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Expression and cellular distribution of estrogen-related receptor beta gamma (ERR beta gamma), estrogen-related receptor gamma (ERR gamma) and progesterone receptor membrane component 1 (PGRMC1) in immature porcine oocytes: confocal microscopy approach[1)]

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Summary

Steroid hormones display a significant role during the regulation of mammalian reproduction. It has been clearly shown that these hormones regulate important stages of ovulation, embryo development and proper implantation during peri-implantation stages. Moreover, the function of steroids has also been shown as activators and/or inhibitors of oocyte maturation. However, the expression of steroid receptors as well as protein distribution within the cell is still poorly recognized in relation to estrogen-related receptors (ERRs) and the progesterone receptor-membrane component (PGRMC1). Therefore, the aim of this study was to analyze ERRβ/γ, ERRγ and PGRMC1 protein expression and localization in porcine immature oocytes using confocal microscopic observations.

The cumulus-oocyte complexes (COCs) were recovered from puberal gilts after ovario-hysterectomy, then morphologically evaluated and fixed using the standard immunofluorescence procedure. The oocytes were stained with primary antibodies against ERRβ/γ, ERRγ and PGRMC1 proteins.

In the results, all investigated proteins were found distributed in cytoplasm of immature porcine oocytes with a similar intensity of expression. We did not find zona pellucida and/or membrane localization of ERRβ/γ, ERRγ and PGRMC1 proteins. Moreover, in the case of ERRβ/γ, an increased expression of this protein was found in granulosa cells which surrounded the cumulus.

It has been shown that major steroid hormone receptors, ERRβ/γ, ERRγ and PGRMC1, were localized in porcine oocyte's cytoplasm, in the stage of a maturity-dependent manner. It is suggested that the maturation stage of porcine oocyte may be the main factor which determines distribution of steroid hormone receptors within the cell. It may display a significant function in sperm-egg interaction during fertilization, where steroid receptors on the oocyte membrane surface may act as sperm-ligand receptors.

Keywords: genes, protein distribution, pig oocyte

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Maturation ability of mammalian oocytes highly determines the chances for a successful monospermic fertilization. Although the *in vitro* cultivation (IVC) procedures, based on supplementation of several substances (proteins, hormones, growth factors), were formulated to produce the media dedicated to *in vitro* maturation (IVM), which significantly mimics the *in vivo* conditions, several differences continue to exist between oocyte maturation *in vivo* and *in vitro* (5, 23, 32). Indeed, in many species of mammals the number of oocytes which *in vitro* reach full competency (MII stage) is still unsatisfactory (4, 21). Therefore, in future investigations the *in vitro* cultivation conditions require modification. Apart from the differences between *in vivo* and *in vitro* conditions, there also exist several differences between mature and immature mammalian oocytes. It has been shown that expression profiling of various genes in immature and *in vitro* maturing oocytes provides information about molecular markers which characterize oocyte maturation potential (11, 13, 14). Moreover, as it was found in our previous studies, that expression and/or cellular distribution of various proteins display differences between immature and mature oocytes (12-14). There are several published data documenting gene expression profile related to proteins responsible for oocyte maturation, such as connexins, cyclin-dependent kinases and growth factors (e.g. transforming growth factor beta superfamily) (20, 26, 28). It is also well known that steroid hormones which control reproductive potential of females and males in parallel influence gametes ability to grow and develop (9, 17, 22). Nevertheless, the gene and/or protein expression profile and cellular distribution of steroid hormones receptors have not been fully recognized. It has been well documented that progesterone receptor (PR) and oestrogen receptor (ER) play a significant role during mammalian oocyte growth and development (8, 18, 24). However, there are only a few studies available related to the role played by progesterone receptor-membrane component (PGRMC1) and estrogen receptor-related protein (ERR) in mammalian oocytes maturation potential (2, 7, 15, 29).

The PGRMC1, also termed the membrane progesterone receptor (MPR), consists of 195 amino acids and is localized in both endoplasmic reticulum and microsomes. As the progesterone receptor, it mediates signals in the progesterone metabolic pathway and activity of this steroid in a variety of tissues, mainly including the female reproductive tract. PGRMC1 is also involved in regulation of embryonic development (7).

The ERR belongs to the subgroup of the steroid/thyroid/retinoid receptors which act as the estrogen receptor by binding similar DNA targets. However, after binding the hormone ERR display no estrogen-related activity of a transcription factor (27).

Although the role of steroid hormones during porcine oocyte maturation was well described, the expression and cellular distribution of PGRMC1 and ERRs in immature female gametes have not been determined yet. Therefore, the aim of this study is to compare the PGRMC1 and ERR protein expression and cellular localization in porcine oocytes immediately after their recovery from ovarian follicles.

Material and methods

Animals. A total of 28 pubertal crossbred Landrace gilts, with the median age of 130 days and median weight of 98 kg, were used in this study. The animals were bred under the same conditions. The experiments were approved by the local Ethical Committee, nr 32/2012.

Collection of porcine ovaries and cumulus-oocyte-complexes (COCs). The ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory within 10 min. at 38°C in 0.9% NaCl. In order to provide optimum conditions for subsequent oocyte maturation *in vitro*, the ovaries of every animal were placed in 5% fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) in PBS (1, 19). Thereafter, individual large follicles (> 5 mm) were opened by puncturing them, using a 5-ml syringe and 20-G needle in a sterile Petri dish, and COCs were recovered. The COCs were washed three times in a modified PBS, supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamycin, and 0.5 mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). COCs were selected under an inverted microscope (Zeiss, Axiovert 35, Lübeck, Germany), counted, and morphologically evaluated with special care, using the scale suggested by Jackowska et al. (10). Only COCs of grade I with a homogeneous ooplasm, uniform and compact cumulus cells were considered for use in the subsequent steps of experiment, resulting in the use of a total of not less than 250 grade I COCs.

Confocal microscope analysis of ERRβγ, ERRγ and PGRMC1 expression and distribution in porcine oocytes. The oocytes (n = 60) were incubated with bovine testicular hyaluronidase (Sigma-Aldrich Co., St. Louis, MO, USA), 300 μg/ml, for 2 min at 38°C to remove cumulus cells. Oocytes were fixed with aceton/methanol mixture at –20°C for 10 min and washed three times in PBS. Oocytes were incubated for 1 hour at room temperature (RT) with rabbit polyclonal ERRβγ-specific (sc-68878), rabbit polyclonal ERRγ-specific (sc-66883), or goat polyclonal PGRMC1-specific (sc-82694) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1 : 500 in PBS with 3% w/v BSA. After several washes with PBS, the samples were incubated for 1 hour at RT with fluorescein isothiocyanate (FITC)-conjugated anti-goat and anti-rabbit IgG, produced in rabbits, diluted to 1 : 500 in PBS. Following a wash in PBS, the oocytes were mounted on glass slides in an anti-fade drop and examined under LSN 510 Olympus microscope (Fluoview 10i) confocal system. FITC was excited at 488 nm by an argon laser, and the emissions

were imaged using a 505-530 nm filter. All confocal microscopic images were analyzed using Imaris 7.2 (BitPlane, Zurich, Switzerland) software.

Statistical analysis. GraphPad Prism version 4.0 (GraphPadSoftware, San Diego, CA) software was used for statistical calculations.

Results and discussion

Using confocal microscopic observations, the cytoplasmic and zona pellucida localization and cellular distribution of ERRβ/γ, ERRγ and PGRMC1 proteins, were analyzed. All investigated proteins were found to be localized rather in oocyte cytoplasm than in zona pellucida. However, significant differences in expression of these proteins were found between porcine immature oocytes. The ERRβ/γ protein was localized in oocyte cytoplasm (Figure 1A). An increased expression of this protein was detected also in cumulus-surrounding granulosa cells (CGCs). Moreover, expression of ERRβ/γ was significantly higher in CGCs as compared to oocyte cytoplasm (Fig. 1A, B, C, D). The ERRγ protein was only distributed in oocyte cytoplasm (Fig. 2A, B) and the intensity of the protein expression was comparable to expression intensity of ERRβ/γ in porcine immature oocytes. Similarly, the expression of PGRMC1 (Fig. 3A, C) was restricted to the cytoplasm of porcine oocytes and was comparable in intensity to expressions of ERRβ/γ and ERRγ. Additionally, in the case of PGRMC1 expression, a DAPI stained nucleus was also detected (3B). The DAPI staining DNA nucleus was also detected in the case of CGCs (Fig. 1).

The steroid hormones display a significant role during ovulation and in the regulation of the entire reproductive cycle in all mammalian species, especially in dogs, in which progesterone (P4) is a dominant hormone (25). Furthermore, the steroids are also involved in appropriate events of embryo development and successful implantation during the peri-implan-

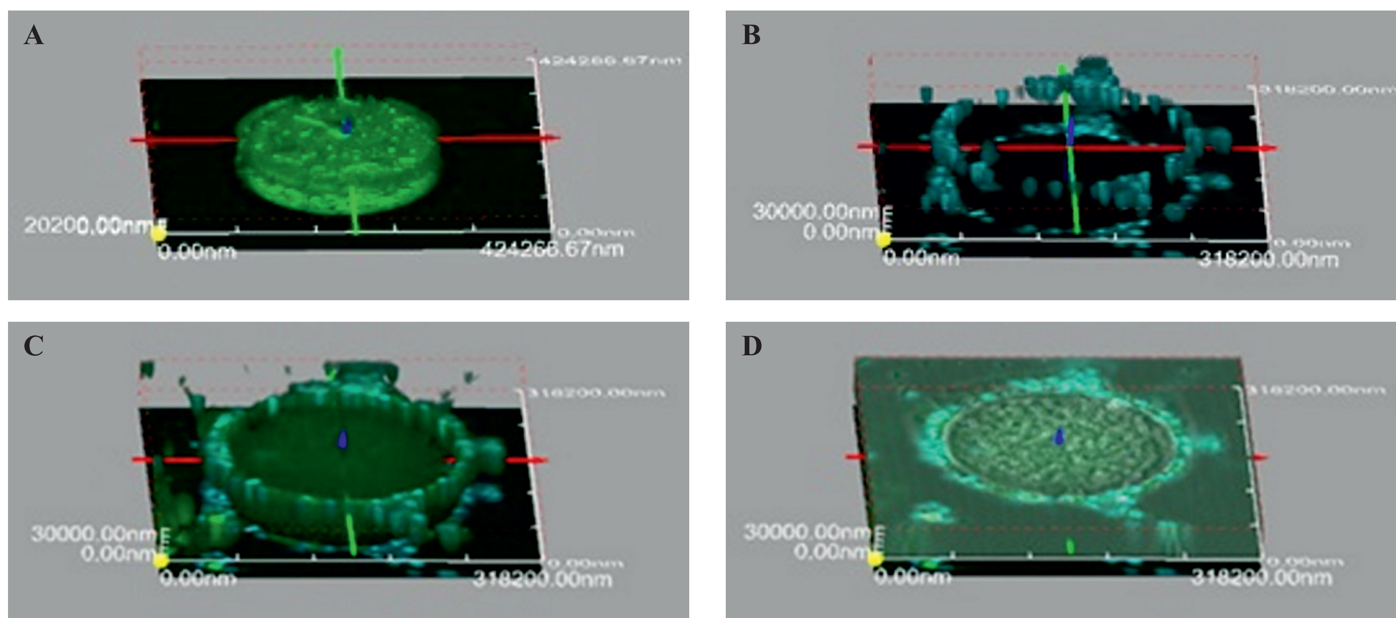

Fig. 1. Confocal microscopic observation of ERRβ/γ protein expression in porcine oocytes
Explanations: The porcine oocytes were stained with porcine rabbit polyclonal anti-ERRβγ (Fig. 1A, B, C, D). The treated oocytes were labeled with FITC-conjugated anti-rabbit IgG. The oocytes were also stained with DAPI for assessment of chromatin configuration of oocytes and nuclear staining of porcine surrounding granulosa cells (1B, C). A-antibody stained, B-DAPI, C, D-merge

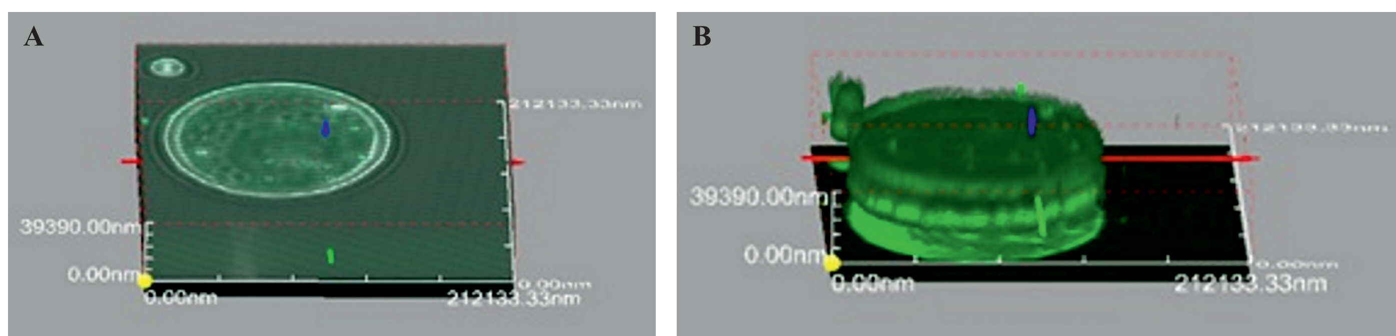

Fig. 2. Confocal microscopic observation of ERRγ protein expression in porcine oocytes
Explanations: The porcine oocytes were stained with porcine rabbit polyclonal anti-ERRγ, (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-23717), (Fig. 2A, B). The treated oocytes were labeled for 1 h with FITC-conjugated anti-rabbit IgG Ab. A-merge, B-antibody stained.

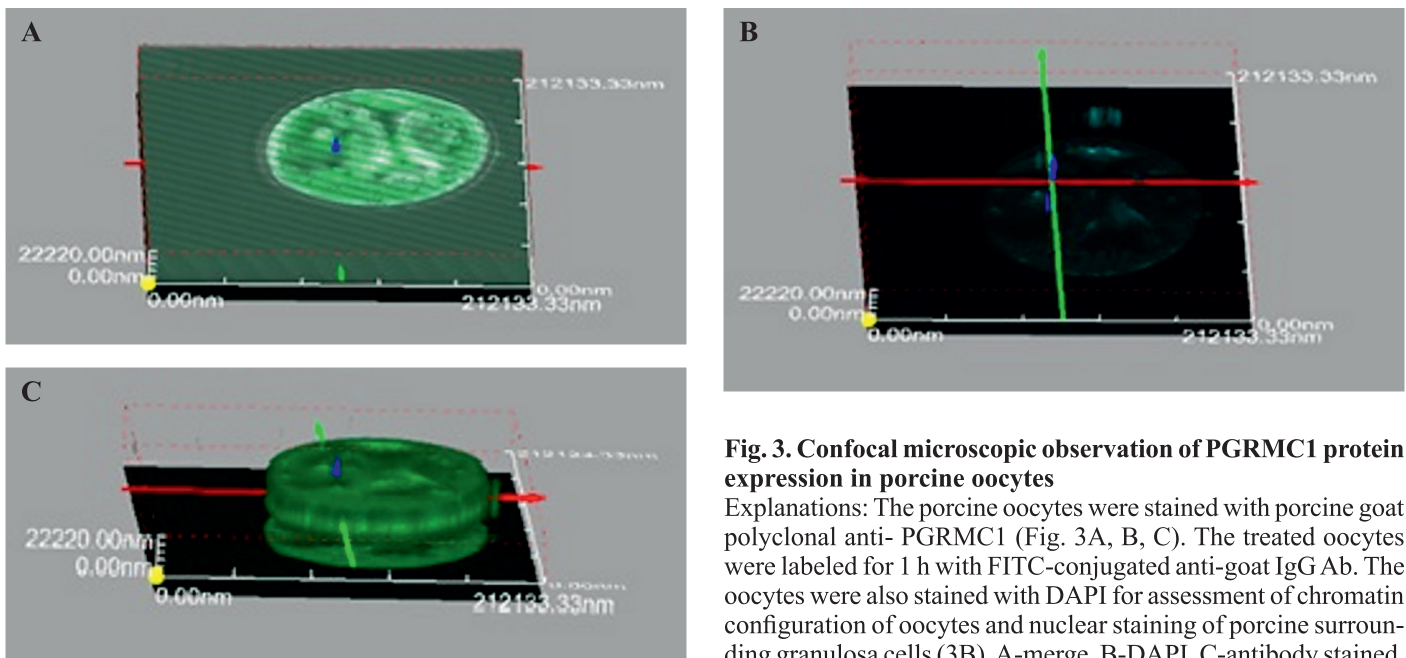

Fig. 3. Confocal microscopic observation of PGRMC1 protein expression in porcine oocytes
Explanations: The porcine oocytes were stained with porcine goat polyclonal anti- PGRMC1 (Fig. 3A, B, C). The treated oocytes were labeled for 1 h with FITC-conjugated anti-goat IgG Ab. The oocytes were also stained with DAPI for assessment of chromatin configuration of oocytes and nuclear staining of porcine surrounding granulosa cells (3B). A-merge, B-DAPI, C-antibody stained.

tation period. However, it has been also shown that the action of steroids and activity of their receptors regulate crucial stages of "oocyte's life" during maturation *in vivo* and/or *in vitro* and fertilization as well (3, 6). Although the role of steroid supplementation in culture media has been significantly explored during mammalian oocyte maturation *in vitro*, the important role of major steroid hormone receptors in immature porcine oocytes remains not fully known as of yet. Therefore, in this study, using confocal microscopic observations we have analysed the nonparametric expression intensity and cellular distribution of major steroid hormone receptors, such as ERRβ/γ, ERRγ and PGRMC1 in porcine oocytes before *in vitro* maturation.

Our recent studies have demonstrated distinct differences between mature and immature mammalian oocytes in the expression of proteins and their cellular distribution within oocyte cytoplasm, zona pellucida, and/or cell membrane. Moreover, it has been determined that the restricted localization of specific proteins, responsible, e.g. for maturation and/or fertilization, is significantly related to the function of the proteins during these processes. Our recent experiments using antibodies against Cx43 and Cdk4 have shown that during *in vitro* maturation of porcine oocytes both of these proteins replace their localization from a nuclear one before IVM to a cytoplasmic one after IVM (our unpublished data). Results presented in this study show distinct cytoplasmic distributions of ERRβ/γ, ERRγ and PGRMC1, which presumably reflect a stage of maturity of the investigated cells. As mentioned above, steroid hormones also regulate crucial steps of fertilization, and may be recognized as the sperm-egg interaction markers. Therefore, we may hypothesize that after IVM in fully mature oocytes all these three receptors become replaced to the zona pellucida, in which they will play a role of oocyte receptors for sperm fusion ligands. In a similar study, Thomas (30) analysed non-genomic rapid response of G protein-coupled receptor 30 (GPR30) and membrane progestin receptors (mPRs) on the surface of oocytes using fish models. He found that both of these receptors are responsible for proper oocyte maturation and development. Moreover, he observed that mPRs were employed in progestin induction of sperm hypermotility. However, he did not investigate cellular distribution of the analyzed steroid receptors. Similar results were obtained by Thomas et al. (31) who analyzed possible mechanisms of G-protein activation by steroids. They found that mPR alpha functions as G-coupled receptors (GPCRs) and plays a significant role during oocyte maturation as well as activation of sperm hyperactivity in fish. Moreover, the GPCRs activity was also shown in relation to the membrane androgen receptor (mAR). The recent study by Luconi et al. (16) also demonstrated that membrane steroid receptors (membrane progesterone receptor-mPR and membrane estrogen receptor-mER) function as stable proteins on the sperm membrane surface and are operative in the non-genomic rapid steroid effects on the sperm. In the study, based on human spermatozoa, two types of antibodies were used, including c262 and H222 specific for PR and ER. After western blot analysis, two isoforms of proteins were identified: 57 kDa for PR and of 29 kDa for ER, which presumably function as membrane components of genomic-classic steroid receptors.

In conclusion, the entire evidence strongly supports the hypothesis that steroid membrane components may be common for both oocytes and spermatozoa, where they function as inducers of capacitation, acrosome reaction and/or maturation and development stability, respectively. Our results add a new dimension to data on oocyte's maturation ability, providing proof for an oocyte's steroid-dependent non-genomic pathway.

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