**EFFECTS OF SEX STEROID RECEPTOR AGONISTS
AND ANTAGONISTS ON THE EXPRESSION OF THE FOXL2
TRANSCRIPTION FACTOR AND ITS TARGET GENES *AMH*
AND *CYP19A1* IN THE NEONATAL PORCINE OVARY***

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Abstract

Recently, we have demonstrated that neonatal exposure to androgen and estrogen agonists or antagonists influenced the number of ovarian follicles in piglets. Since the FOXL2 transcription factor is required for proper ovarian follicle formation and activation, the objective of the study was to examine effects of exposure of the neonatal porcine ovary to testosterone propionate (TP; an androgen), flutamide (FLU; an antiandrogen), 4-*tert*-octylphenol (OP; compound with estrogenic activity), ICI 182,780 (ICI; an antiestrogen), and methoxychlor (MXC; compound with estrogenic, antiestrogenic and antiandrogenic properties) on FOXL2 expression and expression of its target genes, *AMH* and *CYP19A1*. Piglets were injected subcutaneously with TP, FLU, OP, ICI, MXC, or corn oil (control) between postnatal days 1 and 10 ($n = 4$ /each group). Ovaries were excised from the 11-day-old piglets and the expression of FOXL2, AMH and CYP19A1 was examined using immunohistochemistry and/or real-time PCR and Western blot. FOXL2 was localized in stroma cells surrounding egg nests and in granulosa cells. TP, OP and MXC increased both *FOXL2* and *AMH* mRNAs, while FLU and ICI decreased *CYP19A1* mRNA. The increased FOXL2 protein abundance was found in all examined groups. In addition, TP, OP, ICI and MXC increased AMH protein abundance, while TP, FLU and OP decreased CYP19A1 protein abundance. In conclusion, neonatal exposure to sex steroid receptor agonists and antagonists increased FOXL2 expression at mRNA and/or protein levels and affected FOXL2 target genes in the ovaries of 11-day-old piglets. Therefore, it seems that impaired ovarian folliculogenesis induced by altered steroid milieu during the neonatal development period in pigs may, at least in part, involve FOXL2.

Key words: pig, ovary, endocrine-active chemicals, FOXL2

Formation of primordial follicles and their activation to growth, which ultimately terminates in ovulation or follicular atresia, are complex and tightly regulated processes mediated by intra- and extra-follicular factors (Pepling, 2012). In mammals, the setting up of the pool of primordial follicles, known as ovarian reserve, occurs pre-birth or in the early postnatal period, and is clearly species specific. In pigs, the ovarian reserve formation begins during the prenatal period and most primordial follicles are generated until postnatal day 25 (Monniaux et al., 2014). In recent years, there has been a growing interest in the study of environmental chemicals – both natural and man-made, which may alter the endocrine system and affect ovarian development during the prenatal or neonatal period. These chemicals, known as endocrine-active chemicals (EACs), interfere with the synthesis, secretion, transport, metabolism, receptor binding or elimination of endogenous hormones (Lauretta et al., 2019). Recently, we have confirmed that the neonatal period is essential for ovarian folliculogenesis and female reproductive potency by demonstrating impacts of EACs displaying androgenic/antiandrogenic and/or estrogenic/antiestrogenic activities on these processes in a porcine animal model. Pigs serve as a relevant model organism for biomedical research, since they reflect human biology more closely than other animal models (Bendixen et al., 2010). We demonstrated that neonatal exposure to androgen and estrogen agonists or antagonists, such as testosterone propionate (TP; a synthetic androgen), flutamide (FLU; a nonsteroidal antiandrogen), 4-*tert*-octylphenol (OP; compound with estrogenic activity), ICI 182,780 (ICI; a pure antiestrogen), and methoxychlor (MXC; compound with estrogenic, antiestrogenic and antiandrogenic properties), influenced the number of primordial and primary follicles in piglets (Knapczyk-Stwora et al., 2018). Moreover, using a RNA sequencing approach, we showed that neonatal exposure of piglets to FLU and OP altered the expression of genes involved in ovarian cell proliferation, apoptosis, and steroidogenesis (Knapczyk-Stwora et al., 2019, 2020 a). Thus, both androgens and estrogens are important for the proper follicle assembly and activation in neonatal porcine ovaries.

One of the crucial molecules involved in ovarian development and function is the forkhead box protein L2 (FOXL2). FOXL2 is a transcription factor expressed in ovarian stromal cells and granulosa cells from the early stage of development, and it regulates the differentiation and maintenance of granulosa cells (Georges et al., 2014). Ovaries of FOXL2 knockout mice and humans with mutations in FOXL2 exhibit early depletion of ovarian follicles demonstrating that FOXL2 is critical for proper folliculogenesis (Crisponi et al., 2001; Uda et al., 2004). FOXL2 regulates the expression of genes involved in steroidogenesis, proliferation, apoptosis, differentiation, and the stress response. For example, it was shown to regulate the expression of *CYP19A1*, which encodes aromatase, the enzyme converting androgens to estrogens (Wang et al., 2007). Moreover, anti-Müllerian hormone (AMH) has been identified as a direct ovarian target gene regulated by FOXL2 (Park et al., 2014). It is well known that AMH regulates the recruitment rate of primordial follicles into the expanding follicle pool in order to prevent exhaustion of the follicular reserve in ovaries (Durlinger et al., 2002). The size of ovarian reserve and their premature exhaustion is known to have impacts on female fertility and reproductive longevity. Wang

et al. (2012) demonstrated upregulation of FOXL2 expression in fish after exposure to bisphenol A, a compound with estrogenic activity, which may lead to altered functioning of the gene encoding CYP19A and, subsequently, estrogen levels. Therefore, in the current study we aimed to examine effects of exposure of the neonatal porcine ovary to TP, FLU, OP, ICI and MXC on FOXL2 expression and expression of its target genes, *AMH* and *CYP19A1*. Towards this goal, immunohistochemical localization of FOXL2 was performed and *FOXL2*, *AMH* and *CYP19A1* expression was assessed for mRNAs by real-time quantitative PCR and for protein levels by Western blot analysis of untreated and EACs-treated ovarian tissues of porcine neonates.

Material and methods

Animals and tissue collection

All animal manipulations were carried out with the approval of the Ethics Committee of the Jagiellonian University in Kraków, Poland (permit number 150/2013, 122/2014, 187/2014 and 188/2014), and under the supervision of a veterinarian. Twenty-four female piglets (Large White × Polish Landrace) were included in the study. Neonatal piglets from different litters were randomly allocated into six groups ($n = 4$ /each group) and injected subcutaneously with: (1) testosterone propionate, TP (20 mg/kg body weight [bw]; Sigma-Aldrich, St. Louis, MO, USA), (2) flutamide, FLU (50 mg/kg bw; Sigma-Aldrich), (3) 4-*tert*-octylphenol, OP (100 mg/kg bw; Sigma-Aldrich), (4) ICI 182,780, ICI (400 µg/kg bw; Sigma-Aldrich), methoxychlor, MXC (100 mg/kg bw; Sigma-Aldrich), or (5) vehicle only (corn oil), which served as a control group (CTR). Chemicals were administered daily between postnatal days 1 and 10 and the effective doses were chosen on the basis of literature (Aydoğan and Barlas, 2006; Armenti et al., 2008; Kummer et al., 2008; Tyndall et al., 2012) and our previous experiments (Knapczyk-Stwora et al., 2013). During the entire experiment all piglets were housed with their own mothers and siblings. One day after the last injection (postnatal day 11), the ovaries were excised from each animal and one gonad was fixed in Bouin's solution for immunohistochemistry, while the contralateral ovary was frozen in liquid nitrogen for RNA and protein extractions.

Immunohistochemistry

Immunohistochemical localization of FOXL2 was conducted on Bouin-fixed, paraffin-embedded, 5 µm-thick ovarian sections. After deparaffinization and rehydration, 0.01 M citrate buffer (pH 6.0) was used for antigen retrieval using microwave. Endogenous peroxidase activity was eliminated by 0.3% hydrogen peroxide in Tris-buffered saline. Horse serum (10%) was used to block nonspecific binding sites followed by an incubation with the primary polyclonal goat anti-FOXL2 antibody (1:250, OAEB02240, Aviva Systems Biology), overnight at 4°C in a humidified chamber. On the next day, the sections were incubated for 1.5 h at room temperature with the horse anti-goat biotinylated secondary antibody (1:300; Vector Laboratories) followed by the incubation with avidin-biotin-peroxidase complex (StreptABCom-

plex-HRP, Vector Laboratories) for 40 min. Finally, the antigen-antibody complex was visualized using 3,3'-diaminobenzidine (Sigma-Aldrich) as a chromogen. Sections were then counterstained with Hematoxylin QS (Vector Laboratories), dehydrated, fixed in xylene, and mounted using DPX (Sigma-Aldrich) and coverslips. For the negative control reaction, sections were incubated with non-immune goat IgG instead of primary antibody and processed as above. The stained sections were examined and photographed under a Nikon Eclipse Ni-U microscope using Nikon Digital DS-Fi1-U3 camera (Nikon, Tokyo, Japan) and Nikon NIS-ELEMENT Image Software.

RNA extraction, reverse transcription and real-time PCR

Total RNA from the collected ovaries was extracted using TRI Reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Concentration and purity of the total RNA samples were measured spectrophotometrically using the NanoDrop ND2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). One microgram of RNA was reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions, and the cDNA was subjected to real-time PCR analysis. A TaqMan probe-based real-time PCR was performed as described previously (Knapczyk-Stwora et al., 2019) using a StepOne™ Real-Time PCR System (Applied Biosystems) and the porcine-specific TaqMan Gene Expression Assays (Applied Biosystems) for: *AMH* (assay ID: Ss03383931_m1), *CYP19A1* (assay ID: Ss03384876_u1), and *FOXL2* (assay ID: Ss04328236_s1). Appropriate no-reverse transcriptase and non-template controls were included in each PCR run. All real-time PCR reactions were carried out in duplicates. Relative mRNA quantification data were analyzed using the real-time PCR Miner algorithm (Zhao and Fernald, 2005), which calculates real-time PCR expression values using parameters such as reaction efficiency and the fractional cycle number at the threshold. The real-time PCR data obtained for AMH, CYP19A1, and FOXL2 were normalized to endogenous control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; assay ID: Ss03373286_u1). Data were expressed as the overall mean $\pm$ SEM.

Protein extraction and Western blot

The abundance of AMH, CYP19A1, and FOXL2 proteins in the collected ovaries was determined by Western blot following protein extraction in cold Tris/EDTA buffer (50 mM Tris, 1mM EDTA, pH 7.5) containing 10 μ l/ml Protease Inhibitor Cocktail (Sigma-Aldrich) as previously described (Grzesiak et al., 2014). After sonication, the supernatant was collected by centrifugation (10000 $\times$ g, 20 min, 4°C), and the protein concentration was determined using Bradford method (Bradford, 1976). Equal amounts of protein (20 μ g) were resolved using SDS-PAGE and transferred onto PVDF membranes. Following protein transfer, the membrane was blocked with 5% non-fat milk in Tris-buffered saline (0.05M Tris-HCl and 150 mM NaCl, pH 7.4) containing 0.1% Tween 20 (TBST) for 1 h at room temperature and then probed with the following primary antibodies: polyclonal rabbit anti-AMH (1:1000, TA336233, Acris Antibodies GmbH, Herford, Germany), monoclonal mouse anti-CYP19A1 (1:500, MCA2077S; Bio-Rad Laboratories Inc., GmbH, Munich, Ger-

many), or polyclonal goat anti-FOXL2 (1:1000, OAEB02240, Aviva Systems Biology, San Diego, CA, USA), overnight at 4°C. Next the membrane was probed with the HRP-conjugated corresponding secondary antibody (1:10,000; Jackson ImmunoResearch, Cambridge, UK) for 1 h at room temperature, and the signal was detected by chemiluminescence using Western Bright Quantum substrate (Advanta, Menlo Park, CA, USA) and ChemiDoc-It 410 Imaging System (UVP, Upland, CA, USA). Subsequently, each membrane was stripped and reprobed with monoclonal mouse anti- β -actin antibody (1:3000; Sigma-Aldrich) followed by HRP-conjugated anti-mouse IgG (1:10,000; Jackson ImmunoResearch). Bands were quantitated densitometrically using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Quantitative data from the immunoreactive bands revealed with the anti-AMH, anti-CYP19A1, and anti-FOXL2 sera were normalized against the corresponding β -actin values measured on the same blots.

Statistical analysis

Data, expressed as mean $\pm$ standard error of the mean (SEM), were analyzed using Statistica v.13.1 software (StatSoft, Inc, Tulsa, OK, USA). The Shapiro-Wilk and Lilliefors tests were applied to test the distribution of data. Due to a lack of normal distribution the nonparametric Mann-Whitney U test was used to determine changes of mRNA and protein expression between CTR (untreated) and the TP-, FLU-, OP-, ICI- and MXC-treated groups. All results were considered statistically significant at the 95% confidence level ($P < 0.05$).

Results

Effect of exposure to sex steroid receptors agonists and antagonists on FOXL2 immunoexpression

The positive FOXL2 staining was found in all examined ovarian sections (Figure 1). FOXL2 nuclear immunostaining was observed only in somatic cells, whereas germ cells did not exhibit any staining (Figure 1). In the ovaries of 11-day-old piglets of all groups, FOXL2 was localized in stroma cells surrounding egg nests (Figure 1 A–F), and in granulosa cells of primordial and primary follicles (Figure 1 A'–F'). Negative control was presented as inset in Figure 1 A.

Effects of exposure to sex steroid receptors agonists and antagonists on the FOXL2, AMH, and CYP19A1 mRNA expression

The mRNA expression levels of FOXL2, AMH, and CYP19A1 in the ovaries of 11-day-old piglets after treatment with TP, FLU, OP, ICI or MXC were examined by quantitative real-time PCR and depicted in Figure 2. The expression levels of both *FOXL2* and *AMH* were significantly higher ($P < 0.05$, $P < 0.01$, respectively) in the TP-, OP-, and MXC-treated groups compared with the control group (Figure 2 A and 2 B, respectively). The expression levels of *CYP19A1* were significantly lower in the FLU- and ICI-treated groups ($P < 0.05$ and $P < 0.01$, respectively) than in the control group (Figure 2 C).

Figure 1. FOXL2 immunostaining in the ovaries obtained from control (A, A'), testosterone propionate- (B, B'), flutamide- (C, C'), 4-*tert*-octylphenol- (D, D'), ICI 182,780- (E, E'), and methoxychlor-treated (F, F') 11-day-old piglets. FOXL2-positive staining was detected in stroma cells surrounding egg nests (arrowheads) (A-F), and granulosa cells of primordial and primary follicles (arrows) (A'-F') in all examined groups. All sections were counterstained with Hematoxylin QS. Negative control sections did not exhibit any positive staining (A, inset). Bars = 50 μ m

Figure 2. The relative expression levels of *FOXL2* (A), *AMH* (B) and *CYP19A1* (C) in the ovaries obtained from control (CTR), testosterone propionate- (TP), flutamide- (FLU), 4-*tert*-octylphenol- (OP), ICI 182,780- (ICI), and methoxychlor-treated (MXC) 11-day-old piglets were checked by real-time PCR. The expression levels of *FOXL2*, *AMH* and *CYP19A1* were normalized against glyceraldehyde-3-phosphate dehydrogenase levels. The results are expressed as mean values $\pm$ SEM. Asterisks denote significant differences between control and treated animals, *P<0.05, **P<0.01, Mann-Whitney U test

Effects of exposure to sex steroid receptors agonists and antagonists on the FOXL2, AMH, and CYP19A1 protein abundance

The protein abundance of FOXL2, AMH, and CYP19A1 in the ovaries of 11-day-old piglets after treatment with TP, FLU, OP, ICI or MXC were examined by Western blot analysis and depicted in Figure 3. As shown in Figure 3D, examined proteins were detected in the ovaries obtained from all examined groups. The approximately observed molecular weights were: ~50 kDa (FOXL2), ~61 kDa (AMH), and ~55 kDa (CYP19A1). FOXL2 protein abundance was markedly greater in all groups treated with sex steroid receptors agonists and antagonists compared with the control group (Figure 3 A; $P<0.05$ for TP-, FLU-, OP-, and ICI-treated groups; $P<0.01$ for MXC-treated group). Administration of TP, OP, ICI or MXC resulted in a significantly higher protein abundance of AMH compared to the control group (Figure 3 B; $P<0.05$ for TP- and OP-treated groups; $P<0.01$ for ICI- and MXC-treated groups). CYP19A1 protein abundance was markedly lower in the TP-, FLU-, and OP-treated groups than in the control group (Figure 3 C; $P<0.05$).

Figure 3. Protein abundance of FOXL2 (A), AMH (B) and CYP19A1 (C) in the ovaries obtained from control (CTR), testosterone propionate- (TP), flutamide- (FLU), 4-*tert*-octylphenol- (OP), ICI 182,780- (ICI), and methoxychlor-treated (MXC) 11-day-old piglets were analysed by Western blot. Relative abundance of protein was evaluated densitometrically and expressed as the ratio relative to β -actin abundance. The results are expressed as mean values $\pm$ SEM. Asterisks denote significant differences between control and treated animals, * $P<0.05$, ** $P<0.01$, Mann-Whitney U test. (D) Representative Western blots showing FOXL2, AMH, CYP19A1, and β -actin (loading control) in the ovaries obtained from all examined groups

Discussion

Recently, we demonstrated prominent effects of TP and FLU on oocyte nest breakdown and follicle assembly in 11-day-old piglets, whereas OP, ICI and

MXC were found to influence the subsequent stages of follicle initial recruitment and growth, confirming that EACs affect proper ovarian development (Knapczyk-Stwora et al., 2018). To extend these lines of research, we hypothesized that EACs affect the expression of FOXL2, a transcription factor, which is required for proper oocyte nest breakdown, ovarian follicle formation and activation as well as granulosa cell function (Elzaia et al., 2017). Therefore, we present data concerning the consequences of neonatal exposure to TP, FLU, OP, ICI and MXC on the expression of FOXL2 and its target genes *AMH* and *CYP19A1* in the ovaries of 11-day-old piglets.

We demonstrated nuclear localization of FOXL2 in ovarian somatic cells of 11-day-old piglets. FOXL2 was localized in stroma cells surrounding egg nests and in granulosa cells of primordial and primary follicles, which is consistent with previous research showing that FOXL2 is expressed in mammalian ovarian somatic cells from the early stage of development to adult life (for review see Bertho et al., 2016). Although neonatal treatment with EACs, which were examined here, did not alter the immunostaining pattern, the FOXL2 mRNA level and protein abundance in the porcine ovarian tissue were affected. We found increased FOXL2 mRNA levels upon neonatal TP, OP and MXC administration, and also, increased FOXL2 protein abundance in all groups treated with examined sex steroid receptors agonists and antagonists. It is well known that mutations in the *FOXL2* gene are associated with blepharophimosis-ptosis-epicanthus syndrome (BPES) in humans, which among others is characterized by premature loss of ovarian function (Crisponi et al., 2001). Moreover, depletion of murine *Foxl2* in mature granulosa cells led to transdifferentiation of granulosa cells into Sertoli-like cells (Uhlenhaut et al., 2009). Thus, the FOXL2 transcription factor is crucial for ovarian development and function (Georges et al., 2014) and its role in ovarian differentiation seems to be highly conserved among vertebrates (Cocquet et al., 2002). The effect of EACs on ovarian FOXL2 expression has been extensively studied in fish, and upregulation of FOXL2 has been demonstrated after exposure of ovaries to compounds with estrogenic activity (Wang et al., 2012; Cervantes-Camacho et al., 2020). Of note, it was shown that FOXL2 regulates, directly and indirectly, genes involved in cell proliferation and apoptosis. Kim et al. (2011) demonstrated upregulation of pro-apoptotic genes when FOXL2 was overexpressed in adult-type granulosa cell tumors. In addition, 17 β -estradiol increased FOXL2 expression in ovarian-like granulosa (KGN) cells at both mRNA and protein levels leading to the inhibition of cell migration and proliferation, as well as enhanced cell apoptosis (Wu et al., 2019). Interestingly, our previous results revealed that EACs-induced disruption of androgen and estrogen signalling in neonates differentially affected ovarian cell proliferation and apoptosis in pigs, i.e. TP increased ovarian apoptosis, FLU decreased granulosa cells proliferation, while ICI is involved in both processes (Knapczyk-Stwora et al., 2018). Considering results presented herein, the elevated FOXL2 mRNA and/or protein expression, arising from neonatal exposure to examined sex steroid receptor agonists and antagonists may, in part, explain molecular mechanisms underlying the EACs action in the neonatal ovary, i.e., altered cell proliferation and apoptosis, which in turn affects follicle formation and activation in pigs.

FOXL2 regulates the expression of many factors involved in ovarian somatic cells function, including differentiation, proliferation, steroidogenesis and apoptosis (Georges et al., 2014). Similarly to other lineage-determining transcription factors, the effect of FOXL2 on gene regulation often entails interaction with β -catenin signalling (Elzaia et al., 2017) and steroid hormone nuclear receptors, such as progesterone receptors and estrogen receptors (ERs) (Ghochani et al., 2012; Uhlenhaut et al., 2009). Hirano et al. (2017) demonstrated that FOXL2 repressed the canonical ERE-mediated transcription of ER β . Moreover, a positive feedback loop between FOXL2 and AMH, in which FOXL2-regulated AMH positively stimulates FOXL2 transcription, was described by Park et al. (2014). Since FOXL2 plays an important part in estrogen signalling and regulates, directly and indirectly, many target genes, we hypothesized that neonatal sex steroid receptor agonists and antagonists influenced the expression of *AMH*, which is a direct ovarian target gene regulated by FOXL2. As here revealed from the present study, similarly to the FOXL2 expression pattern, levels of AMH mRNAs were higher in response to TP, OP, and MXC administration. In addition, neonatal exposure to TP, OP, ICI and MXC resulted in a higher AMH protein abundance. It is well known that AMH produced by granulosa cells of ovarian follicles from the primary to antral stage is a key factor regulating the recruitment of primary follicles to growth. AMH inhibits initial recruitment of primordial follicles into the growing pool, which prevents exhaustion of the follicular reserve in ovaries (Durlinger et al., 2002). Uzumcu et al. (2006) reported that MXC exposure of mice during the primordial-to-primary follicle transition increased AMH production and inhibited folliculogenesis. Our findings suggest that the previously observed decrease in the percentage of developing follicles in porcine ovaries in response to neonatal ICI and MXC administration (Knapczyk-Stwora et al., 2018) may arise from the FOXL2-mediated stimulation of AMH expression. On the other hand, we previously observed an increase in the percentage of developing follicles upon TP and OP neonatal administration (Knapczyk-Stwora et al., 2018). Thus, we propose that the elevated FOXL2 and AMH expression in those groups may not exert their inhibitory effects on ovarian reserve. Instead, they might be due to the inhibitory effects of TP and OP on AMH signalling. Further research in support of this proposition is needed.

Besides the stimulatory role of FOXL2 in AMH expression, the positive regulation of a rate-limiting enzyme in estrogen production, namely CYP19A1, via FOXL2 was initially described in goat and mouse ovaries as well as in human cells (Pannetier et al., 2006; Uhlenhaut et al., 2009; Fleming et al., 2010). On the contrary, the role of human FOXL2 function as a transcriptional repressor of *CYP19A1* has been reported using Chinese hamster ovary (CHO) cells (Kuo et al., 2011). Similarly, mouse *Foxl2* maintained repression of FSH-dependent genes, including *Cyp19a1* in the mouse granulosa cell, which confirms the transcriptional repression of key granulosa cell genes by FOXL2, and blockade of premature differentiation and/or proliferation of granulosa cells (Kuo et al., 2012). Interestingly, in the present study, neonatal administration of either FLU or ICI resulted in lower CYP19A1 mRNA expression in the ovaries of 11-day-old piglets. Moreover, CYP19A1 protein abundance was lower upon neonatal TP, FLU, and OP administration. Thus, it seems that elevated

FOXL2 expression in neonatal porcine ovaries, arising from treatment with EACs having androgenic, antiandrogenic and estrogenic activities, entails downregulation of *CYP19A1* which, as a consequence, may affect granulosa cells differentiation/proliferation, and ovarian folliculogenesis. Moreover, it is known that the *CYP19A1* expression is controlled in a cell-specific, temporal, and spatial manner by different factors, including androgens and estrogens (Stocco, 2008). Thus, the direct role of androgen and estrogen receptor agonists and antagonists on the *CYP19A1* in the neonatal porcine ovary cannot be excluded.

Surprisingly, the similar effect of androgen and estrogen receptor agonists and antagonists on the expression pattern of *FOXL2*, *AMH* and *CYP19A1* was observed. These findings indicate that neonatal period of ovarian development in pigs is sensitive to changes in the hormonal status (i.e., excess or deficiency of either androgens or estrogens). It confirms our earlier results showing that neonatal disruption of androgen signalling induced by either androgen excess or deficiency promoted structural luteolysis (Knapczyk-Stwora et al., 2020 b). Of note, in a current study the effect of EACs on the expression of *FOXL2*, *AMH* and *CYP19A1* was examined only in the preantral follicles. Since androgen and estrogen action within ovarian follicles depends on the follicle stage development, it seems that the regulation mechanism of sex steroid receptor agonists and antagonists actions on the expression of the *FOXL2* transcription factor and its target genes may be stage-dependent. In addition, *FOXL2* activity is modulated by post-translational modifications such as SUMOylation, phosphorylation and ubiquitylation (Leung et al., 2016), and it is thus plausible that the *FOXL2* effect on target gene expression can be modulated by other pathways.

To summarize, all examined EACs increased *FOXL2* expression at mRNA or/and protein levels and affected *FOXL2* target genes, namely *AMH* and *CYP19A1*, in the ovaries of 11-day-old piglets. However, the prominent effect of the expression of both of these *FOXL2* target genes was observed only in response to compounds with androgenic and estrogenic activity. Considering our results, we confirm that the exposure to compounds affecting androgen and estrogen action during the neonatal window of porcine development affects ovarian folliculogenesis, and this effect may be, at least in part, mediated by altered *FOXL2* expression. All these results point to a crucial role of steroid milieu during the neonatal development period in pigs, and possible consequences of commonly used EACs, which may further lead to impaired female fertility in adulthood.

Conflict of interest

None of the authors have any conflict of interest to declare.

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