Original Article

Characterization of Porcine Granulosa Cell Line AVG-16

(pig / cell line / AVG-16 / steroidogenesis)

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Abstract. Commercially available, but not yet characterized, the AVG-16 granulosa cell line was established from granulosa cells of medium porcine follicles. To examine the suitability of the AVG-16 cell line for studying the molecular mechanism of action of various environmental oestrogens, we investigated: 1/ cell morphology (by standard haematoxylin and eosin (HE) staining); 2/ basal and follicle-stimulating hormone (FSH) or luteinizing hormone (LH) -stimulated steroid hormone (progesterone; P_4 and 17β-oestradiol; E_2) secretion (by radioimmunoassay) and 3/ expression of receptors involved in the regulation of granulosa cell function: FSH receptor (FSHR), LH receptor (LHR), oestrogen receptor α (ERα), oestrogen receptor β (ERβ) and aryl hydrocarbon receptor (AhR). mRNA and protein expression was determined by RT-PCR and fluorescence immunocytochemistry, respectively. The secretion of P_4 and E_2 by AVG-16 cells was in the range of steroid hormone secretion by porcine cultured primary granulosa cells. Neither FSH (100 ng/ml) nor LH (100 ng/ml) affected P_4 and E_2 secretion by AVG-16 cells. The presence of FSHR and LHR at both mRNA and protein level was not demonstrated in the cells. However, AVG-16 cells were found to express mRNA and protein of ERα, ERβ and AhR. The results of our study showed that AVG-16 cells possess the capability of steroid hormone production, and both oestrogen receptors and AhR are present in these cells. Therefore, AVG-16 cells may serve as an unlimited source of homogenous porcine granulosa cells useful for studying the effects of environmental oestrogens on ovarian physiology.

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Abbreviations: AhR – aryl hydrocarbon receptor, bFGF – fibroblast growth factor-basic human, DMEM – Dulbecco's modified Eagle's medium, E_2 – 17β-oestradiol, ECACC – European Collection of Cell Cultures, ERα – oestrogen receptor α, ERβ – oestrogen receptor β, FBS – foetal bovine serum, FSHR – follicle-stimulating hormone receptor, HE – haematoxylin and eosin staining, LHR – luteinizing hormone receptor, NEAA – MEM non-essential amino acid solution, P_4 – progesterone, PBS – phosphate-buffered saline, PCR – polymerase chain reaction, TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Introduction

Granulosa cells are the most intensively studied cells in the female reproductive system due to their involvement in the regulation of follicular development, ovulation and subsequent corpus luteum function (Stokłosowa et al., 1982; Veldhuis et al., 1985; Skinner 2005; Ciereszko et al., 2007). The broad introduction of molecular methods into reproductive sciences further increased the researchers' interest in these cells. Numerous studies have been performed on primary cultures of isolated porcine or bovine granulosa cells obtained from a slaughterhouse (Nynca et al., 2013a, b; Castro et al., 2014; Piasecka-Srader et al., 2014). Primary granulosa cells cultured *in vitro,* however, have some limitations. First, researchers have to collect the ovaries and isolate the cells for each experiment. In addition, usually more than one cell type is obtained during the cell isolation (e.g., granulosa, theca, endothelial cells) (Stokłosowa, 2004; Freshney, 2005). Finally, the use of primary granulosa cells may lead to receiving highly variable results, e.g., of progesterone (P_4) secretion, due to intra-species differences and spontaneous luteinization of cultured cells (Channing and Seymour, 1970; Orly et al., 1980; Lowsky and Farookhi, 1989). Thus, granulosa cell lines have become an attractive alternative for *in vitro* model examination of the follicular function.

There are two main types of cell lines: primary cell lines (with a limited life-span) and continuous cell lines (Mather and Roberts, 1998; Wójtowicz, 2004). Primary

cell lines are established by continuous culturing of primary cells. They are usually conditionally immortalized and cells die in the absence of growth factors in the culture medium. Continuous cell lines are derived from tumour cells (e.g., HeLa, MCF-7, PC3, Saos-2 cells) or they are established by transformation. Transformation may occur spontaneously as a result of too high cell density in the culture (Shay et al., 1993) or may be induced by: 1/ a viral vector containing an oncogene (Zeleznik et al., 1979; Rosendahl et al., 2014), 2/ radiation (Terzaghi and Little, 1976) or 3/ chemicals (Kang and Shadduck, 1977; Chow et al., 2003; Tsujiuchi et al., 2005). The first granulosa cell line (CHO; Chinese Hamster Ovary) was developed in the 1960s from Chinese hamster ovaries (reviewed by Wójtowicz, 2004). Since then, granulosa cell lines have been derived from murine (Zeleznik et al., 1979; Briers et al., 1993; Yanagihara et al., 1995; Li et al., 1997), bovine (Bernath et al., 1990), porcine (Leighton et al., 1993; Kwan et al., 1996; Chedrese et al., 1998; Lin, 2005) and primate (Ishiwata et al., 1984; Zhang et al., 2000; Nishi et al., 2001; Husen et al., 2002) granulosa cells. The development of granulosa cell lines provides easily available and reproducible material for cytological, biochemical and molecular studies (Havelock et al., 2004). Granulosa cell lines create a suitable model for studying the molecular mechanisms of steroidogenesis as well as all aspects of signal transduction involved in the hormonal regulation of folliculogenesis (Stokłosowa, 2004).

Since the pig is a purported source of organs, tissues and cells in human therapy, it may provide an excellent animal model for studying the effects of various endocrine disruptors on human health. Furthermore, pork is commonly consumed by humans and toxic lipophilic substances (such as dioxins) accumulate in fat tissue, so that pork consumers are continuously exposed to the detrimental action of these compounds. Since it is known that dioxins (e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) influence porcine ovarian function, granulosa cell lines seem to be a good choice to study the mechanism of TCDD action and aryl hydrocarbon receptor (AhR) biology in the ovary of the pig. Presently, porcine granulosa cell line AVG-16 (Horisberger, 2006) is the only porcine granulosa cell line available commercially (European Collection of Cell Cultures; ECACC; UK), and the literature concerning the subject is limited to only one reference (Horisberger, 2006). The author reported that AVG-16 cells produced measurable amounts of P_4 and displayed normal karyotypes (2n = 38) after numerous passages. In the present study, we examined the suitability of AVG-16 cell line for studying the effects of TCDD and possibly other environmental oestrogens on ovarian function in pigs. For that purpose we investigated: 1/ cell morphology, 2/ basal and FSH- or LH-stimulated steroid hormone (P_4 and 17β-oestradiol; E_2) secretion and 3/ expression of receptors involved in the regulation of granulosa cell function: follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), oestrogen receptor α (ERα), oestrogen receptor β (ERβ) and AhR.

Material and Methods

Materials

In the current study, the following reagents were used for: 1/ cell culture: trypan blue dye (Chemapol, Prague, Czech Republic); gentamycin (KRKA, Novo Mesto, Slovenia); AVG-16 cell line, Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), MEM non-essential amino acid solution (NEAA), L-glutamine, fibroblast growth factor-basic human (bFGF), trypsin (Sigma, St. Louis, MO); 2/ histological staining: paraformaldehyde (ABChem, Olsztyn, Poland), haematoxylin, eosin (Sigma); 3/ radioimmunoassay: [2,4,6,7-^{3}H]-E_2 and [1,2,6,7-^{3}H]-P_4 (Amersham, Little Chalfont, UK); anti-P_4 and anti-E_2 antibodies (Department of Animal Physiology, University of Warmia and Mazury in Olsztyn, Poland); 4/ RT-PCR: RNase OUT™ recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA); Omniscript RT Kit, *Taq* PCR Master Mix Kit (Qiagen, Hilden, Germany) peqGold TriFast (*Peqlab* Biotechnologie GmbH, Erlangen, Germany); oligo$(dT)_{15}$ primers (Roche, Basel, Switzerland); hexanucleotide primers (Sigma) and 5/ immunocytochemistry: polyclonal rabbit anti-AhR antibodies (EnzoScientific, Farmingdale, NY); polyclonal rabbit anti-ERα antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal mouse anti-ERβ antibodies (Serotec, Oxford, United Kingdom); polyclonal goat anti-FSHR antibodies (a gift from prof. Maria Słomczyńska, Jagiellonian University, Krakow, Poland); polyclonal rabbit anti-LHR antibodies (a gift from prof. Nafis Rahman, University of Turku, Finland); fluoromount aqueous mounting medium, propidium iodide (Sigma), goat anti-rabbit biotinylated secondary antibodies, horse anti-mouse biotinylated secondary antibodies, rabbit anti-goat biotinylated secondary antibodies and FITC-conjugated streptavidin (Vector Laboratories, Burlingame, CA).

Culture of porcine granulosa cells

AVG-16 cells after 4th passage were obtained from the ECACC bank. To thaw the AVG-16 cells, the cryotube with the cells was placed in a beaker with warm water (37 °C). The thawed cells were transferred to a centrifuging tube containing cold culture medium: DMEM with 2 mM L-glutamine, 10% FBS, 0.1 mM NEAA, 2.5 ng/ml bFGF, 0.05 mg/ml gentamycin, and centrifuged (180 × *g*, 10 min, 4 °C). Next, the supernatant was removed and the cells were resuspended in warm culture medium (DMEM with 2 mM L-glutamine, 10% FBS, 0.1 mM NEAA, 2.5 ng/ml bFGF, 0.05 mg/ml gentamycin). Cells were counted and cell viability (≥ 97 %) was determined by 0.4% trypan blue dye exclusion. The AVG-16 cells were seeded in 25 cm^2 flasks with 12.5 ml medium (DMEM with 2 mM L-glutamine, 10% FBS, 0.1 mM NEAA, 2.5 ng/ml growth factor (bFGF),

0.05 mg/ml gentamycin) at 37 °C with a 5% CO_2 atmosphere. After reaching 90 to 95 % confluency, the cells were trypsinized. The granulosa cells were washed twice with sterile phosphate-buffered saline (PBS) and then 0.25% trypsin solution was added for 5 min at 37 °C. To inhibit the trypsin action, DMEM supplemented with 10% FBS was added. The cells were washed by centrifugation (180 × *g*, 10 min at RT). To perform target experiments, after 7th passage, granulosa cells were cultured in six-well plates (seeding density: 1×10^6 cells/3 ml medium) to measure steroid secretion and demonstrate FSHR, LHR, ERα, ERβ and AhR mRNA expression, and in 8-well plates (seeding density: 1.5×10^5 cells/ 0.3 ml medium) to analyse cell morphology and to detect FSHR, LHR, ERα, ERβ and AhR protein expression.

In addition to performing AVG-16 cell cultures, cultures of porcine primary granulosa cells were also carried out, according to our previously reported procedures (Jablonska et al., 2011a, b, 2014; Nynca et al., 2009, 2013a, b; Piasecka-Srader et al., 2014). Since the examined parameters were extensively studied, the results of these studies are available in numerous publications (Table 1). The porcine primary granulosa cells were cultured only to demonstrate the presence of LHR and FSHR mRNA and LHR protein in these cells. Such approach enabled us to compare the AVG-16 cell line characteristics with those of porcine primary cell cultures without performing re-trials.

Histological staining of the cells

Cells were allowed to attach, and after reaching 90-95 % confluency (approx. 72 h), the medium was removed and the cells were fixed in 4% formaldehyde for 25 min at 4 °C. Morphological analysis was performed by standard haematoxylin and eosin (HE) staining. The morphology of stained AVG-16 cells was analysed under a light microscope (Nikon Eclipse TS100; Nikon, Tokyo, Japan) and images were archived by a digital camera (ELWD 0.3 T1-SNCP; Nikon).

The effects of gonadotropins on steroidogenesis

To estimate P_4 and E_2 secretion, after reaching 60-65 % confluency (approx. 48 h), granulosa cells were cultured for 48 h with or without (as a control) FSH (100 ng/ml medium; N = 4) or LH (100 ng/ml medium; N = 3). The concentration of steroid hormones was measured by a radioimmunoassay as validated and described previously (Ciereszko et al., 1998). The antibody characteristics were reported previously (Ciereszko et al., 2001; Szafranska et al., 2002). Intra- and inter-assay coefficients of variation for P_4 were 3.64 % and 3.63 %, respectively. The sensitivity of the assay was 5 pg/ml. Intra- and inter-assay coefficients of variation for E_2 were 5.74 % and 3.74 %, respectively, and the sensitivity of the assay was 1 pg/ml. Serial dilutions of medium samples showed parallelism with the standard curves of the examined steroids. All analyses were performed in triplicate.

Statistical analysis was performed using Student's *t*-test (Statistica program; StatSoft Inc., Tulsa, OH). Data were expressed as mean ± SEM. A P value of < 0.05 was considered significant.

Receptor mRNA expression in the cells

FSHR, LHR, ERα, ERβ and AhR mRNA expression levels were analysed in AVG-16 cells after reaching 90 to 95 % confluency (approx. 72 h). Total RNA was extracted from the granulosa cells using TriFast following the manufacturer's recommendations. For cDNA syn-

Table 1. The selected parameters of the AVG-16 and porcine primary granulosa cell cultures examined in the current study or in previous studies.

PARAMETERS		PORCINE AVG-16 CELL LINE	PRIMARY PORCINE GRANULOSA CELLS
CELL MORPHOLOGY		+	Jablonska et al., 2014*
HORMONE SECRETION	P_4	+	Nynca et al., 2013b*; Jablonska et al., 2014*; Piasecka-Srader et al., 2014*
	E_2	+	Nynca et al., 2013b*; Jablonska et al., 2014*; Piasecka-Srader et al., 2014*
mRNA EXPRESSION	FSHR	+	+
	LHR	+	+
	ERα	+	Nynca et al., 2013a*
	ERβ	+	Nynca et al., 2013a*
	AhR	+	Jablonska et al., 2013*
PROTEIN	FSHR	+	Knapczyk-Stwora et al., 2013
	LHR	+	+
	ERα	+	Nynca et al., 2013a*
	ERβ	+	Nynca et al., 2013a*
	AhR	+	Jablonska et al., 2013*

+ – the parameters examined in the current study
* – the parameters examined in the previous studies of the authors of the current study

Table 2. Primer characteristics and PCR conditions used in the current study.

GENE	PRIMER SEQUENCES (SENSE, ANTISENSE)	PRODUCT LENGHT	GENEBANK ACC NUMBER	ANNEALING TEMPERATURE	NUMBER OF CYCLES
FSHR	5' AGCCTCTGGACCAGTCATTC 3' 5' CACCATCTTCTGCCAGAGAC 3'	308 bp	NM_214386	55 °C	36
LHR	5' GCCTCAGCCGACTATCACTC 3' 5' GGAAGGCGTCATTGTGCATC 3'	507 bp	NM_214449	56 °C	36
ERα	5' AGGGAGAGGAGTTTGTGTG 3' 5' TCTCCAGCAGCAGGTCATAG 3'	305 bp	AF035775	57 °C	40
ERβ	5' GCTTCGTGGAGCTCAGCCTG 3' 5' AGGATCATGGCCTTGACACAGA 3'	262 bp	AF164957	58 °C	38
AhR	5' AGAGAGTGGCATGATAGTGTTC 3' 5' GCCTAGGTGTTTCATAATGTTG 3'	603 bp	HM488957	54 °C	36
GAPDH	5' ATGGTGAAGGTCGGAGTGAA 3' 5' CTTGGCAGCGCCGGTAGAAGC 3'	681 bp	AF017079	55 °C (coamplification with FSHR) 57 °C (coamplification with ERα) 58 °C (coamplification with ERβ)	25 (coamplification with FSHR) 30 (coamplification with ERα) 26 (coamplification with ERβ)
L19	5' AGCCTGTGACTGTCCATTC 3' 5' TCAGCTTGTGGATGTGCTC 3'	279 bp	XM_003131509	54 °C (coamplification with AhR) 56 °C (coamplification with LHR)	27 (coamplification with AhR) 25 (coamplification with LHR)

thesis, 1 µg of RNA was reverse transcribed in a 20 µl reaction volume with 0.5 µM oligo(dT)$_{15}$ primer, 1 µM hexamers, 10U RNAse Out, using the Omniscript RT Kit. Complementary DNA was amplified by polymerase chain reaction (PCR) using 40 pmol of porcine FSHR, LHR, ERα, ERβ and AhR sense and antisense primer pairs (Table 2). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; for FSHR and ER mRNA expression) and ribosomal protein L19 (for LHR and AhR mRNA expression) were used as reference genes. PCR was performed in one tube for the examined gene and the reference gene. Primers for housekeeping genes were added during the reaction at appropriate cycle number, specific for the gene. Amplification conditions for each gene were optimized based on the primer-dropping method (Wong et al., 1994). The optimal number of cycles and cycling conditions are demonstrated in Table 2. Aliquots of PCR reaction products were separated by electrophoresis in 1.5% agarose gel and visualized under ultraviolet illumination.

Receptor protein expression in the cells

FSHR, LHR, ERα, ERβ and AhR protein expression was detected in AVG-16 cells plated in 8-well LabTek Chamber Slide Systems (Nunc, Denmark) after reaching 90 to 95 % confluency (approx. 72 h). Granulosa cells were fixed in 4% paraformaldehyde and incubated with appropriate antibodies (12 h, Table 3). Primary antibody was omitted in a negative control. Next, the cells were incubated with secondary antibodies (Table 3) for one hour and treated with FITC-conjugated streptavidin (1 : 50). The images of cell preparations were captured with a Nikon Eclipse 80i fluorescence microscope and a Nikon Digital Sight DS-SMC camera (Nikon). Fluorescence intensity of stained cells was determined using the NIS-Elements 3.0 Imaging System software (Nikon).

Table 3. The characteristic of primary and secondary antibodies used for immunocytochemistry.

PROTEIN	PRIMARY ANTIBODIES	SECONDARY ANTIBODIES
FSHR	polyclonal goat anti- FSHR antibodies (1 : 100; a gift from prof. Maria Słomczyńska (Department of Endocrinology, Jagiellonian University)	rabbit anti-goat biotinylated secondary antibodies (1 : 100; Vector Laboratories, Burlingame, CA)
LHR	polyclonal rabbit anti-LHR antibodies (1 : 300; a gift from prof. Nafis Rahman (Department of Physiology, University of Turku)	goat anti-rabbit biotinylated secondary antibodies (1 : 100; Vector Laboratories)
ERα	polyclonal rabbit anti- ERα antibodies (1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA)	goat anti-rabbit biotinylated secondary antibodies (1 : 100; Vector Laboratories)
ERβ	monoclonal mouse anti-ERβ antibodies (1 : 50; Serotec, Oxford, United Kingdom)	horse anti-mouse biotinylated secondary antibodies (1 : 100; Vector Laboratories)
AhR	polyclonal rabbit anti-AhR antibodies (1 : 100; EnzoScientific, Farmingdale, NY)	goat anti-rabbit biotinylated secondary antibodies (1 : 100; Vector Laboratories)

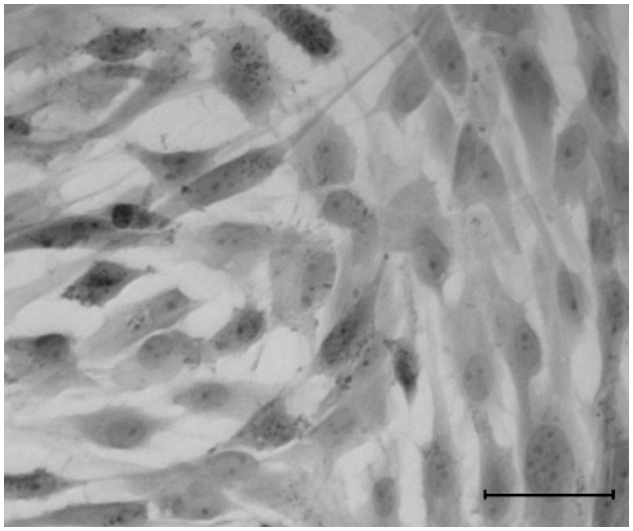

Fig. 1. Representative image of AVG-16 granulosa cells in culture. Cells were cultured in DMEM with 2 mM L-glutamine, 10% foetal bovine serum (FBS), 0.1 mM NEAA, 2.5 ng/ml growth factor (bFGF), 0.05 mg/ml gentamycin, until reaching 90–95 % confluency. Morphological analysis was performed by standard haematoxylin and eosin (HE) staining.
Scale bar = 20 μm.

Results

The morphology of cultured AVG-16 cells (confluency 80–90 %) was very similar to that of primary granulosa cells isolated from medium porcine follicles and cultured for 48 h (confluency 80–90 %). AVG-16 cells grew as an adherent monolayer and covered the entire surface of the culture plate. Cells latched on to the culture dish by flattened insets similar to those observed in fibroblast cells. After reaching 80–90 % confluency, the cells had a flattened elongated shape, typical of epithelial cells (Fig. 1).

The AVG-16 cell line produced 175.11 ± 18.86 ng/ml of P_4 and 112.70 ± 9.63 pg/ml of E_2 after 48 h of culture (seeding density: 3.3×10^5 cells/ml medium). FSH (100 ng/ml) did not affect the secretion of P_4 (Fig. 2A) and E_2 (Fig. 2B) by AVG-16 cells. Moreover, there were no significant differences in P_4 (Fig. 3A) and E_2 (Fig. 3B) secretion between control cells and cells treated with 100 ng/ml of LH.

No expression of FSHR (Fig. 4) and LHR (Fig. 5) either at the mRNA or protein level was demonstrated in the AVG-16 cell line. Transcripts for both gonadotropin receptors, however, were found in primary granulosa cells (Figs. 4 and 5). Similarly, the presence of FSHR

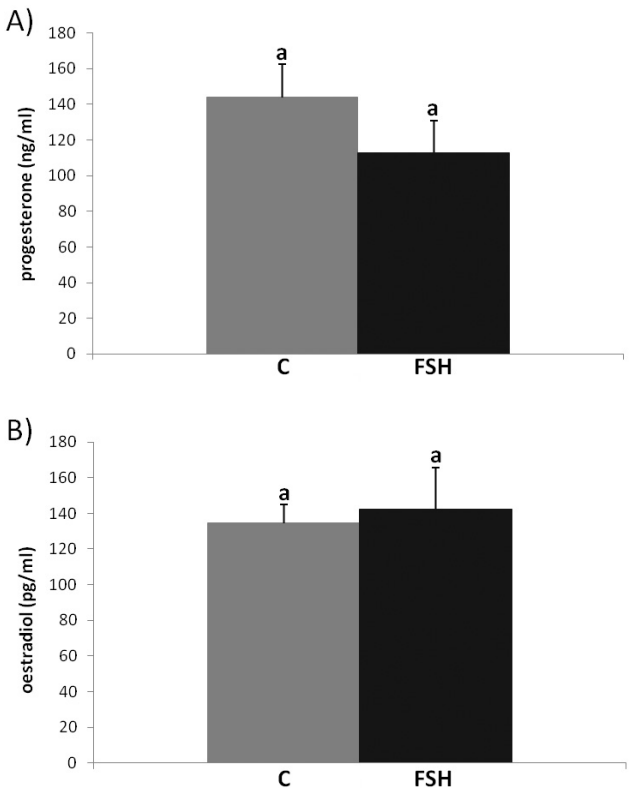

Fig. 2. The effects of follicle-stimulating hormone (FSH) on **A**) progesterone (P_4) and **B**) oestradiol (E_2) secretion (mean ± SEM) by AVG-16 granulosa cell line. After reaching 60 to 65 % confluency, cells were cultured for 48 h with 0 ng/ml (control, C) of FSH or 100 ng/ml of FSH. Statistical analysis was performed using Student's *t*-test. Different superscripts designate statistical differences ($P < 0.05$).

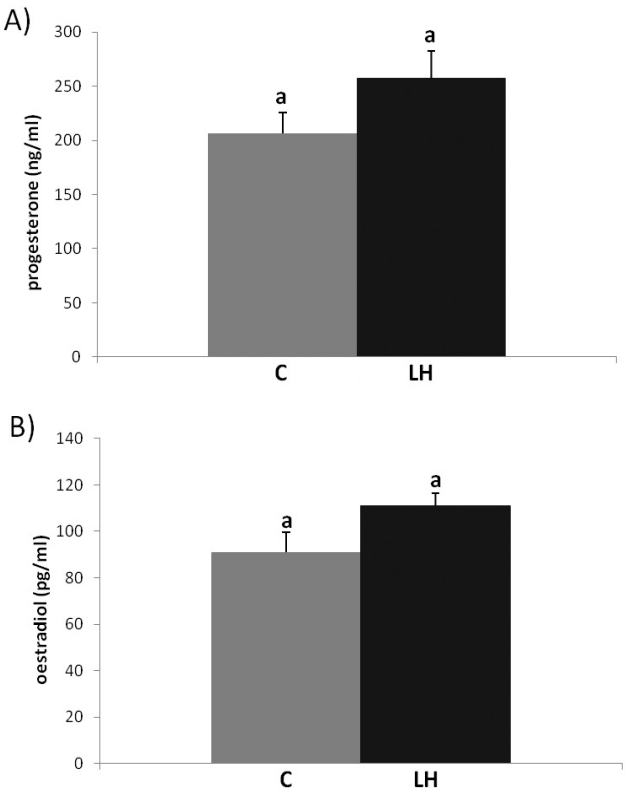

Fig. 3. The effects of luteinizing hormone (LH) on **A**) progesterone (P_4) and **B**) oestradiol (E_2) secretion (mean ± SEM) by AVG-16 granulosa cell line. After reaching 60 to 65 % confluency, cells were cultured for 48 h with 0 ng/ml (control, C) of LH or 100 ng/ml of LH. Statistical analysis was performed using Student's *t*-test. Different superscripts designate statistical differences ($P < 0.05$).

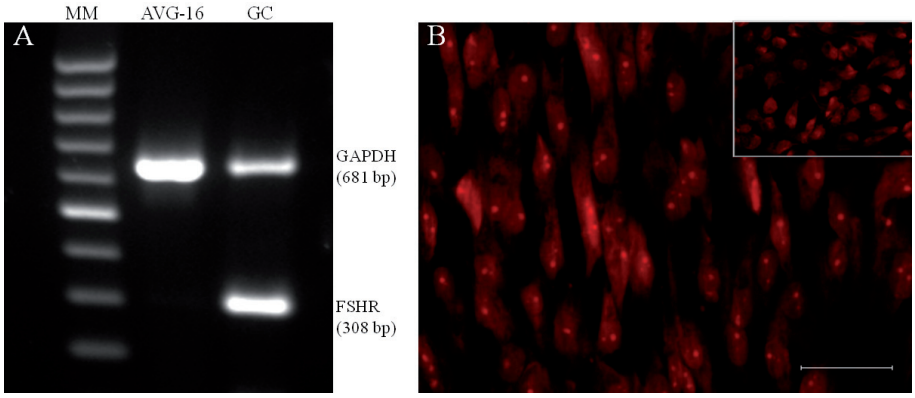

Fig. 4. Representative images of follicle-stimulating hormone receptor (FSHR) (**A**) mRNA expression determined by RT-PCR and (**B**) protein immunostaining determined by immunocytofluorescence in AVG-16 granulosa cell line. Red colour – granulosa cells stained with propidium iodide. The inset presents a negative control.
MM – molecular marker, GC – primary granulosa cells (a positive control), GAPDH – glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), bp – base pair. Scale bar = 20 μm.

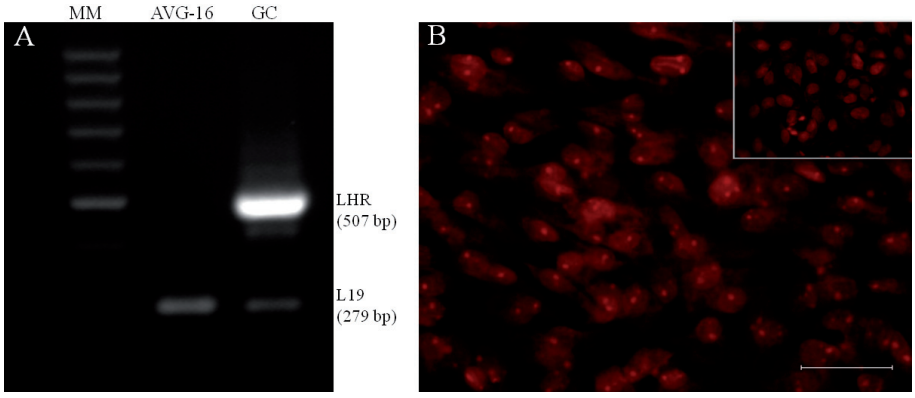

Fig. 5. Representative images of luteinizing hormone receptor (LHR) (**A**) mRNA expression determined by RT-PCR and (**B**) protein immunostaining determined by immunocytofluorescence in AVG-16 granulosa cell line. Red colour – granulosa cells stained with propidium iodide. The inset presents a negative control.
MM – molecular marker, GC – primary granulosa cells (a positive control), L19 – ribosomal protein L19, bp – base pair. Scale bar = 20 μm.

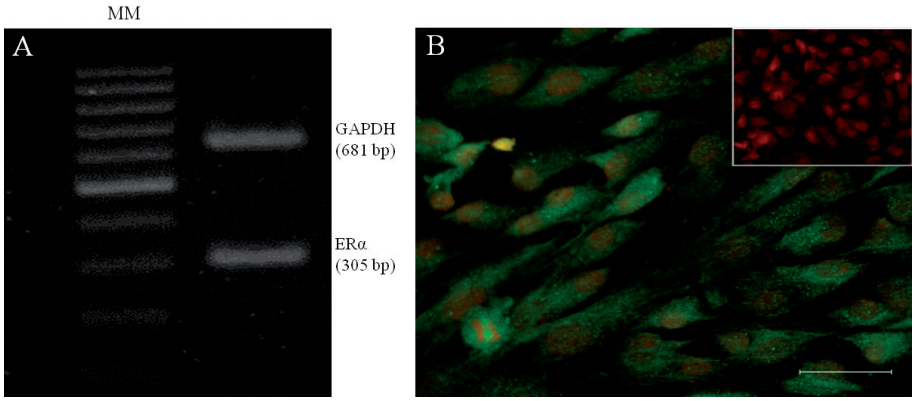

Fig. 6. Representative images of oestrogen receptor α (ERα) (**A**) mRNA expression determined by RT-PCR and (**B**) ERα protein immunostaining determined by immunocytofluorescence in AVG-16 granulosa cell line. Green colour – ERα immunopositive cells, red colour – granulosa cells stained with propidium iodide. The inset presents a negative control.
MM – molecular marker, GAPDH - glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), bp – base pair. Scale bar = 20 μm.

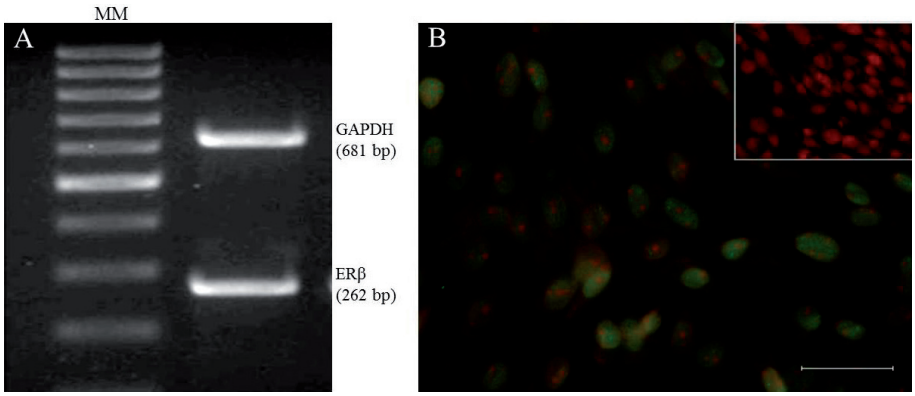

Fig. 7. Representative images of oestrogen receptor β (ERβ) (**A**) mRNA expression determined by RT-PCR and (**B**) ERβ protein immunostaining determined by immunocytofluorescence in AVG-16 granulosa cell line. Green colour – ERβ immunopositive cells, red colour – granulosa cells stained with propidium iodide. The inset presents a negative control.
MM – molecular marker, GAPDH – glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), bp – base pair. Scale bar = 20 μm.

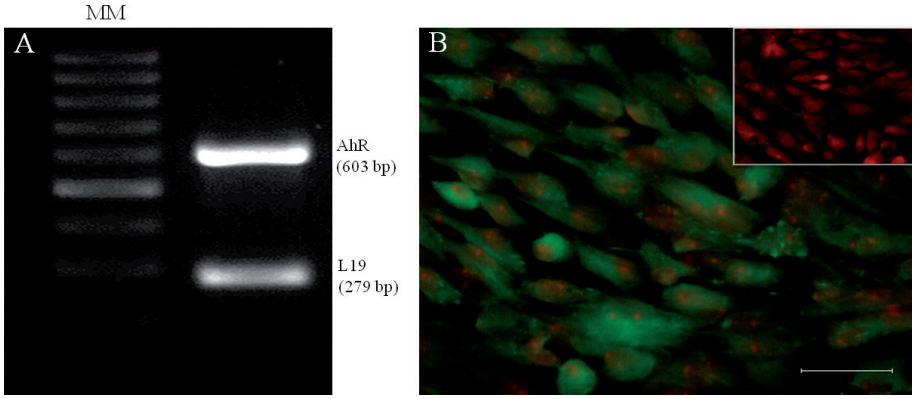

Fig. 8. Representative images of aryl hydrocarbon receptor (AhR) (**A**) mRNA expression determined by RT-PCR and (**B**) AhR protein immunostaining determined by immunocytofluorescence in AVG-16 granulosa cell line. Green colour – AhR immunopositive cells, red colour – granulosa cells stained with propidium iodide. The inset presents a negative control.
MM – molecular marker, L19 – ribosomal protein L19, bp – base pair. Scale bar = 20 μm.

and LHR proteins was demonstrated in porcine follicular sections and in primary granulosa cells, respectively (data not shown). However, AVG-16 cells were found to express mRNA and protein of ERα (Fig. 6), ERβ (Fig. 7) and AhR (Fig. 8). The ERβ protein was localized mainly in the nuclei, whereas AhR and ERα were detected in both the nuclei and the cytoplasm.

Discussion

In view of the apparent lack of information on the AVG-16 cell line (Horisberger, 2006), a comparison between cultured AVG-16 cells and primary cultures of porcine granulosa cells or other granulosa cell lines is needed for gaining an appropriate *in vitro* model to examine AhR biology and its involvement in the regulation of ovarian function. To characterize the AVG-16 cell line for its suitability to study the molecular mechanism of TCDD (and other AhR ligands) action, we investigated the cell morphology, steroid hormone (P_4 and E_2) secretion, and expression of receptors involved in the regulation of granulosa cell function: FSHR, LHR, ERα, ERβ and AhR.

In the current study, AVG-16 cells grew as a monolayer and covered the surface of the culture plate, reaching 95 % confluency after approximately 72 h of culture. At this stage, the cells demonstrated a phenotype typical of epithelial cells. A similar shape was assumed by primary porcine granulosa cells cultured for 4–6 days (Stokłosowa et al., 1982; Picton et al., 1999). Cells from miscellaneous granulosa cell lines behaved similarly to AVG-16 cells during culture. Cultured PGV and PGC-2 (porcine granulosa cell lines; Kwan et al., 1996; Lin, 2005) as well as BGC-1 cells (bovine granulosa cell line; Bernath et al., 1990) formed a monolayer consisting of morphologically homogenous cells characterized by an elongated and fibroblastic shape.

AVG-16 cells after 48 h of culture produced approximately 175 ng/ml of P_4. In cultures of primary porcine granulosa cells obtained from medium and large folli-

cles (Tiemann et al., 2007; Nynca et al., 2013b; Jablonska et al., 2014; Piasecka-Srader et al., 2014) P_4 secretion ranged from 0.25 ng/ml to 140 ng/ml. It is known that in pigs, granulosal P_4 secretion is highly variable and individual-dependent (Channing and Seymour, 1970). The AVG-16 cell line demonstrated higher P_4 secretion than the other porcine granulosa cell lines (jc-410 cell line: 0.372 ± 0.35 ng/10^5 cells after 24 h of culture; PGC-2 cell line: 5.6 ng/3 × 10^5 cells/48 h; Kwan et al., 1996; Chedrese et al., 1998). Interestingly, porcine (PGV) and bovine (BGC-1) granulosa cell lines did not produce P_4 at all (Lerner et al., 1995; Lin 2005).

AVG-16 cells after 48 h of culture produced approximately 112 pg/ml of E_2. In primary cultures of granulosa cells isolated from medium porcine follicles the amount of E_2 in the medium after 48 h of culture ranged from 18 to 30 pg/ml (Nynca et al., 2013b; Jablonska et al., 2014; Piasecka-Srader et al., 2014).The same type of cells, but cultured with androstenedione and seeded at a higher density (5 × 10^5/ml), produced more E_2 (100 pg/ml; Tiemann et al., 2007). Basal E_2 secretion by porcine granulosa cells derived from large follicles ranged from 25 to 45 pg/ml (Nynca et al., 2013b; Jablonska et al., 2014; Piasecka-Srader et al., 2014) and in the presence of androstenedione the production of E_2 was much higher (3.7 ± 0.6 ng/ml; Basini et al., 2010). The ability of AVG-16 cells to secrete E_2 was higher than that of cultured primary granulosa cells derived from both medium and large follicles. In contrast to AVG-16, oestradiol secretion by jc-410 and PGC-2 porcine cell lines was detected only in the presence of androstenedione in the culture medium (Kwan et al., 1996; Chedrese et al., 1998). Due to the ability to secrete P_4 and E_2, the AVG-16 cell line may provide a good model to study the regulation of steroidogenesis.

In the current study, FSH did not alter P_4 and E_2 production by AVG-16 cells. In contrast, in primary cell cultures, FSH stimulated P_4 production by granulosa cells isolated from small (May and Schomberg, 1981; Veldhuis et al., 1982b), medium (Thanki and Channing 1978; Nynca et al., 2013b) and large follicles (Thanki and Channing, 1978; Stokłosowa et al., 1982; Nynca and Ciereszko, 2006) and increased E_2 secretion by cells from large porcine follicles (Stokłosowa et al., 1982). E_2 secretion by the granulosa cells isolated from small (Picton et al., 1999) and medium (Nynca and Ciereszko, 2006) follicles was, in turn, not affected by FSH. Similarly to AVG-16, FSH did not alter P_4 and E_2 production by other porcine granulosa cell lines (jc-410 and PGC-2; Kwan et al., 1996; Chedrese et al., 1998). However, the addition of FSH to cultured BGC-1 cells caused a significant increase in E_2 secretion (Bernath et al., 1990).

In the current study, LH had no effect on either P_4 or E_2 secretion by the AVG-16 cell line. In primary cultures, however, LH stimulated P_4 and E_2 production by granulosa cells isolated from large follicles (Stoklosowa et al., 1982; Veldhuis et al., 1982a; Nynca et al., 2009). Similarly to the AVG-16 cell line, there was no effect of LH on P_4 production in the other porcine granulosa cell lines (jc-410 and PGC-2; Kwan et al., 1996; Chedrese et al., 1998). It seems that the lack of responsiveness to gonadotropins is a common characteristic of both porcine (the current study; Kwan et al., 1996; Chedrese et al., 1998) and rodent (reviewed by Amsterdam et al., 1993) granulosa cell lines.

Due to the fact that AVG-16 granulosa cells did not respond to FSH or LH stimulation, the gonadotropin receptor expression was also examined in the current study. Neither mRNA nor protein of FSH and LH receptors were found in AVG-16 cells. Similarly, gonadotropin receptors (mRNA and protein) were not demonstrated in other granulosa cell lines derived from humans (Zhang et al., 2000), rodents (Zilberstein et al., 1989; Vandersticheie et al., 1994) and pigs (Kwan et al., 1996). On the other hand, both mRNA and protein expression of FSHR and LHR was reported in cultured primary porcine granulosa cells (Kempisty et al., 2014). Amsterdam and Selvaraj (1977) suggested that the loss of FSHR may be one of the symptoms of granulosa cell differentiation during the establishment of the cell lines.

Since the effects of oestrogens in granulosa cells are mediated *via* ERα and ERβ, the presence of ERs in AVG-16 cells was studied next. The expression of both ERα and ERβ mRNA as well as protein was demonstrated in the AVG-16 cell line. The ERα protein was found in the nuclei and cytoplasm, while ERβ was found only in the nuclei. The presence of mRNA for ERα and ERβ was reported for primary granulosa cells of pigs (Chronowska and Kott, 2012; Nynca et al., 2013b). In contrast, only ERβ protein expression was found in these cells and it was mostly limited to the nuclei (Nynca et al., 2013b). On the other hand, Slomczynska et al. (2001) demonstrated ERα immunoexpression only in granulosa cells of preovulatory follicles and ERβ in medium and large follicles. The presence of both ERs in AVG-16 cells increases the potential of these cells to serve as a good model to study the mechanisms underlying the regulation of steroidogenesis, folliculogenesis as well as the involvement of environmental oestrogens in this regulation.

It is well known that environmental pollutants can affect granulosa cells not only by activation of oestrogen receptors, but also *via* activation of AhR (Abel and Haarmann-Stemmann, 2010). In the present study, we found that AhR mRNA and protein were expressed in AVG-16 cells. The presence of AhR was also reported both for primary cultured granulosa cells and cells directly isolated from medium and large porcine follicles, with the highest expression of the AhR protein in cells isolated from preovulatory follicles (Wójtowicz et al., 2005; Jablonska et al., 2011b; Jablonska and Ciereszko 2013).

In conclusion, characterization of the AVG-16 cell line showed that AVG-16 cells may be an excellent alternative for primary porcine granulosa cell cultures since they retained the capability of basal steroid hormone production. Moreover, this cell line may serve as an unlimited source of a homogenous porcine granulosa cell

population useful for studying the molecular mechanisms induced by environmental oestrogens in the ovary.

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