimulated with FSH/LH.
Effect of lapatinib on cumulus expansion assessed after 24 or 44 h.

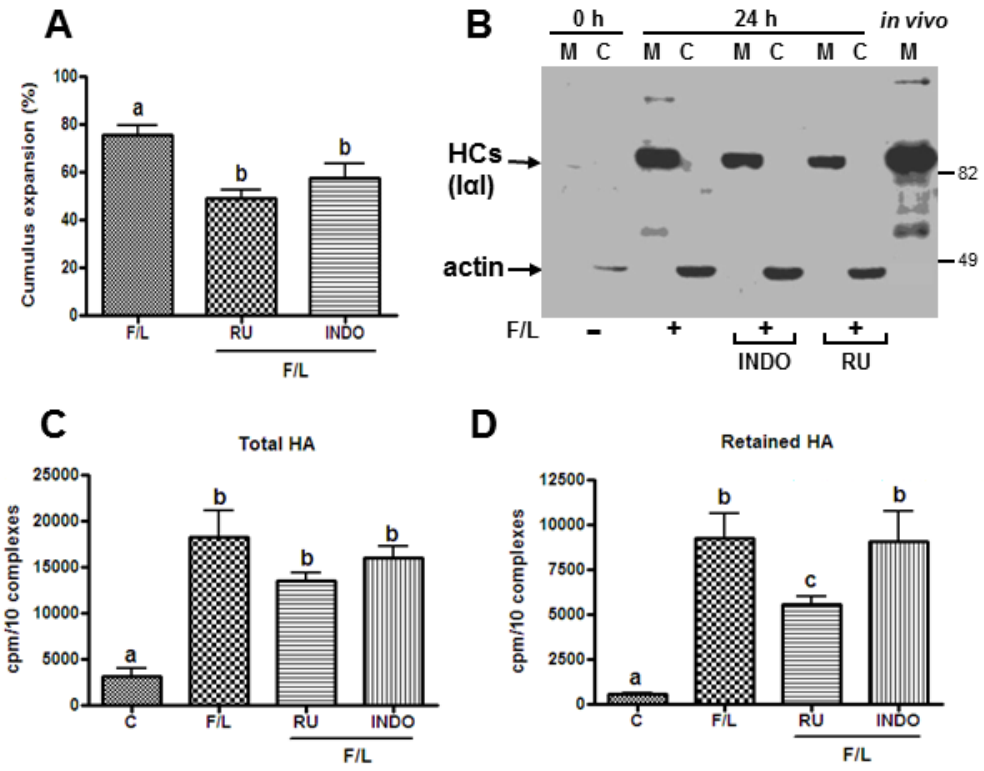
XI

Effects of RU486 and indomethacin on meiotic maturation, formation of extracellular matrix, and progesterone production by porcine oocyte cumulus complexes

Nagyova Eva, Scsukova Sona, Kalous Jaroslav, Mlynarcikova Alzbeta.

We investigated whether inhibition of either progesterone receptor (PR) by PR antagonist, RU486, or cyclooxygenase-2 (COX-2) by indomethacin, affects FSH/LH-induced oocyte maturation in pig. We confirmed findings of several investigators showing that PR-signaling pathway is involved in regulation of oocyte maturation. Importantly, we have shown that PR and COX-2 do not appear to play a role in FSH/LH-induced total HA synthesis by porcine OCC or in formation of cumulus ECM (Fig.1). However, both PR and COX-2 activity affect FSH/LH-regulated progesterone production by porcine granulosa cells and OCC.

Figure 1



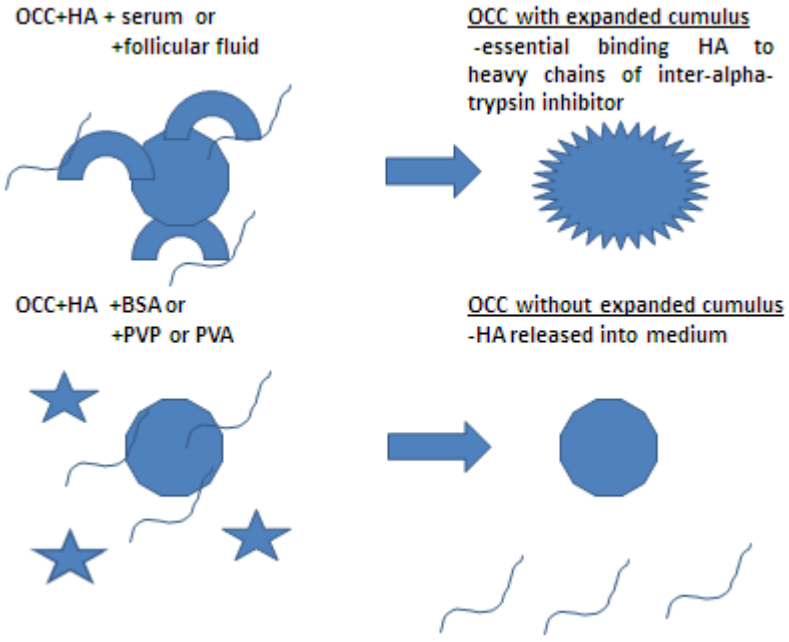
XII

Organization of the expanded cumulus -extracellular matrix in preovulatory follicles: a role for inter-alpha-trypsin inhibitor

Nagyova Eva

The present review pointing out that: 1/ formation of expanded HA-rich oocyte-cumulus ECM is dependent on the presence of intact IαI molecules (Fig.1), 2/the heavy chains of IαI molecules identified in the serum/follicular fluid are covalently linked to HA during cumulus expansion in mouse and pig 3/the family of IαI molecules can freely cross the blood barrier and the follicular fluid collected at any stage of folliculogenesis can be successfully used instead of serum to form expanded cumulus ECM in pig. Moreover, we confirmed the role of TNFAIP6 in porcine preovulatory follicle, since we have shown that TNFAIP6 is expressed in porcine preovulatory follicle soon after following LH/hCG surge and the OCC and MGC display the similar pattern of TNFAIP6 expression that is also comparable under *in vivo* and *in vitro* conditions. Finally, this review provides clear evidence that TNFAIP6 mediates the transfer of heavy chains of IαI to HA in porcine cumulus ECM.

Figure 1



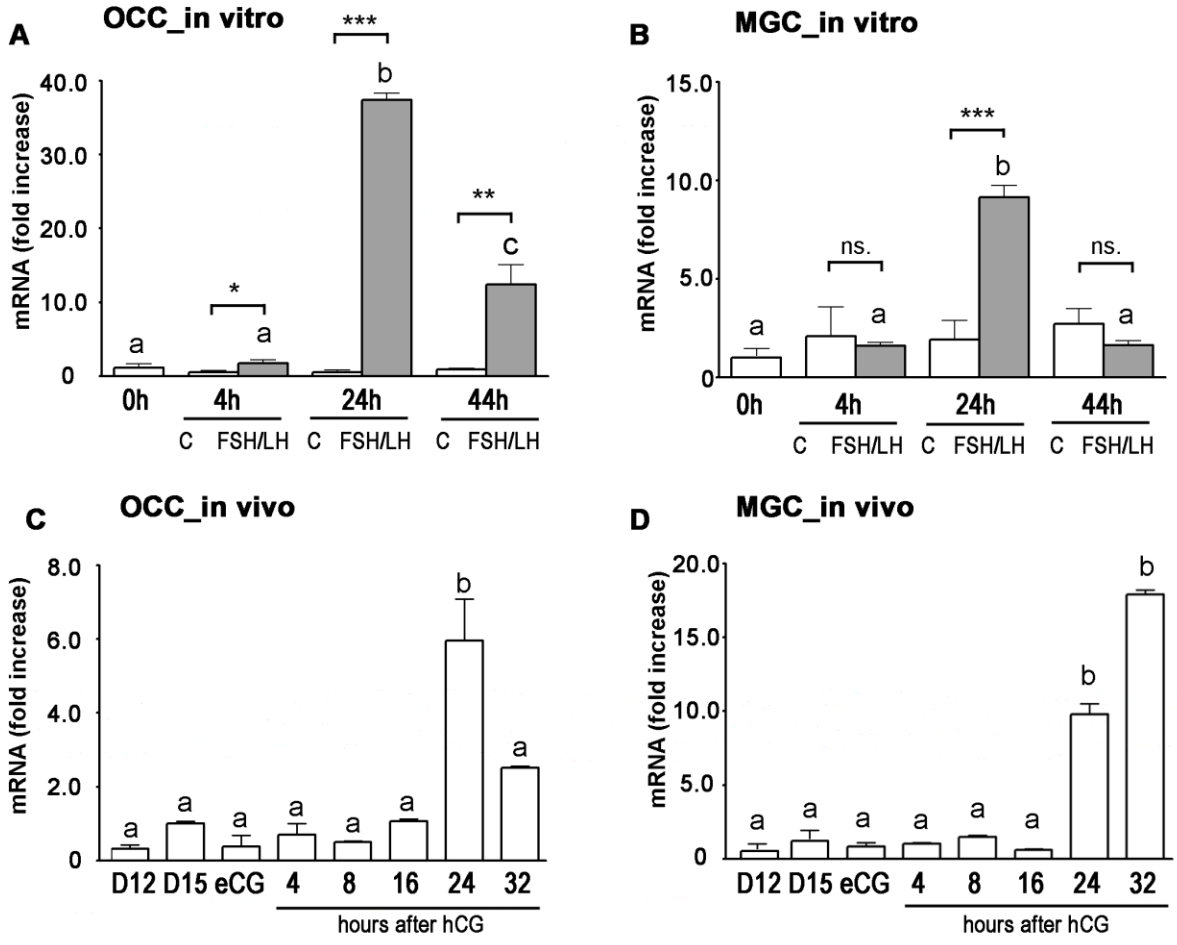
XIII

Increased expression of pentraxin 3 after *in vivo* and *in vitro* stimulation with gonadotropins in porcine oocyte-cumulus complexes and granulosa cells

Nagyova E, Kalous J and Nemcova L

We investigated the expression of pentraxin 3 (PTX3) in porcine preovulatory follicle. We found that the expression of *PTX3* transcripts was significantly increased 24 h after either *in vivo* hCG stimulation or *in vitro* FSH/LH treatment in both OCC and mural granulosa cells (Fig.1). Western blot analysis with PTX3 antibody revealed that not only matrix extracts from *in vivo* stimulated gilts contain high levels of PTX3 protein but also matrix extracts of FSH/LH stimulated OCC that were cultured in medium supplemented either with follicular fluid or with porcine serum. Moreover, localization of PTX3 protein in the OCC was confirmed by immunostaining.

Figure 1



3/Methods involved in the thesis.

Stručné vyjádření základních metod.

Isolation and Culture of Oocyte-Cumulus Complexes and Granulosa cells

Porcine ovaries were collected at a local abattoir and immediately transported to the laboratory. OCCs were aspirated from medium-sized antral follicles about 3-5 mm in diameter. Culture medium was M199 (Sevac Prague, Czech Republic) supplemented with 20 mM $NaHCO_3$, 6.25 mM HEPES, 0.91 mM sodium pyruvate, 1.62 mM calcium lactate and antibiotics. Incubation of OCCs was carried out in 96-well dishes (Nunc) at 38.5°C in the atmosphere of 5% CO_2. Groups of 10 OCCs were incubated in 100 μl media/well with or without the tested chemicals (SB431542, Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany; SIS3, Merck KGaA, Darmstadt, Germany; AG1478, Sigma, Prague, Czech Republic). For PCR experiments groups of 30 OCCs were cultured in 4-well dishes (Nunclon, Roskilde, Denmark). Stock solutions of 10 mM SB431542, SIS3 and AG1478 were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium prior to addition to culture wells to obtain final concentrations: 10 μM for SB431542, 20 μM for SIS3 and 10 μM for AG1478. Final concentration of DMSO in culture media was 0.1 and 0.2 % (vol/vol) and DMSO was also added to control groups. After 1 hr pretreatment of OCCs with the tested inhibitors (SB431542, SIS3, and AG1478), rhFSH (100 ng/ml; Organon, Oss, The Netherlands) or EGF (100 ng/ml; Sigma, Prague, Czech Republic) and fetal bovine serum (FBS, 5%; GIBCO, UK) were added to the culture medium and the OCCs were further incubated for 23 hr.

Granulosa cells (GC) were aspirated from medium follicles (3-5 mm in diameter), washed three times in medium M199 with Earl's salt and Hepes buffer and dispersed in medium M199 supplemented with L-glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and

fungizone (0.25 μg/ml). Incubation of GC was carried out in 24-well plates (TPP AG, Switzerland) at 37 ˚C in the atmosphere of 5% CO_2. The density and cell viability were determined in a haemocytometer by trypan blue exclusion. The cell viability ranged from 80% to 90%. Cells were incubated at the density of 1-1.2 x 10^6 viable cells/0.5 ml of culture medium supplemented with 5% FBS (Kolena and Channing, 1985). After 1 hr pretreatment of GC with the tested inhibitors (SB431542, SIS3, AG1478), FSH (1 μg/ml) or EGF (100 ng/ml) was added to the culture medium and GC were further cultured for 71 hr.

Oocyte - Granulosa Cell Coculture

Denuded oocytes were prepared by vortexing isolated OCCs in 1 ml of medium for 5 min. Granulosa cells were seeded into each individual well of a 24-well plate at a density of 2.5 x 10^5 cells/0.5 ml of culture medium supplemented with 5% FBS in the presence or absence of 20 denuded oocytes per well. After 1 hr pretreatment of GC with SB431542 (10 μM), FSH (1 μg/ml) was added to the culture medium and granulosa cells were further cultured for 47 hr.

Progesterone Assay

At the end of the incubation period, the cultured media were collected for progesterone radioimmunoassay determination (RIA kit, Institute of Isotopes, Budapest, Hungary). The cross-reactivity of the progesterone antibody was less than 13% with other progestins, and less than 0.01% with the androgens and estrogens tested. The intra- and inter-assay coefficients of variation were below or equal to 10.2% and 11.8%, respectively.

Hyaluronic Acid Synthesis

Synthesis of hyaluronic acid was measured as described previously (Nagyova et al., 1999). Briefly, groups of 10 porcine OCCs were cultured for 24 hr in the FSH- or EGF-supplemented medium with or without inhibitors (SB431542, SIS3, AG1478) in the presence of 2.5 μCi of D- [6-^{3}H] glucosamine hydrochloride (MGP, Zlin, Czech Republic). The cultures were

terminated by adding 10 µl of a solution containing 50 mg/ml pronase (Sigma, Prague, Czech Republic) and 10% Triton X-100 in 0.2 M Tris buffer, pH 7.8. The samples were incubated for 1 hr at 38.5 °C and then transferred to Whatman 3MM filter paper circles. The circles were air-dried and then washed through three changes of solution containing 0.5% cetylpyridinium chloride and 10 mM nonradioactive glucosamine hydrochloride (Sigma, Prague, Czech Republic) for 45 min each. The circles were dried once again and radioactivity was measured using a liquid scintillation counter. Synthesis of HA was measured either in medium plus OCC (total HA) or within the complexes alone (retained HA).

Assessment of Oocyte Maturation

Treated OCCs were denuded and mounted on slides. Oocytes were then fixed for 48 hr in acetic acid/ethanol (1:3, v/v), stained with 1% orcein, and examined under a phase-contrast microscope. Oocytes cultured in control, FSH-, or EGF- supplemented medium in the absence or presence inhibitors for 24 hr were scored for germinal vesicle breakdown (GVBD).

Real-Time Reverse Transcription-Polymerase Chain Reaction

The total RNA from 30 OCCs was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration of the RNA in the samples was measured by a spectrophotometer Nanodrop ND-1000, (NanoDrop Technologies, Wilmington, DE).

Real-time RT-PCR was performed on the RotorGene 3000 cycler (Corbett Research, Sydney, Australia) by a One-Step RT-PCR Kit (Qiagen) with gene specific primers (Table 1). The 25 µl total reaction volume contained QIAGEN OneStep RT-PCR Buffer (1x), dNTP Mix (400 µmol/l of each), reverse and forward primers (both 400 µmol/l), SybrGreenI (1 µl of 1:1000x stock solution, Molecular Probes, Oregon), RNasine inhibitor (5 IU, Promega), QIAGEN OneStep RT-PCR Enzyme Mix (1 µl) and template RNA (3 µl). The reaction conditions were as follows: reverse transcription at 50 ºC for 30 min, initial activation at 95 ºC

for 15 min, cycling: denaturation at 94 ºC for 15 s, annealing at temperature specific for each set of primers for 20 s, extension at 72 ºC for 20 s. Fluorescence data were acquired at approx. 3 ºC below the melting temperature of products to distinguish the possible primer dimers. Products were verified by melting analysis and gel electrophoresis on 1.5% agarose gel with ethidium bromide staining. The relative abundance (RA) of templates in different samples was determined using comparative analysis software (Corbett Research). The RAs for individual target genes were normalized according to the relative concentration of the internal standard. The ratios of the target gene concentration to the *ACTB* concentration were estimated in each sample.

To confirm the identity of the RT-PCR products with *HAS2, TNFAIP6* and *PTX3*, DNA was isolated from the gel using MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using Big Dye Terminator kit V.3.1 (Applied Biosystems, Foster City, CA) and ABI Prism 310 (Applied Biosystems). The resulting sequences were identified using the Basic Local Alignment Search Tool (BLAST).

Immunofluorescence

The OCC cultured in vitro for 24 h were fixed at room temperature (RT) with 2% (w/vol) paraformaldehyde-PBS (pH 7.4). After 40 min, the OCC were washed three times in 0.1 M PBS with 0.3% PVP, and blocked in 0.1 M PBS containing 5% normal goat serum for 40 min. To detect expression of cumulus-related proteins such as TNFAIP6, PTX3 and HABP, the fixed OCC were incubated for 72 h at 4°C with either rabbit polyclonal anti-human TNFAIP6 antibody (Santa Cruz Biotechnology, 1:100 dilution), rabbit polyclonal anti-human PTX3 antibody (Abcam, Cambridge, UK; 1:100 dilution) or biotinylated HA-binding protein (HABP; Merck-Calbiochem, Prague, Czech Republic; 1:20 dilution). After washing three times with 0.1 M PBS containing 0.3% PVP and 0.1% Tween, the OCC were incubated for 2 h at RT with either fluorescent Alexa 488 goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands; 1:500)

for TNFAIP6 and PTX3 detection or Alexa Fluor 488 streptavidin-conjugate (Molecular Probes, Leiden, The Netherlands; 1:80) for HABP detection. Primary antibodies were omitted as a control for the specificity of immunostaining. DNA was stained with 2.5 µg/mL of 4,6-diamidino-2-phenylindole (DAPI). Finally, the OCC were washed three times in 0.1 M PBS with 0.3% PVP and 0.1% Tween, mounted in fluorescent mounting medium (Dako, Carpenteria, CA) and examined with an Olympus AX70 microscope equipped with a DP30BW CCD camera. Image files were edited with Adobe Photoshop computer software.

Western Blot Analysis

Groups of twenty complexes obtained at 0 h or after 24 h of in vitro culture were washed in PBS containing a cocktail of protease inhibitors (PBS-PI) (Complete^{TM}, Mini, Boeringher Mannheim, Germany) and then digested with 1 IU of Streptomyces hyaluronidase (Merck-Calbiochem, Prague, Czech Republic) in 20 µL of PBS-PI for 2 h at 37°C. The digested samples were centrifuged at 300 X g for 5 min to separate the matrix extract from the cell pellet. The in vivo complexes were isolated from naturally cycling pigs, and matrix and cell pellet extracts were prepared from three OCCs for each lane. Reducing Laemmli buffer was added to each fraction. All samples were boiled for 4 min at 99°C, separated in 7.5 % acrylamide/SDS gels and transferred to Hybond-P membranes (Amersham-Pharmacia, Prague, Czech Republic). Membranes were blocked with 5% non-fat dry milk in TBS for 2 h at RT. Primary and secondary antibodies were diluted in TBS containing 5% BSA and 0.05% Tween 20. Proteins of the IαI family were detected using rabbit anti- human IαI antibody (1:2000, Dako, Carpenteria, CA) after overnight incubation at 4°C, followed by rabbit anti-actin antibody (1:500; Sigma-Aldrich, Prague, Czech Republic) incubated 1 h at RT. Donkey anti-rabbit IgG horseradish peroxidase-linked F (ab')^2 fragment (1:10000; Amersham-Pharmacia, Prague, Czech Republic) was used as

the second antibody for 1 h at RT. After primary and secondary antibody, 3 X 10 min washes were made in TBS containing 0.1% Tween 20. The positive reaction was detected by enhanced chemiluminescence (ECL, Amersham-Pharmacia, Prague, Czech Republic). To compare sample loading, the expression of actin protein with a positive band at ~42 kDa was analyzed [10].

4/ Summary

Stručné vyjádření závěrů disertace

The ovulatory process can be regarded as a series of biochemical and morphological changes ultimately leading to the release of a mature oocyte and the transformation of the Graafian follicle into the corpus luteum. This process involves acute changes in steroidogenesis, resumption of oocyte meiosis and finally rupture of the follicular wall and luteinization of the granulosa cells. Normally, all of these changes are induced synchronously by the pre-ovulatory LH surge. Experimentally, however, these changes in steroidogenesis, oocyte maturation and follicular rupture can be dissociated from each other showing that the LH effect is mediated via different cellular messengers (Channing et al. 1978). The main subject of this thesis was analysis of cellular and molecular events associated with oocyte maturation in porcine preovulatory follicles. Experimentally, we assessed the changes in steroidogenesis and oocyte maturation in porcine preovulatory follicles. Moreover we analyzed the effect of oocyte factors on cumulus expansion and progesterone production, identified components related to cumulus extracellular matrix, determined several signaling pathways associated with oocyte maturation and organization of cumulus ECM. Finally, we put together following important conclusions to demonstrate that:

-The direct contact of the mouse denuded or cumulus -enclosed oocytes with PMG is essential to prevent resumption of meiosis and the inhibitory activity of PMG is not species-specific. We have shown the drop of cAMP levels in oocytes blocked at the GV stage by PMG.

- *In vitro* synthesis of HA by porcine OCC in serum supplemented medium is induced by FSH and the oocytectomy does not change the HA synthetic capacity of the cumulus cells or the ability to retain HA within the complex. In serum-free medium porcine cumulus cells are still capable of responding to FSH and synthesizing HA-but that the levels generated are reduced, both in total and with respect to retention within the complexes. The ability of IGF1 to enhance cumulus expansion in chemically defined media is associated with normal capabilities of the cumulus cells to both synthesize HA and retain HA within the complex.

- Porcine oocytes produce at least two factors that contribute to the production and stability of the preovulatory matrix. Secretion of both factors occurred only in GV stage oocytes and during the GV to MI transition. Oocytes that progressed to and beyond MI appeared to produce a factor or factors that enabled production of HA after stimulation of cumulus cells with FSH but not its retention within expanded complexes. Both factors produced by porcine oocytes could be stored frozen, while maintaining both their expansion-promoting activity and ability to retain HA within the expanded cumulus extracellular matrix.

- The heavy chains of inter-alpha-trypsin inhibitor family proteins (IαI) are covalently linked to HA in porcine OCC expanded *in vivo* and *in vitro*, thereby directly participating to the formation of cumulus extracellular matrix (ECM). We identified IαI molecules in porcine serum and follicular fluid. We show that IαI molecules can freely cross the blood-follicle barrier and that follicular fluid collected at any stage of folliculogenesis can be successfully used instead of serum for stabilizing *in vitro* -expanded oocyte cumulus ECM.

- The tumor-necrosis factor alpha-induced protein 6 (TNFAIP6) is present in porcine expanding OCC and catalyzes the formation of heavy chains (IαI) - HA complex. Western blot analysis with

anti-TNFAIP6 antibody revealed intense positive signals at 120 kDa and 35 kDa, corresponding to the heavy chains (IαI)-TNFAIP6 complex and the free TNFAIP6, respectively. We found that porcine follicular fluid collected from follicles 24 h after hCG stimulation contains heavy chains (IαI)-HA coupling activity.

-The *TNFAIP6* expression in the pig, like other species, increases in preovulatory follicles soon after the LH (hCG) surge. The OCC and MGC display similar patterns of *TNFAIP6* expression under both *in vivo* and *in vitro* conditions.

- The SMAD2/3 signaling pathway is involved in regulating expansion and HA synthesis in porcine OCC. The SMAD2/3 activation by GDF9/TGFβ growth factors produced by the oocyte and/or cumulus cells contributes to limiting gonadotropin induction of progesterone synthesis by porcine cumulus cells. In FSH-stimulated porcine OCC inhibition of EGFR kinase activity by AG1478 strongly decreases HA synthesis and its retention in the matrix, as well as progesterone synthesis. Together, the cross talk between FSH/EGFR and TGFβ/GDF9 signaling pathways is essential for functional activities of porcine OCC.

- The treatment with MG132 (a specific proteasomal inhibitor) reduced the expression of cumulus matrix-related transcripts: *TNFAIP6* and *HAS2*. Concomitantly HA was detected with biotinylated HABP within FSH/LH-stimulated OCC, but not in those treated with MG132. The FSH/LH-stimulated progesterone production was significantly suppressed by MG132 in OCC and granulosa cells.

- The lapatinib, through the EGF receptor signaling pathway inhibits oocyte maturation. The lapatinib reduced the expression of cumulus expansion-related transcripts (*TNFAIP6, PTGS2*), synthesis of HA, cumulus expansion, and progesterone secretion by OCC that were cultured in FSH/LH-supplemented medium. Together, the present data indicate that porcine OCC are sensitive to the lapatinib -mediated inhibition of the EGFR pathway, since the lapatinib almost completely blocked nuclear maturation of porcine OCC cultured in vitro.

- The progesterone receptor (PR)-signaling pathway is involved in regulation of oocyte maturation. The PR and COX-2 do not appear to play a role in FSH/LH-induced total HA synthesis by porcine OCC or in formation of cumulus ECM. However, both PR and COX-2 activity affect FSH/LH-regulated progesterone production by porcine granulosa cells and OCC.

-The formation of expanded HA-rich oocyte-cumulus ECM is dependent on the presence of intact IαI molecules and that the heavy chains of IαI molecules identified in the serum/follicular fluid are covalently linked to HA during cumulus expansion in mouse and pig. The TNFAIP6 is expressed in porcine preovulatory follicle soon after following LH/hCG surge and that TNFAIP6 catalyzes the formation of heavy chains (of IαI) - HA complexes in pig.

- The expression of *PTX3* transcripts was significantly increased 24 h after either *in vivo* hCG stimulation or *in vitro* FSH/LH treatment in both OCC and mural granulosa cells. Western blot analysis with PTX3 antibody revealed high levels of PTX3 protein in matrix extracts of hCG or FSH/LH stimulated OCC. The localization of PTX3 in porcine OCC was confirmed also by immunostaining.

5/Selected papers for this thesis: Eva Nagyova

Seznam vlastních vědeckých prací, které jsou podkladem disertace

Is cAMP decrease essential for resumption of meiosis in mouse oocytes?
By: **Nagyova, E;** Kalous, J; Sutovsky, P; et al. REPRODUCTION NUTRITION DEVELOPMENT Volume: 33 Issues: 5 Pages: 419-428 Published: 1993
Times Cited: 5
IF: 0

Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: Retention of hyaluronic acid after insulin-like growth factor-I treatment in serum-free medium.
By: **Nagyova, E;** Prochazka, R; Vanderhyden, BC BIOLOGY OF REPRODUCTION Volume: 61 Issues: 3 Pages: 569-574 Published: SEP 1999
Times Cited: 29
IF: 3.318

Secretion of paracrine factors enabling expansion of cumulus cells is developmentally regulated in pig oocytes.
By: **Nagyova, E;** Vanderhyden, BC; Prochazka, R. BIOLOGY OF REPRODUCTION Volume: 63 Issues: 4 Pages: 1149-1156 Published: OCT 2000
Times Cited: 29
IF: 3.318

Covalent transfer of heavy chains of inter-alpha-trypsin inhibitor family proteins to hyaluronan in in vivo and in vitro expanded porcine oocyte-cumulus complexes.
By: **Nagyova, E;** Camaioni, A; Prochazka, R; et al. BIOLOGY OF REPRODUCTION Volume: 71 Issue: 6 Pages: 1838-1843 Published: DEC 2004
Times Cited: 29
IF: 3.318

Synthesis of tumor necrosis factor alpha-induced protein 6 in porcine preovulatory follicles: A study with A38 antibody.
By: **Nagyova, Eva**; Camaioni, Antonella; Prochazka, Radek; et al. BIOLOGY OF REPRODUCTION Volume: 78 Issues: 5 Pages: 903-909 Published: MAY 2008
Times Cited: 9
IF: 3.318

Expression of Tumor Necrosis Factor Alpha-Induced Protein 6 Messenger RNA in Porcine Preovulatory Ovarian Follicles.
By: **Nagyova, Eva**; Nemcova, Lucie; Prochazka, Radek JOURNAL OF REPRODUCTION AND DEVELOPMENT Volume: 55 Issues: 3 Pages: 231-235 Published: JUN 2009
Times Cited: 12
IF: 1.515

Activation of Cumulus Cell SMAD2/3 and Epidermal Growth Factor Receptor Pathways Are Involved in Porcine Oocyte-Cumulus Cell Expansion and Steroidogenesis.
By: **Nagyova, Eva**; Camaioni, Antonella; Scsukova, Sona; et al. MOLECULAR REPRODUCTION AND DEVELOPMENT Volume: 78 Issue: 6 Pages: 391-402 Published: JUN 2011
Times Cited: 14
IF: 2.527
Inhibition of proteasomal proteolysis affects expression of extracellular matrix components and steroidogenesis in porcine oocyte-cumulus complexes
By: **Nagyova, E.;** Scsukova, S.; Nemcova, L.; et al. DOMESTIC ANIMAL ENDOCRINOLOGY Volume: 42 Issues: 1 Page: 50-62 Published: JAN 2012
Times Cited: 8
IF: 2.171
Regulation of cumulus expansion and hyaluronan synthesis in porcine oocyte-cumulus complexes during in vitro maturation.
By: **Nagyova, E** ENDOCRINE REGULATIONS Volume: 46 Issues: 4 Pages: 225-35 Published: 2012-Oct
Times Cited: 5
IF: 0
Lapatinib inhibits meiotic maturation of porcine oocyte-cumulus complexes cultured in vitro in gonadotropin-supplemented medium
By: **Nagyova, Eva;** Nemcova, Lucie; Mlynarcikova, Alzbeta; et al. FERTILITY AND STERILITY Volume: 99 Issue: 6 Pages: 1739-1748 Published: MAY 2013
Times Cited: 1
IF: 4.590
Effects of RU486 and indomethacin on meiotic maturation, formation of extracellular matrix, and progesterone production by, porcine oocyte-cumulus complexes.
By: **Nagyova, E.;** Scsukova, S.; Kalous, J.; et al. DOMESTIC ANIMAL ENDOCRINOLOGY Volume: 48 Pages: 7-14 Published: JUL 2014
Times Cited: 1
IF: 2.171
Organization of the expanded cumulus-extracellular matrix in preovulatory follicles: arole for inter-alpha-trypsin inhibitor.
By: **Nagyova, E** ENDOCRINE REGULATIONS Volume: 49 Issues: 1 Page: 37-45 Published: 2015
Times Cited: 0
IF: 0
Increased expression of pentraxin 3 after in vivo and in vitro stimulation with gonadotropins in porcine oocyte-cumulus complexes.
By: **Nagyova, E;** Kalous, J; Nemcova, L; Submitted to Domestic Animal Endocrinology, August 28, 2015

6/List of all published results /WOS/

Seznam všech výsledků /WOS/

1.

Organization of the expanded cumulus-extracellular matrix in preovulatory follicles: arole for inter-alpha-trypsin inhibitor. By: Nagyova, E Endocrine regulations Volume: 49 Issue: 1 Pages: 37-45 Published: 2015

Times Cited: 0

2.

Effects of RU486 and indomethacin on meiotic maturation, formation of extracellular matrix, and progesterone production by, porcine oocyte-cumulus complexes. By: Nagyova, E.; Scsukova, S.; Kalous, J.; et al. DOMESTIC ANIMAL ENDOCRINOLOGY Volume: 48 Pages: 7-14 Published: JUL 2014

Times Cited: 1

3.

Lapatinib inhibits meiotic maturation of porcine oocyte-cumulus complexes cultured in vitro in gonadotropin-supplemented medium By: Nagyova, Eva; Nemcova, Lucie; Mlynarcikova, Alzbeta; et al. FERTILITY AND STERILITY Volume: 99 Issue: 6 Pages: 1739-1748 Published: MAY 2013

Times Cited: 1

4.

Regulation of cumulus expansion and hyaluronan synthesis in porcine oocyte-cumulus complexes during in vitro maturation. By: Nagyova, E Endocrine regulations Volume: 46 Issue: 4 Pages: 225-35 Published: 2012-Oct

Times Cited: 5

5.

Inhibition of proteasomal proteolysis affects expression of extracellular matrix components and steroidogenesis in porcine oocyte-cumulus complexes By: Nagyova, E.; Scsukova, S.; Nemcova, L.; et al.DOMESTIC ANIMAL ENDOCRINOLOGY Volume: 42 Issues: 1 Page: 50-62 Published: JAN 2012

Times Cited: 8

6.

Activation of Cumulus Cell SMAD2/3 and Epidermal Growth Factor Receptor Pathways Are Involved in Porcine Oocyte-Cumulus Cell Expansion and Steroidogenesis. By: Nagyova, Eva; Camaioni, Antonella;

Scsukova, Sona; et al. MOLECULAR REPRODUCTION AND DEVELOPMENT Volume: 78 Issue: 6 Pages: 391-402 Published: JUN 2011

Times Cited: 13

7.

Effect of epidermal growth factor-like peptides on pig cumulus cell expansion, oocyte maturation, and acquisition of developmental competence in vitro: comparison with gonadotropins. By: Prochazka, Radek; Petlach, Michal; Nagyova, Eva; et al. REPRODUCTION Volume: 141 Issues: 4 Pages: 425-435 Published: APR 2011

Times Cited: 27

8.

Involvement of EGFR and Smad2/3 Activation in FSH-Induced Porcine Oocytes-Cumulus Cell Complex Maturation. By: Nagyova, Eva; Prochazka, Radek; Nemcova, Lucie; et al. Conference: 43rd Annual Meeting of the Society-for-the-Study-of-Reproduction Location: Milwaukee, WI Date: JUL 31-AUG 03, 2010; BIOLOGY OF REPRODUCTION Supplement: S Pages: 187-187 Meeting Abstract: 640 Published: 2010

Times Cited: 0

9.

Development of Functional LH Receptors on Pig Cumulus-Oocyte Complexes Cultured In Vitro by a Novel Two-Step Culture System. By: Prochazka, Radek; Nemcova, Lucie; Nagyova, Eva; et al. MOLECULAR REPRODUCTION AND DEVELOPMENT Volume: 76 Issue: 8 Pages: 751-761 Published: AUG 2009

Times Cited: 6

10.Expression of Tumor Necrosis Factor Alpha-Induced Protein 6 Messenger RNA in Porcine Preovulatory Ovarian Follicles. By: Nagyova, Eva; Nemcova, Lucie; Prochazka, Radek JOURNAL OF REPRODUCTION AND DEVELOPMENT Volume: 55 Issues: 3 Pages: 231-235 Published: JUN 2009

Times Cited: 12

11.

Effects of selected endocrine disruptors on meiotic maturation, cumulus expansion, synthesis of hyaluronan and progesterone by porcine oocyte-cumulus complexes. By: Mlynarcikova, Alzbeta; Nagyova, Eva; Fickova, Maria; et al. TOXICOLOGY IN VITRO Volume: 23 Issues: 3 Pages: 371-377 Published: APR 2009

Times Cited: 27

12.

Altered organization of the oocyte-cumulus extracellular matrix in the presence of proteasomal inhibitor MG132. By: Nagyova, E.; Nemcova, L.; Sutovsky, P. et al. Conference: Joint Conference of the 33rd FEBS Congress/11th IUBMB Conference Location: Athens, GREECE Date: JUN 28-JUL 03, 2008. FEBS JOURNAL Volume: 275 Supplements: 1 Page: 261-261 Published: JUN 2008

Times Cited: 0

13.

Synthesis of tumor necrosis factor alpha-induced protein 6 in porcine preovulatory follicles: A study with A38 antibody. By: Nagyova, Eva; Camaioni, Antonella; Prochazka, Radek; et al. BIOLOGY OF REPRODUCTION Volume: 78 Issues: 5 Pages: 903-909 Published: MAY 2008

Times Cited: 9

14.

Proteolytic activity of the 26s proteasome is required for the meiotic resumption, germinal vesicle breakdown, and cumulus expansion of porcine cumulus-oocyte complexes matured in vitro .By: Yi, Young-Joo; Nagyova, Eva; Manandhar, Gaurishankar; et al. BIOLOGY OF REPRODUCTION Volume: 78 Issue: 1 Pages: 115-126 Published: JAN 2008

Times Cited: 12

15.

Molecular mechanisms of insulin-like growth factor 1 promoted synthesis and retention of hyaluronic acid in porcine oocyte-cumulus complexes. By: Nemcova, Lucie; Nagyova, Eva; Petlach, Michal; et al.

BIOLOGY OF REPRODUCTION Volume: 76 Issues: 6 Pages: 1016-1024 Published: JUN 2007

Times Cited: 14

16.

Proteasomal activity is involved in oocyte meiosis and cumulus expansion during in vitro maturation of porcine oocytes.By: Yi, Young-Joo; Nagyova, Eva; Manandhar, Gaurishankar; et al. Conference: 39th Annual Meeting of the Society-for-the-Study-of-Reproduction Location: Univ Nebraska Med Ctr, Omaha, NE Date: JUL 29-AUG 01, 2006. BIOLOGY OF REPRODUCTION Special Issue: SI Pages: 177-177 Published: 2006

Times Cited: 0

17.

Synthesis of tumor necrosis factor-stimulated gene-6 protein by cumulus and granulosa cells in porcine preovulatory follicle. By: Nagyova, E; Camaioni, A; Prochazka, R; et al. Conference: 38th Annual Meeting of the Society-for-the-Study-of-Reproduction Location: Ctr Rech Biol Reproduct, Quebec City, CANADA Date: JUL 24-27, 2005. BIOLOGY OF REPRODUCTION Special Issue: SI Pages: 159-159 Published: 2005

Times Cited: 0

18.

Covalent transfer of heavy chains of inter-alpha-trypsin inhibitor family proteins to hyaluronan in in vivo and in vitro expanded porcine oocyte-cumulus complexes. By: Nagyova, E; Camaioni, A; Prochazka, R; et al. BIOLOGY OF REPRODUCTION Volume: 71 Issue: 6 Pages: 1838-1843 Published: DEC 2004

Times Cited: 29

19.

Expression of growth differentiation factor 9 messenger RNA in porcine growing and preovulatory ovarian follicles. By: Prochazka, R; Nemcova, L; Nagyova, E; et al. BIOLOGY OF REPRODUCTION Volume: 71 Issues: 4 Pages: 1290-1295 Published: OCT 2004

Times Cited: 40

20.

Inhibitory effect of cadmium and tobacco alkaloids on expansion of porcine oocyte-cumulus complexes. By: Mlynarcikova, A.; Scsukova, S.; Vrsanska, S.; et al. Central European Journal of Public Health Volume: 12 Issues: Suppl. S Pages: S62-S64 Published: MAR 2004

Times Cited: 4

21.

Epidermal growth factor-receptor tyrosine kinase activity regulates expansion of porcine oocyte-cumulus cell complexes in vitro. By: Prochazka, R; Kalab, P; Nagyova, E BIOLOGY OF REPRODUCTION Volume: 68 Issues: 3 Pages: 797-803 Published: MAR 2003

Times Cited: 44

22.

Covalent transfer of inter-alpha-trypsin inhibitor heavy chains to hyaluronan in porcine oocyte-cumulus complexes cultured in vitro. By: Nagyova, E; Camaioni, A; Prochazka, R; et al. Conference: 36th Annual Meeting of the Society-for-the-Study-of-Reproduction Location: CINCINNATI, OHIO Date: JUL 19-22, 2003

BIOLOGY OF REPRODUCTION Volume: 68 Supplements: 1 Page: 261-261 Meeting Abstract: 362 Published: 2003

Times Cited: 0

23.

Components of cigarette smoke inhibit expansion of oocyte-cumulus complexes from porcine follicles. By: Vrsanska, S; Nagyova, E; Mlynarcikova, A; et al. PHYSIOLOGICAL RESEARCH Volume: 52 Issue: 3 Pages: 383-387 Published: 2003

Times Cited: 28

24.

Evaluation of members of the TGFbeta superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis. By: Vanderhyden, B C; Macdonald, E A; Nagyova, E; et al. Reproduction (Cambridge, England) Supplement Volume: 61 Pages: 55-70 Published: 2003

Times Cited: 42

25.

Insulin-like growth factor-I as intraovarian regulator of hyaluronic acid synthesis. By: Nagyova, E.; Prochazka, R.Conference: Annual Conference of the Society for Reproduction and Fertility Location: Leeds, UK Date: July, 2002. Reproduction Abstract Series (Cambridge) Issue: 28 Pages: 31 Published: July 2002

Times Cited: 0

26.

Evaluation of members of the TGF beta superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis. By: Vanderhyden, BC; Macdonald, EA; Nagyova, E; et al. Conference: 6th International Symposium on Reproduction in Domestic Ruminants Location: CRIEFF, SCOTLAND Date: AUG 14-17, 2002. REPRODUCTION Supplement: 61 Pages: 55-70 Published: 2002

Times Cited: 0

27.

Effect of intraovarian factors on porcine follicular cells: cumulus expansion, granulosa and cumulus cell progesterone production. By: Jezova, M; Scsukova, S; Nagyova, E; et al. ANIMAL REPRODUCTION SCIENCE Volume: 65 Issues: 1-2 Pages: 115-126 Published: JAN 31 2001

Times Cited: 22

28.

Effects of TGF-beta and GDF-9 on cumulus expansion and progesterone production by oocytectomized oocyte-cumulus cell complexes. By: Vanderhyden, BC; Nagyova, E; Dhawan, BIOLOGY OF REPRODUCTION Volume: 64 Supplements: 1 Page: 211-211 Published: 2001

Times Cited: 0

29.

Gossypol inhibits follicle-stimulating hormone- and epidermal growth factor-stimulated expansion of oocyte-cumulus complexes from porcine preovulatory follicles. By: Kolena, J; Vrsanska, S; Nagyova, E; et al. PHYSIOLOGICAL RESEARCH Volume: 50 Issues: 6 Pages: 627-630 Published: 2001

Times Cited: 4

30.

Secretion of paracrine factors enabling expansion of cumulus cells is developmentally regulated in pig oocytes. By: Nagyova, E; Vanderhyden, BC; Prochazka, R. BIOLOGY OF REPRODUCTION Volume: 63 Issues: 4 Pages: 1149-1156 Published: OCT 2000

Times Cited: 28

31.

Epidermal growth factor and insulin growth factor I increase FSH-stimulated expansion of porcine cumulus cells in serum-free medium. By: Prochazka, R.; Nagyova, E.; Srsen, V.Conference: Joint Summer Meeting of the Society for the Study of Fertility, the British Andrology Society and the British Fertility Society Location: Edinburgh, Scotland, UK Date: July, 2000 Journal of Reproduction and Fertility Abstract Series Issue: 25 Pages: 64 Published: July, 2000

Times Cited: 0

32.

Developmental regulation of effect of epidermal growth factor on porcine oocyte-cumulus cell complexes: Nuclear maturation, expansion, and F-actin remodeling. By: Prochazka, R; Srsen, V; Nagyova, E; et al. MOLECULAR REPRODUCTION AND DEVELOPMENT Volume: 56 Issue: 1 Pages: 63-73 Published: MAY 2000

Times Cited: 47

33.

Effect of porcine oocytes at different stages of development on synthesis of hyaluronic acid by mouse oocytectomized complexes By: Nagyova, E.; Prochazka, R.; Vanderhyden, B. C. Conference: Proceedings of the Annual Conference of the International Embryo Transfer Society Location: Maastricht,

Netherlands Date: January 09-11, 2000. Theriogenology Volume: 53 Issues: 1 Page: 462 Published: January 1, 2000

Times Cited: 2

34.

Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: Retention of hyaluronic acid after insulin-like growth factor-I treatment in serum-free medium. By: Nagyova, E; Prochazka, R; Vanderhyden, BC BIOLOGY OF REPRODUCTION Volume: 61 Issues: 3 Pages: 569-574 Published: SEP 1999

Times Cited: 29

35.

Effects of follicle-stimulating hormone, bovine somototrophin and okadaic acid on cumulus expansion and nuclear maturation of Blue fox (Alopex lagopus) oocytes in vitro. By: Srsen, V; Kalous, J; Nagyova, E; et al. ZYGOTE Volume: 6 Issues: 4 Pages: 299-309 Published: NOV 1998

Times Cited: 37

36.

Paracrine and autocrine regulation of cumulus expansion in porcine follicles. By: Motlik, J; Prochazka, R; Nagyova, E; et al. Conference: 1st Conference of the European-Society-for-Domestic-Animal-Reproduction Location: NEUSTADT, GERMANY Date: NOV 27-29, 1997.REPRODUCTION IN DOMESTIC ANIMALS Volume: 33 Issue: 3-4 Pages: 181-185 Published: JUN 1998

Times Cited: 0

37.

Secretion of cumulus expansion-enabling factor (CEEF) in porcine follicles. By: Prochazka, R; Nagyova, E; Brem, G; et al. MOLECULAR REPRODUCTION AND DEVELOPMENT Volume: 49 Issue: 2 Pages: 141-149 Published: FEB 1998

Times Cited: 31

38.

Acquisition of meiotic competence is related to the functionality of the phosphoinositide/calcium signaling pathway in the mouse oocyte. By: Lefevre, B; Nagyova, E; Pesty, A; et al. EXPERIMENTAL CELL RESEARCH Volume: 236 Issue: 1 Pages: 193-200 Published: OCT 10 1997

Times Cited: 21

39.

Porcine oocytes produce CEEF only during their growth period and transition to metaphase I.By: Nagyova, E.; Prochazka, R.; Motlik, J.Conference: Annual Conference of the International Embryo Transfer Society Location: Nice, France Date: January 12-14, 1997.Theriogenology Volume: 47 Issue: 1 Pages: 197 Published: 1997 SIOLOGICAL RESEARCH Volume: 50 Issue: 6 Pages: 627-630 Published: 2001

Times Cited: 7

40.

The effect of butyrolactone I, a specific inhibitor of cdk-kinases on GVBD and chromosome condensation during the first meiotic division of pig and cattle oocytes. By: Kubelka, M; Kalab, P; Kalous, J; Nagyova E et al. Conference: Symposium on Oocyte Maturation amd Early Embryonic Development under the Influence of Biologically Active Substances, at 1st Central European Conf on Animal Reproduction Location: KUHLUNGSBORN, GERMANY Date: SEP 22-23, 1996. ARCHIV FUR TIERZUCHT-ARCHIVES OF ANIMAL BREEDING Volume: 39 Special Issues: SI Pages: 53-53 Published: 1996

Times Cited: 3

41.

Porcine cumulus and mural granulosa cells secrete cumulus expansion enabling factor (CEEF).By: Prochazka, R; Nagyova, E; Motlik, J.Conference: Symposium on Oocyte Maturation amd Early Embryonic Development under the Influence of Biologically Active Substances, at 1st Central European Conf on Animal Reproduction Location: KUHLUNGSBORN, GERMANY Date: SEP 22-23, 1996. ARCHIV FUR TIERZUCHT-ARCHIVES OF ANIMAL BREEDING Volume: 39 Special Issues: SI Pages: 86-86 Published: 1996

Times Cited: 0

42.

Resumption of meiosis in Blue fox oocytes is controlled by cumulus granulosa cells. By: Srsen, V; Kalous, J; Nagyova, E; et al.Conference: Symposium on Oocyte Maturation amd Early Embryonic Development under the Influence of Biologically Active Substances, at 1st Central European Conf on Animal Reproduction Location: KUHLUNGSBORN, GERMANY Date: SEP 22-23, 1996. ARCHIV FUR TIERZUCHT-ARCHIVES OF ANIMAL BREEDING Volume: 39 Special Issues: SI Pages: 89-89 Published: 1996

Times Cited: 0

43.

IN-VITRO TECHNIQUES IN FOX REPRODUCTION. By: FARSTAD, W; KROGENAES, A; NAGYOVA, E; et al.

Conference: WORKSHOP ON BIOTECHNOLOGY AND ANIMAL WELFARE Location: ZEIST, NETHERLANDS Date: NOV 08-10, 1992. LIVESTOCK PRODUCTION SCIENCE Volume: 36 Issues: 1 Page: 23-27 Published: JUL 1993

Times Cited: 9

44.

IS CAMP DECREASE ESSENTIAL FOR RESUMPTION OF MEIOSIS IN MOUSE OOCYTES.By: NAGYOVA, E; KALOUS, J; SUTOVSKY, P; et al. Conference: 5th Franco / Czechoslovak Meeting - From Maturation to Cloning: In Vitro Techniques Location: JOUY, FRANCE Date: DEC 14-15, 1992. REPRODUCTION NUTRITION DEVELOPMENT Volume: 33 Issues: 5 Pages: 419-428 Published: 1993

Times Cited: 5

45.

LACK OF EFFECT OF OOCYTECTOMY ON EXPANSION OF THE PORCINE CUMULUS. By: PROCHAZKA, R; NAGYOVA, E; RIMKEVICOVA, Z; et al. JOURNAL OF REPRODUCTION AND FERTILITY Volume: 93 Issue: 2 Pages: 569-576 Published: NOV 1991

Times Cited: 61

7/Abbreviations

LH	Luteinizing hormone
FSH	Follicle stimulating hormone
ECM	Extracellular matrix
HA	Hyaluronan /Hyaluronic acid
cAMP	cyclic adenosine monophosphate
dbcAMP	dibutyryl cAMP
8-Br-cAMP	8-bromo cAMP
PMG	Porcine membrane granulosa
CEEF	Cumulus -expansion enabling factor
OCC	Oocyte- cumulus complexes
OOX	Oocytectomized complexes / removed oocytes from complexes/
PVP	Polyvinylpyrrolidone
IGF-1	Insulin-like growth factor 1
PKA	Protein kinase A
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
LD	Late diakinesis
MI	Metaphase I
MII	Metaphase II
TGFβ	Transforming growth factor beta
GDF9	Growth differentiation factor 9
EGF	Epidermal growth factor
HER2	Human EGF receptor type 2
EGFR	Epidermal growth factor receptor

AG1478	Inhibitor of the EGFR tyrosine kinase activity
SB431542	An inhibitor of ALK 4/5/7 –dependent SMAD2 and SMAD3 phosphorylation
TNFAIP6	Tumor necrosis factor alpha-induced protein 6
TSG6	Tumor necrosis factor stimulated gene 6
IαI	Inter-alpha-trypsin inhibitor
HCs	Heavy chains (of IαI)
RA	Relative abundance
MGC	Mural granulosa cells
PTGS2	Prostaglandin-endoperoxide synthase 2
COX2	Cyclooxygenase 2
HAS2	Hyaluronan synthase type 2
MG132	Proteasomal inhibitor
HABP	Hyaluronan binding proteins
PR	Progesterone receptor
RU486	PR antagonist
PTX3	Pentraxin 3
GC	Granulosa cells

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9/References

Suh CS, Sonntag B, Erickson GF 2002 The ovarian Life cycle: A contemporary View. Review in Endocrine &Metabolic disorders; 3: 5-12.

Eppig JJ 2001 Oocyte control of ovarian follicular development and function in mammals. Reproduction 122:829-838.

Eppig JJ, Wigglesworth K, Pendola F, Hirao Y.1997 Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biol Reprod 56(4):976-84.

Lawrence TS, Dekel N, Beers WH.1980 Binding of human chorion gonadotropin by rat cumuli oophori and granulosa cells: a comparative study. Endocrinology 106(4):1114-8.

Peng XR, Hsueh AJ, LaPolt PS, Bjersing L, Ny T 1991 Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. Endocrinology 129(6):3200-7.

Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M.2004 EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 30; 303(5658):682-4.

Conti M, Hsieh M, Park JY, Su YQ 2006 Role of the epidermal growth network in ovarian follicles. Mol Endocrinol. 20(4):715-23. Review

Russell DL, Salustri A.2006 Extracellular matrix of the cumulus –oocyte complex. Semin Reprod Med 24(4):217-27. Review

Eppig JJ.1980 Regulation of cumulus oophorus expansion by gonadotropins in vivo and in vitro. Biol Reprod 23(3):545-52.

Salustri A, Yanagishita M, Hascall VC.1989 Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone –induced mucification.J Biol Chem 15;264(23):13840-7.

Salustri A, Yanagishita M, Underhill CB, Laurent TC, Hascall VC1992 Localization and synthesis of hyaluronic acid in the cumulus cell and mural granulosa cells of the preovulatory follicle. Dev Biol 151(2):541-51.

Nagyova E; Prochazka R; Vanderhyden BC 1999 Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: Retention of hyaluronic acid after insulin-like growth factor-I treatment in serum-free medium. Biol Reprod 61 (3): 569-574.

Nagyova E 2015 Organization of the expanded cumulus-extracellular matrix in preovulatory follicles: a role for inter-alpha-trypsin inhibitor. Endocrine regulations 49 (1): 37-45. Review

Nagyova E, Prochazka R 2002 Insulin-like growth factor-I as intraovarian regulator of hyaluronic acid synthesis. .Conference: Annual Conference of the Society for Reproduction and Fertility Location: Leeds, UK Reproduction Abstract Series (Cambridge) Issue: 28 (31).

Nemcova L, Nagyova E, Petlach M et al.2007 Molecular mechanisms of insulin-like growth factor 1 promoted synthesis and retention of hyaluronic acid in porcine oocyte-cumulus complexes. Biol Reprod 76 (6): 1016-1024.

Vanderhyden BC; Macdonald EA, Nagyova E et al. 2003 Evaluation of members of the TGFbeta superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis. Reproduction 61: 55-70.

Prochazka, R; Nemcova, L; Nagyova, E; et al. 2004 Expression of growth differentiation factor 9 messenger RNA in porcine growing and preovulatory ovarian follicles. Biol Reprod 71 (4): 1290-1295.

Channing CP, **Hillensjo** T, Schaerf FW.1978 Hormonal control of oocyte meiosis, ovulation and luteinization. Clin Endocrinol Metab 7(3):601-24. Review

Kolena J, **Channing** CP. 1985 Stimulatory action of follicular fluid components on maturation of granulosa cells from small porcine follicles. Horm Res 21(3):185-98.