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Temporal changes in the transcriptomic profile of granulosa cells of pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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ABSTRACT

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) compound is an environmental chemical adversely affecting reproductive processes. Intracellular TCDD effects are mediated via aryl hydrocarbon receptor (AhR). The aim of the current study was to identify genes linking the AhR pathway with phenotypic consequences of TCDD action in granulosa cells of pigs. By applying multifactorial analysis, with TCDD and incubation time as factors, it was possible to determine temporal changes induced by TCDD in the cell transcriptome. Among the identified 144 differentially expressed genes (DEGs; $P_{adjusted} < 0.05$, **log2** fold change (FC) ≥ 1), 111 DEGs were classified as sustained genes (FC values changing between 3 and 24 h). Eighty six DEGs were classified as early genes and only nine as late genes (FC changes observed between 3 and 12 h or 12 and 24 h, respectively). The sustained gene category included genes related to TCDD mechanism of action (*AHR, ARNTL, CYP1A1*), cell proliferation (*TGFβ3*), follicular development and ovulation (*PTGS2*) as well as stress response (*NR3C1*). The early gene category contained DEGs associated with cell proliferation (*DUSP4, TAB1*) and cellular response to stress (*DHX34*). The *CYP1A1* gene was the only DEG classified as an early, late and sustained gene. The multifactorial approach allowed for statistically analyzing TCDD-induced changes over time in the gene expression in granulosa cells of pigs. Changes over time in the granulosal transcriptome profile indicated the involvement of stress related molecules in the cellular response to TCDD and TCDD effects on ovulation. The TCDD effects were particularly evident during the early stage of action by this compound.

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1. Introduction

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) compound, a highly toxic representative of polychlorinated dibenzo-*p*-dioxins (PCDDs), is a persistent environmental pollutant characterized by great lipophilicity and great resistance to degradation. The TCDD compound interferes with the endocrine system of humans and animals (Poland and Knutson, 1982; Diamanti-Kandarakis et al., 2009; Petroff et al., 2011), adversely affecting ovarian physiology, sexual behavior and fertility (for review see: Mandal, 2005). Actions of TCDD compromise ovarian luteal and follicular functions across species (for review see: Gregoraszczuk, 2002). Specifically, TCDD disrupts ovarian steroidogenesis in humans (Morán et al., 2000; 2003) and rats (Franczak et al., 2006; Shi et al., 2007; Jablonska et al., 2010). In the pig, the adverse effects of TCDD on progesterone and estradiol secretion have also been reported (Piekło et al., 2000; Gregoraszczuk et al., 2001; Grochowalski et al., 2001; Jablonska et al., 2011, 2014).

The cellular effects of TCDD are primarily mediated via the activation of the aryl hydrocarbon receptor (AhR) pathway - i.e., AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) (Pocar et al., 2005). The TCDD-AhR-ARNT complex binds to specific DNA sequences known as dioxin response elements, affecting the expression of specific genes (e.g., cytochrome P450, family 1, subfamily A, polypeptide 1; *CYP1A1*) (Rifkind, 2006; Hankinson, 2016). The induction of *CYP1A1* transcription is commonly used as a marker of TCDD action in various tissues. There was previously detection of the presence of both AhR and ARNT in granulosa cells of pigs (Jablonska and Ciereszko, 2013; Sadowska et al., 2017), indicating these cells are responsive to TCDD. Furthermore, TCDD induced transcription of *CYP1A1* (Sadowska et al., 2017) and increased CYP1A1 activity (T. Molcan and R.E. Ciereszko, personal information) in granulosa cells of pigs.

Molecular mechanisms underlying the TCDD disruption of ovarian physiology have been previously studied in pigs (Jablonska et al., 2011; Piasecka-Srader et al., 2016) and other species (Heiden et al., 2008; Magre et al., 2012) with the use of methods allowing for simultaneous analysis of no more than a few genes or proteins. This limitation can be currently overcome by using microarrays and RNA sequencing (RNA-Seq). The latter method allows researchers to examine the effects of TCDD on the entire cell/tissue transcriptome. In view of the fact that TCDD was reported to induce time-dependent changes in ovaries (Karman et al., 2012; Piasecka-Srader et al., 2016; Sadowska et al., 2017; Ruszkowska et al., 2018), such global transcriptomic analysis may be difficult. Because the expression of particular genes peak or reach a nadir at different times after the treatment, a one-time-point-study does not allow for ascertaining of some important changes in gene expression as well as does not allow for proper investigation of over-time changes in the transcriptomic profile.

Previously, similar to what occurred in other studies, the effect of TCDD on the transcriptome of granulosa cells of pigs was investigated at single time points (Sadowska et al., 2017). The novelty of the current study is that both TCDD and incubation time (3, 12 and 24 h) were assumed as the factors in the applied multifactorial analysis. Such an approach allowed for identification of temporal changes (3 compared with 12 h, 3 compared with 24 h, 12 compared with 24 h) in the transcriptomic profile of TCDD-treated granulosa cells of pigs. In addition, the TCDD-induced differentially expressed genes (DEGs) were evaluated for the physiological functions, positive or negative correlations with other DEGs as well as for the occurrence of alternative splicing events.

2. Material and methods

2.1. Materials

The porcine granulosa cell line (AVG-16) was purchased from The European Collection of Authenticated Cell Cultures (ECACC; 06062701, Salisbury, UK). The reagents used for the cell culture were obtained from Sigma Aldrich (St. Louis, MO, USA). PeqGold TriFast was purchased from Peqlab Biotechnologie GmbH (Erlangen, Germany) and TruSeq RNA Sample Preparation Kit from Illumina (San Diego, CA, USA). KAPA Library Quantification Kit was obtained from KapaBiosystem (Wilmington, MA, USA) and DNA High Sensitivity LabChip kit from Agilent Technologies (Santa Clara, CA, USA).

2.2. Culture of granulosa cells

Granulosa cells of pigs were cultured and passaged as previously described (Sadowska et al., 2017). Briefly, granulosa cells were seeded in six-well plates (1×10^6 cells/ 3 mL Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 10% fetal bovine serum, 0.1 mM MEM non-essential amino acid solution, 2.5 ng/mL fibroblast growth factor-basic human, 100 U penicillin, 100 µg streptomycin and 0.25 µg amphotericin B/mL). After there was 60%–65% confluence, the cells were treated with TCDD (100 nM) for 3, 12 or 24 h ($n=$ two/time point). To assure the potential of TCDD to transduce intracellular signaling in the examined cells, the selected dose of TCDD moderately exceeded its environmentally relevant concentration. Furthermore, the selected TCDD concentration was previously reported (Jablonska et al., 2014) to affect granulosa cell steroidogenesis of pigs without affecting cell viability. After culture, the cells were designated for total RNA isolation.

2.3. Total RNA isolation and evaluation of RNA integrity

Total RNA was isolated from cells using peqGold TriFast. The abundance of RNA and quality were determined spectrophotometrically (NanoVue Plus, GE Healthcare, Little Chalfont, UK). To evaluate RNA integrity, a microfluidic electrophoresis (2100 Bioanalyzer, Agilent Technologies) was used. The samples with a RNA integrity number (RIN; 28 S/18 S ratio) greater than 8.0 were used for RNA-Seq (Sadowska et al., 2017).

2.4. Construction and sequencing of Illumina cDNA libraries

The samples of total RNA were depleted and used to construct cDNA libraries (TruSeq RNA Sample Preparation Kit). Initially, RNA was purified and fragmented, then cDNA strands were synthesized. Subsequently, 3′ ends were adenylated, adapters were ligated and libraries were amplified (PCR). The cDNA library templates were quantified using the KAPA Library Quantification Kit. The estimation of library profiles were performed using the DNA High Sensitivity LabChip kit on the 2100 Bioanalyzer (Agilent Technologies). There was subsequent 100 paired-end sequencing conducted with the use of HiSeq2500 high throughput sequencing instrument (Illumina; Sadowska et al., 2017).

2.5. Bioinformatic analysis of gene expression

Primary processing of raw reads (cDNA fragments obtained after sequencing) was performed as previously described (Sadowska et al., 2017). The sequenced data were submitted to the NCBI BioProject database (http://www.ncbi.nlm.nih.gov/bioproject) under accession number: PRJNA429720. To increase the accuracy of mapping process, the reads were mapped to the *Sus scrofa* genome (Sus_scrofa.Sscrofa10.2; Ensembl database) with the use of two different programs i.e., TopHat2 (Trapnell et al., 2009) and STAR (version 2.4; Dobin and Gingeras, 2015). Pearson's correlation analysis between any two biological replicates of both TCDD-treated and untreated cells was performed to assess the overall similarity between the RNA samples obtained after 3, 12 or 24 h of TCDD treatment. To compare the transcript abundance between samples, the raw counts per transcript were converted to FPKM (fragments per kilobase of exon per million fragments mapped). Differentially expressed genes (DEGs) and the corresponding $P_{adjusted}$ values were determined by means of R statistical software using DESeq2 1.18.1 package (Love et al., 2014) and the results from both mappers. The identified DEGs that were common for both mappers were designated for further analysis. A multifactorial analysis was performed to examine the effects of both TCDD treatment and cell incubation time (3, 12, 24 h) on gene expression. Experimental data were fitted into a linear model: Y = ~ time + treatment + time:treatment, where Y is the expression value of each gene, time is the effect of time shift and treatment is the effect of TCDD action. The changes in abundance of gene expression were considered to be statistically significant only when the following criteria were met: $P_{adjusted} < 0.05$ and log2 fold change (FC) ≥ 1.0. Furthermore, the results of multifactorial gene expression analysis were visualized using Circos software (Krzywinski et al., 2009) combined with custom Perl script. Means of FPKM values have been multiplied by 10 (to avoid negative log values) and log-transformed (log base = 10).

2.6. Functional enrichment analysis (GO and STRING pathways)

Functional analysis of the identified DEGs was performed based on the Gene Ontology (GO) database using the clusterProfiler 3.0.2 (Yu et al., 2012) and DOSE 2.10.2 (Yu et al., 2015) with the established criterion $P_{adjusted} < 0.05$. The DEGs successfully categorized in GO "biological regulation" category (GO:0065007) were submitted to the STRING (Search Tool for the Retrieval of Interacting Genes) *S. scrofa* database to establish possible interactions between the identified genes. The STRING is a large database of known and predicted protein interactions that covers more than 1100 organisms (Franceschini et al., 2013) and includes direct (physical) and indirect (functional) associations. A protein-protein interaction *P*-value (PPI enrichment *P*-value) was calculated and the cut-off criterion of combined score was set as > 0.4. In addition, a false discovery rate (FDR) test was applied to determine if all DEGs were enriched.

2.7. Correlation of gene expression and alternative splicing events

To identify significantly correlated genes ($r > 0.7$ or $r < -0.7$), the gene expression values of the identified DEGs were processed using the custom script in R statistical software ($P < 0.05$). To identify the differential expression of exons and the occurrence of splice junctions (differential usage of exons and junctions) in DEGs of granulosa cells of pigs treated with TCDD, QoRTs (Hartley and Mullikin, 2015) and JunctionSeq (Hartley and Mullikin, 2016) tools were applied. Initially, QoRTs was used to generate raw exon and splice junction counts in DEGs of TCDD-treated cells in comparison to control cells ($P_{adjusted} < 0.05$). Then, JunctionSeq was used to visualize the identified sites of differential usage of exons and splice junction loci (both known and novel).

3. Results

3.1. The identification of differentially expressed genes (DEGs)

The data acquired after RNA-Seq were preprocessed as previously described by Sadowska et al. (2017). The numbers of short-sequence reads as well as of the reads after quality control were previously published (Sadowska et al., 2017). The biological replicates (a, b) were significantly correlated with regards to gene expression profile (Table 1). As depicted in Fig. 1, the genes expressed in cells incubated for 3 h clustered separately from those incubated for 12 and 24 h in both control and TCDD-treated cells. Furthermore, with all incubation times, genes expressed in the cells treated with TCDD clustered separately from those expressed in control cells.

The multifactorial analysis, by taking into account the effects of both TCDD and incubation time, allowed for identification of 144 DEGs ($P_{adjusted} < 0.05$ and log2 fold change (FC) > 1.0; Tab. S1, Fig. S1). The FC value for DEGs ranged from -3.51

Table 1
Correlation coefficients of gene expression levels† between two biological replicates of control and TCDD-treated granulosa cells of pigs.

Incubation time	Control	TCDD
3h	0.9939*	0.9995*
12h	0.9994*	0.9980*
24h	0.9976*	0.9997*

TCDD:2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
†mean normalized read counts.
* $P < 0.0001$.

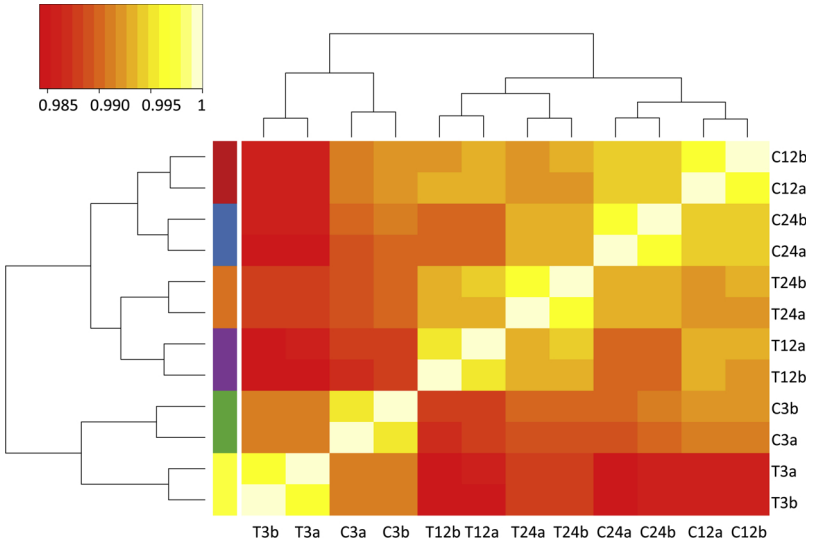

Fig. 1. Distance matrix illustrating the transcriptomic profile of granulosa cells of pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 3, 12 or 24 h; Twelve analyzed samples include: C3a, C3b, C12a, C12b, C24a, C24b and T3a, T3b, T12a, T12b, T24a and T24b. C: control (untreated) cells; T: TCDD-treated cells; 3, 12 and 24: incubation times expressed in hours; a, b: biological replicates; Color scale of the figure represents correlation coefficients, with the most intensive red standing for 0.985 and the most pale yellow standing for 1.

(*ENSSSCG00000021324*) to 3.21 (*CYP1A1*; Tab. S1). The results for temporal comparison of DEG fold change values calculated for granulosa cells treated with TCDD (Tab. S1) indicated there were 86, 111 and 9 differentially expressed genes between 3 and 12 h, 3 and 24 h, as well as 12 and 24 h of cell incubation, respectively (Fig. 2). The DEGs were then classified into three groups: 1/ early genes – where FC increased or decreased between 3 and 12 h, 2/ late genes – where FC increased or decreased between 12 and 24 h and 3/ sustained genes – where FC increased or decreased between 3 and 24 h. The fold change values of 50, 3 and 59 DEGs increased and the FC value of 36, 6 and 52 DEGs decreased in the early, late and sustained gene group, respectively (Fig. 3; Table S1). Furthermore, there were nine different temporal DEG fold change patterns (Fig. 3) in the TCDD-treated cells. The greatest number of DEGs was assigned to patterns F and I (Fig. 3F and I), while only one DEG was classified to pattern E (Fig. 3; *CYP1A1*).

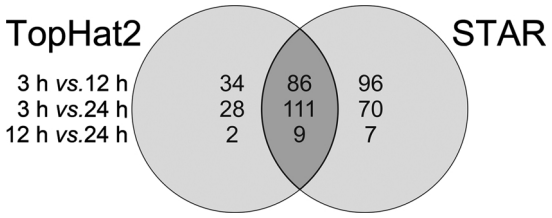

Fig. 2. Venn diagram presenting temporal changes in the number of differentially expressed genes (DEGs; $P_{adjusted} < 0.05$ and log2 fold change ≥ 1.0) identified in granulosa cells of pigs incubated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 3, 12 or 24 h; Presented results were obtained after mapping RNA-Seq reads with two different programs (TopHat2 and STAR). TCDD and incubation time were assumed as factors in the applied multifactorial analysis; Number of DEGs was compared between the 3 h and 12 h, 3 h and 24 h, and 12 h and 24 h of cell incubation.

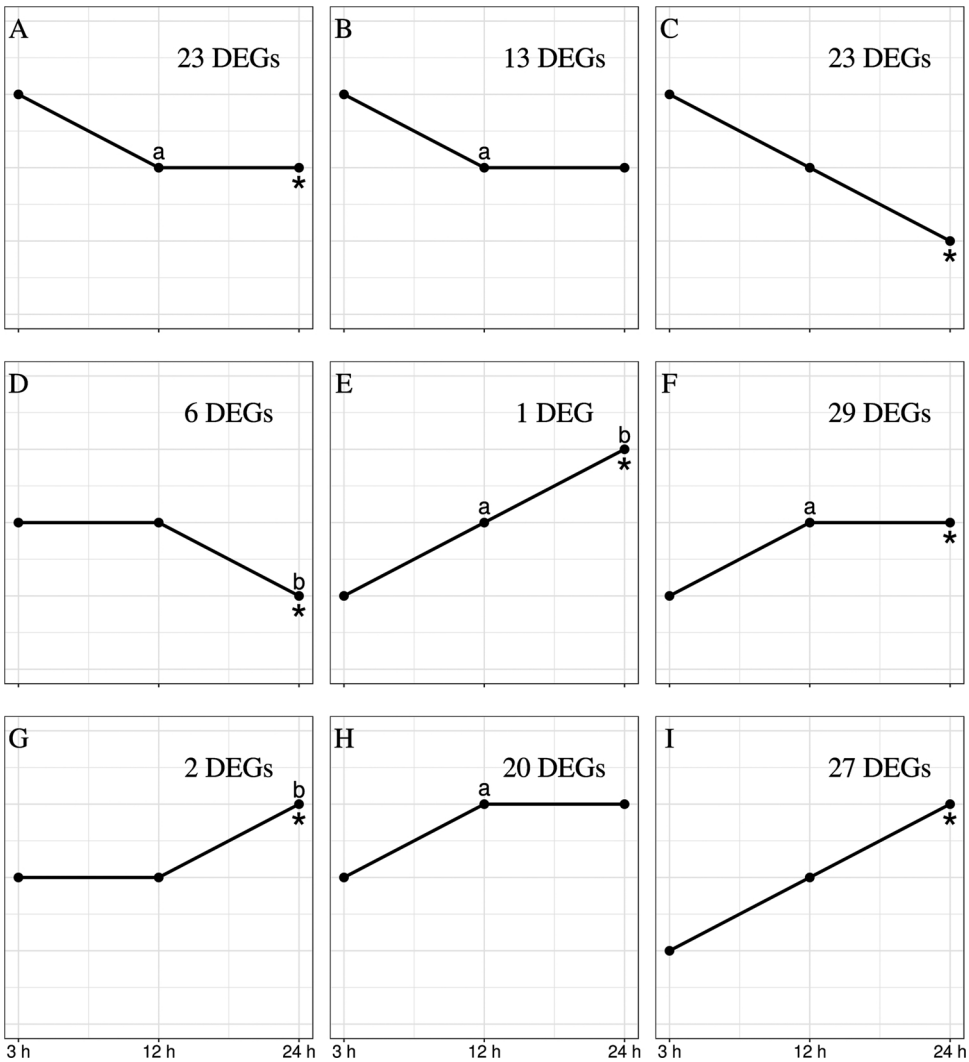

Fig. 3. Temporal fold change patterns for differentially expressed genes (DEGs) of granulosa cells of pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 3, 12 or 24 h; DEGs were identified with the use of multifactorial analysis ($P_{adjusted} < 0.05$) where TCDD and incubation time were the factors; [a]depicts significant differences between 3 and 12 h of TCDD treatment (early genes); [b]depicts significant differences between 12 and 24 h of TCDD treatment (late genes); *depicts significant differences between 3 and 24 h of TCDD treatment (sustained genes); Majority of DEGs (135 genes) had A, B, C, F, H or I fold change patterns, in which the gene fold change significantly increased (76 genes) or decreased (59 genes) between 3 and 12 h as well as between 3 and 24 h of incubation.

3.2. Positive and negative correlation of DEGs

The results for correlation analysis of DEGs indicated all 144 DEGs were positively co-expressed with some other DEGs and 122 DEGs were co-expressed negatively ($r > 0.7$ or $r < -0.7$; $P < 0.05$; Table S2). The co-expression patterns of three exemplary DEGs i.e., *ARNTL* (aryl hydrocarbon receptor nuclear translocator-like), *DUSP4* (dual specificity protein phosphatase 4) and *PTGS1* (prostaglandin-endoperoxide synthase 1) are depicted in Fig. 4 and the co-expressed DEGs are listed in Table S3.

3.3. Physiological functions of DEGs

To recognize the biological significance of DEGs identified in granulosa cells of pigs by means of multifactorial analysis (TCDD and incubation time being the factors), the genes were classified into three main categories (“biological processes”, “molecular function” and “cellular components”) according to GO database. One hundred twenty three genes of 144 DEGs identified by multifactorial analysis ($P_{adjusted} < 0.05$) were assigned to GO terms (Table 2). The genes classified into “biological processes” were annotated to: “biological regulation”, “single-multicellular organism process”, “multicellular organism development”, “biological adhesion”, “cell adhesion” and “response to endogenous stimulus” (Table 2). The “molecular function” of the GO category

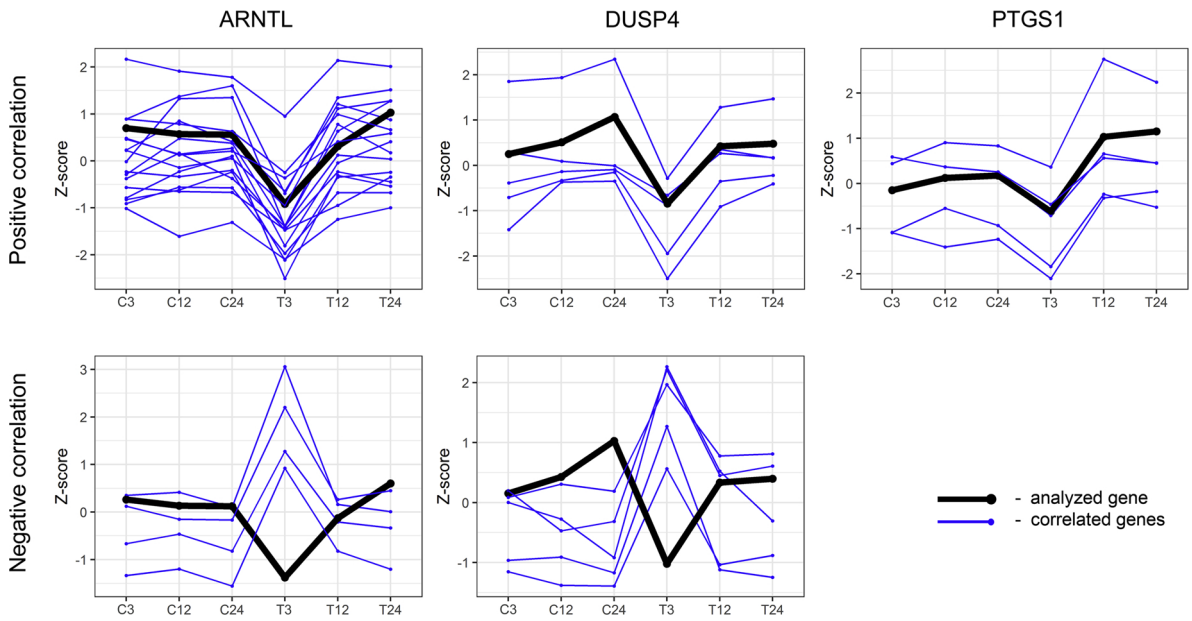

Fig. 4. Effects of correlation analysis performed for three exemplary differentially expressed genes (DEGs) (*ARNTL*: aryl hydrocarbon receptor nuclear translocator-like; *DUSP4*: dual specificity protein phosphatase 4; *PTGS1*: prostaglandin-endoperoxide synthase 1); Expression of DEGs was analyzed in granulosa cells of pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 3, 12 or 24 h by custom script in R packages ($P_{adjusted} < 0.05$ and log2 fold change ≥ 1.0); Expression data are presented as normalized values (Z-scores); Plots for the analyzed genes are depicted as thick lines and for the correlated genes as thin lines; Names of the correlated genes are presented in Supplementary Table 2.

encompassed "nucleic acid binding transcription factor activity", "transcription factor activity", "sequence-specific DNA binding" and "receptor signaling complex scaffold activity". The "cellular components" category linked the analyzed genes to "transcription factor complex".

The majority of the identified DEGs was assigned to "biological regulation" term (87 DEGs). There, therefore, was subsequent functional classification of these DEGs using the STRING database. A confidence gene network interaction (PPI enrichment P-value: 1.55×10^{-5}) with 86 nodes and 44 edges was generated, where nodes were defined as individual genes in the network, and edges were defined as interactions between the genes. The networks included, among others, the TGFβ-signaling pathway (four genes), cytokine-cytokine receptor interactions (nine genes), the MAPK signaling pathway (nine genes) and genes related to prostaglandin synthesis (two genes; Fig. 5). Among the most interacting nodes were *TGFBR2* (TGF-beta receptor type-2, 6 edges), *TAB1* (TGF-beta-activated kinase 1 and MAP3K7-binding protein 1, 5 edges) and *CCL25* (C-C motif chemokine 25, 4 edges).

3.4. Alternative splicing events in DEGs of TCDD-treated cells

Eleven events (sites) of differential usage of exons and splice junctions were identified in DEGs of granulosa cells of pigs after 3 h of TCDD treatment and six events after 12 h (Table S4). There was no differential usage of exons and splice junctions after 24 h of TCDD treatment. Among the identified events, nine and six events were defined as differentially expressed exons after 3 and 12 h of TCDD treatment, respectively. Two events, occurring after 3 h of TCDD treatment, were described as splice junctions. The exemplary JunctionSeq profile plots for *PGST2* and *DUSP4* are depicted in Fig. 6.

4. Discussion

Previously, it was reported that there were TCDD effects at the granulosa cell transcriptome of pigs (Sadowska et al., 2017). The current study extended the focus from the previous studies by including in the analysis, time factor (i.e., the duration of cell treatment with TCDD; 3, 12 or 24 h). The use of multifactorial analysis allowed for proper examination of the temporal changes in TCDD-induced gene expression as well as identification of genes associated with the early/late and transient/sustained response to TCDD. Surprisingly, there are few previous studies of the actions over time of compounds in transcriptome-based assessments. In addition, the identified DEGs were assessed for physiological functions, positive or negative correlations with other DEGs as well as for the occurrence of the alternative splicing events.

In the present study, there was identification of 144 DEGs. Most of DEGs (111) were classified as sustained genes i.e., genes with the fold change (FC) values increasing or decreasing between 3 and 24 h from the time of TCDD treatment initiation. Eighty six DEGs were categorized as early genes with the FC values increasing or decreasing between 3 and 12 h of TCDD treatment. Interestingly, only nine DEGs were assigned to the late gene group with there being significant FC changes only between 12 and 24 h of the

Table 2
Results of GO functional enrichment analysis for DEGs identified in granulosa cells of pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

GO term ID	Term name	Term category	$P_{adjusted}$	Number of all known genes enriched to the GO term	Number of DEGs enriched to GO terms	Number of DEGs enriched to the particular GO term	List of DEGs enriched to the particular GO term
GO:0022610	biological adhesion	BP	0.0258	778	123	17	RND1, PLXNC1, PTPRU, THBS2, CTGF, SMAD6, GCNT1, NR4A3, ENSSSCG00000005494, NOV, THBS3, DCHS2, GATA3, VCAN, PRLR, ITGA2, CLDN1
GO:0007155	cell adhesion	BP	0.023	771	123	17	RND1, PLXNC1, PTPRU, THBS2, CTGF, SMAD6, GCNT1, NR4A3, ENSSSCG00000005494, NOV, THBS3, DCHS2, GATA3, VCAN, PRLR, ITGA2, CLDN1
GO:0009719	response to endogenous stimulus	BP	0.0384	720	123	16	TAB1, GCLC, TGFB3, CTGF, SMAD6, NR4A3, LBH, ANKRD1, CTSL, GATA3, LMO2, NR3C1, NR4A2, PRLR, PTGER2, IGFBP5
GO:0,065,007	biological regulation	BP	0.0099	9441	123	87	TAB1, RND1, PLXNC1, FAM83B, GCLC, TRERF1, ENSSSCG00000002314, ENSSSCG00000002345, TGFB3, DHX34, LDLRAP1, PTPRU, AGO4, ZNF606, THBS2, CTGF, ZNF516, SMAD6, ENSSSCG00000005376, NR4A3, ENSSSCG00000005494, PTGS1, NOV, F5, RGS4, ENSSSCG00000007675, MAPK8IP3, OMD, IL1R2, LBH, TRPC4, DDX60, ANKRD1, DNMBP, CNNM2, GATA3, GRIP2, ACKR4, RCAN1, LMO2, BDNF, ENSSSCG00000013396, ENSSSCG00000013397, ARID3A, S1PR2, ENC1, LVRN, NR3C1, ENSSSCG00000015364, HDAC9, ENSSSCG00000015707, DUSP4, NR4A2, FRZB, TFPI, GPR1, CCL20, ACKR3, LRRN3, PRLR, ITGA2, FAM20A, ERN1, IGFBP4, SLC6A4, MAP3K8, OSGIN2, C3ORF33, ZHX2, PTGER2, GABRP, STK38 L, CBLB, USP53, ETV6, ZNF667, DDIT4L, IGFBP5, SLC45A3, BDKRB2, ENSSSCG00000028004, CLDN1, BHLHE41, SCN4B, CIPC, ENSSSCG00000029816, AHR
GO:0044707	single-multicellular organism process	BP	0.029	3494	123	43	TAB1, RND1, PLXNC1, TGFB3, LDLRAP1, AGO4, THBS2, CTGF, ZNF516, SMAD6, GCNT1, NR4A3, NOV, F5, THBS3, MAPK8IP3, OMD, LBH, DCHS2, TRPC4, DDX60, ANKRD1, CTSL, GATA3, BDNF, S1PR2, ENC1, VCAN, DUSP4, NR4A2, FRZB, ACKR3, LRRN3, PRLR, ITGA2, FAM20A, SLC6A4, ZHX2, ETV6, SLC45A3, CLDN1, BHLHE41, SCN4B
GO:0007275	multicellular organism development	BP	0.0443	2845	123	37	TAB1, RND1, PLXNC1, TGFB3, AGO4, THBS2, CTGF, ZNF516, SMAD6, GCNT1, NR4A3, NOV, THBS3, MAPK8IP3, OMD, LBH, DCHS2, TRPC4, ANKRD1, GATA3, BDNF, S1PR2, ENC1, VCAN, DUSP4, NR4A2, FRZB, ACKR3, LRRN3, PRLR, ITGA2, FAM20A, SLC6A4, ZHX2, ETV6, SLC45A3, CLDN1
GO:0005667	transcription factor complex	CC	0.0438	233	123	9	TRERF1, ENSSSCG00000002345, SMAD6, NR4A3, ANKRD1, GATA3, LMO2, ENSSSCG00000013396, ENSSSCG00000013397
GO:0030159	receptor signaling complex scaffold activity	MF	0.04	9	123	3	LDLRAP1, MAPK8IP3, GRIP2
GO:0001071	nucleic acid binding transcription factor activity	MF	0.0162	916	123	19	TRERF1, ENSSSCG00000002345, ZNF606, ZNF516, SMAD6, NR4A3, GATA3, LMO2, ENSSSCG00000013396, ENSSSCG00000013397, ARID3A, NR3C1, ENSSSCG00000015364, NR4A2, ZHX2, ETV6, BHLHE41, ENSSSCG00000029816, AHR
GO:0003700	transcription factor activity, sequence-specific DNA binding	MF	0.0162	916	123	19	TRERF1, ENSSSCG00000002345, ZNF606, ZNF516, SMAD6, NR4A3, GATA3, LMO2, ENSSSCG00000013396, ENSSSCG00000013397, ARID3A, NR3C1, ENSSSCG00000015364, NR4A2, ZHX2, ETV6, BHLHE41, ENSSSCG00000029816, AHR

BP: Biological process.
CC: Cellular component.
MF: Molecular function.
GO: Gene Ontology.
DEGs: Differentially expressed genes.

treatment. The fact that the overall change (3 compared with 24 h) in the number of DEGs resulted from changes taking place between 3 and 12 h of the initiation of treatment indicate that TCDD effects on gene expression are especially pronounced during the first 12 h of the treatment. The results from *in vivo* (Boverhof et al., 2005) and *in vitro* (Dere et al., 2006) studies of TCDD action on hepatic gene expression in mice indicated the number of TCDD-regulated genes increased between 2 and 12 h of the time of treatment initiation, but not between 12 and 24 h. These previous results, although different with regards to the statistical approach, species and tissue, are consistent with the results in the present study. Results of all cited studies indicate the cellular response to TCDD is initiated early, possibly to alleviate as soon as possible the adverse effects of the dioxin.

Analyzing temporal alterations in TCDD-induced gene expression in granulosa cells of pigs, it was possible to distinguish nine different patterns of DEG fold changes. Each of these patterns contained from one to 29 DEGs. Only one pattern consisted of a single DEG (i.e., *CYP1A1*). The *CYP1A1* gene was also the only DEG with a significant increase in FC between all analyzed time points. Similar to other cellular models (Bofinger et al., 2001; Dasmahapatra et al., 2001), TCDD treatments resulted in an increase in abundance of *CYP1A1* mRNA in granulosa cells of pigs. It should also be noted that the TCDD-induced *CYP1A1* gene expression may be especially important in steroidogenic cells because the CYP1A1 protein is able to induce metabolism of 17β-estradiol and thus modulate endocrine functions of these cells (Safe et al., 2013). Furthermore, TCDD treatment in the current study resulted in temporal changes of *AHR* and *ARNTL* gene expression, confirming the generally accepted mode of TCDD action across species (Black et al., 2012). It appears that TCDD-evoked disruption of ovarian function in pigs is tightly associated with clear and time-dependent expression changes of *AHR*, *ARNTL* (pattern F) and *CYP1A1* (pattern E) genes.

The majority of the identified DEGs was assigned to the "biological regulation" term belonging to the GO "biological processes" category. Among the assigned DEGs, there was the *AHR* gene as well as genes associated with cell proliferation, growth and differentiation (*DUSP4*[1], *NR3C1*, *TAB1, TGFB3, SMAD6, DHX34*), stress response (*DHX34, NR3C1*), cell cycle and apoptosis (*STK38 L, GCLC, DDIT4L*), regulation of cytoskeleton components (*PLXNC1)* as well as prostaglandin (*PTGS1, PTGS2, PTGER2*) and steroid hormone synthesis *(DUSP4, PLXNC1, TRERF1)*. These results are largely consistent with previous findings (Sadowska et al., 2017) indicating TCDD, by inducing temporal changes in the transcriptome, may have a marked effect on functions of ovarian follicles in pigs.

The development of ovarian follicles is closely associated with granulosa cell proliferation, growth and differentiation. These processes are regulated by multiple intracellular signaling pathways. In the present study, TCDD induced changes over-time in the expression profile of several genes involved in ovarian follicular development, especially those of the TGFβ pathway. Treatment with TCDD increased the fold change (FC) of *DUSP4* and *TAB1* gene expression between 3 and 12 h (pattern H) and decreased the FC of the *TGFβ3* gene between 3 and 24 h (pattern C) of cell incubation. The *DUSP4* gene is known to encode a phosphatase implicated in the inhibition of the ERK/MAPK pathway (Misra-Press et al., 1995). The *TAB1* gene, in turn, encodes a protein responsible for dephosphorylation of TGFβ-activated kinase 1 (TAK1), a stimulator of both, the MAPK pathway (Roh et al., 2014) and granulosa cell proliferation and survival. These actions of TCDD reflect its inhibitory effects on follicular development in pigs. The involvement of the *DUSP4* gene in this inhibition is supported by the fact that TCDD induced a differential usage of the exon 4 in *DUSP4* gene after 3 h of TCDD treatment. In addition, there were changes in the FC of *DUSP4* and *TAB1* gene expression that correlated positively with the changes in the FC of *AHR* and *ARNTL* gene expression, respectively. The inhibitory effects of TCDD on the expression profile of genes linked to the TGFβ pathway were also reported previously (Sadowska et al., 2017).

The results of previous *in vivo* studies support the transcriptomic findings of the *in vitro* studies performed on granulosa cells of pigs (Sadowska et al., 2017; the current study). *iIn vivo* treatment with TCDD resulted in a reduced follicular growth and increased follicular atresia in female zebrafish (Heiden et al., 2006) as well as in a disrupted estrous cyclicity in rats (Jablonska et al., 2010). Results from *in vitro* studies performed on cultured granulosa cells of humans (Ernst et al., 2014) and pigs (Jablonska et al., 2014) indicated that TCDD had no effect on cell proliferation. It should be noted that the *in vivo* experiments lasted much longer (weeks) than those performed on cultured granulosa cells (hours) which allowed for assessment of the long-term effects of TCDD on follicular processes.

Female reproduction is dependent, not only on follicular development, but also on a properly regulated ovulation process. Treatment with TCDD interrupted ovulation in some species (Petroff et al., 2001; Pocar et al., 2003). The TCDD-induced blockage of ovulation is associated with the changes in the expression of some genes, including prostaglandin-endoperoxide synthase 2 (*PTGS2)*. The enzyme catalyzes the formation of prostaglandin E_2 which functions in the preovulatory follicular rupture at the time of ovulation in pigs and other species (Joyce et al., 2001; Blaha et al., 2017). Pharmacological *in vivo* and *in vitro* selective blockage of PTGS2 was reported to prevent ovulation (Mikuni et al., 1998). In the current study, treatment with TCDD decreased the FC of *PTGS2* (pattern C) between 3 and 24 h of the treatment. Furthermore, there was an increased expression of the *PTGS2* gene in exon 15 after 12 h of TCDD treatment. Similarly, other authors reported that *PTGS2* gene expression in rat granulosa cells was reduced when there was treatment with TCDD (Mizuyachi et al., 2002) and bifenthrin (Liu et al., 2011). It is of interest that treatment with TCDD also increased the FC of *PTGS1* (pattern F) between 3 and 12 h as well as between 3 and 24 h of the treatment periods. The enhanced expression of the *PTGS1* gene seems to be associated with pathological processes in the ovary such as inflammation and cancerogenesis (Hales et al., 2008). The results of the study, therefore, confirm the hypothesis that TCDD may disrupt ovarian physiology by, among other actions, affecting the production of prostaglandins.

The adverse effects of TCDD on reproductive tissues is often associated with stress-related intracellular pathways. Treatment of rat uterine and ovarian tissues with TCDD resulted in the production of reactive oxygen species (Melekoglu et al., 2016). The cellular

[1] The full names of the DEGs are presented in Supplementary Table 1.

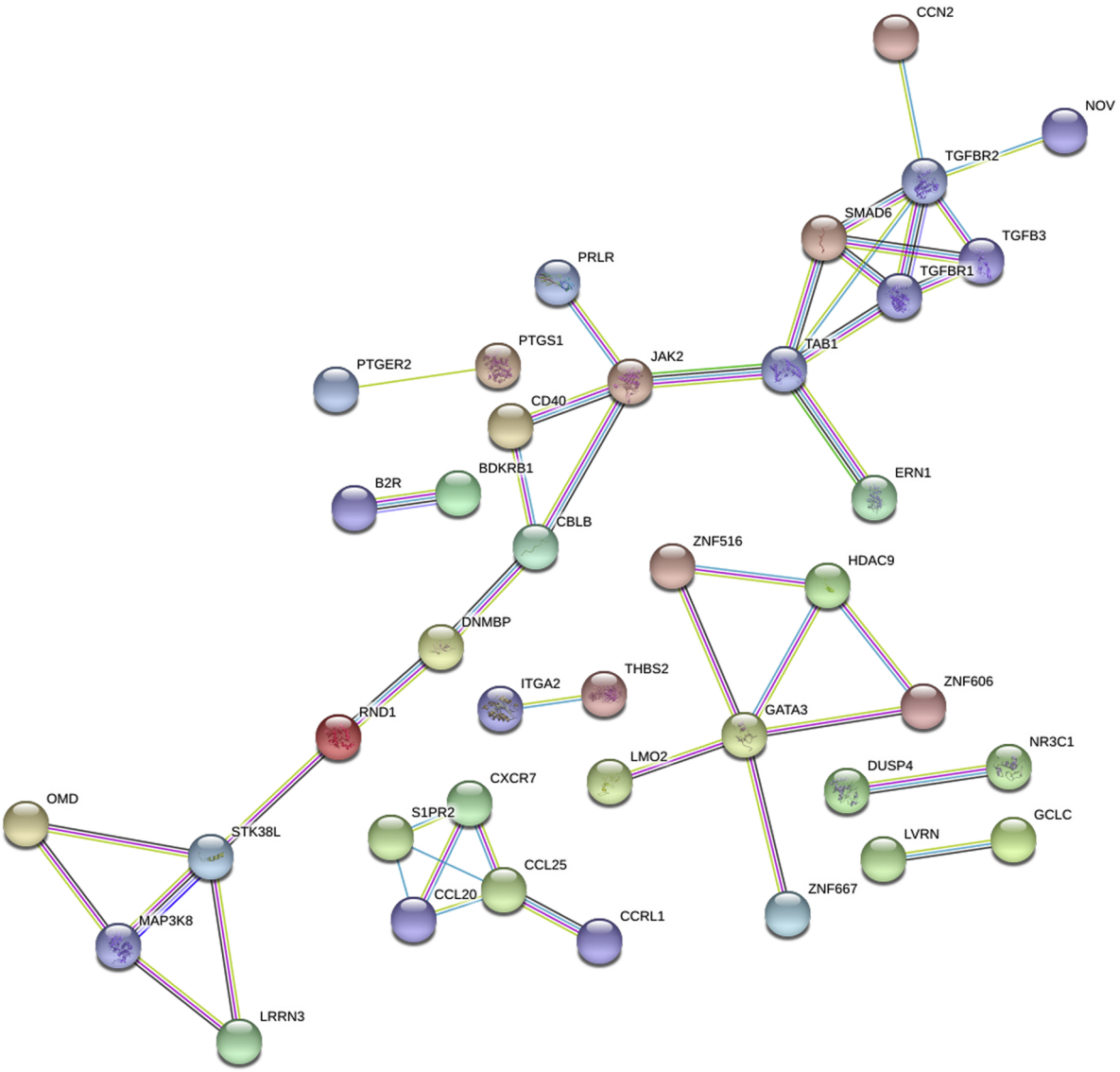

Fig. 5. Gene interaction network of differentially expressed genes (DEGs; $P_{adjusted} < 0.05$ and log2 fold change ≥ 1.0) identified in granulosa cells of pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 3, 12 or 24 h; DEGs presented in the figure were obtained by multifactorial analysis, enriched in "biological regulation" Gene Ontology category (87 DEGs) and submitted to STRING analysis; The more links were found between any two genes (nodes), the stronger the evidence of the associations.

response to stress involves a plethora of mechanisms including glucocorticoid signaling or nonsense-mediated mRNA decay (NMD) pathways (Sonneveld et al., 2007; Longman et al., 2013). In the present study, treatment with TCDD decreased the FC of glucocorticoid receptor *(NR3C1)* between 3 and 12 of the treatment period as well as 3 and 24 h (pattern A) and increased the FC of RNA helicase *(DHX34)* between 3 and 12 h (pattern H) of cell incubation. Furthermore, there was a change in the FC of *NR3C1* gene expression that was negatively correlated with the changes in the FC of *ARNTL* and *DUSP4* gene expression. The glucocorticoid receptor is considered to be a stress-related transcription factor and glucocorticoids functioned to enhance the response of hepatoma cell lines to TCDD (Sonneveld et al., 2007). The NMD, in turn, is an mRNA quality-control mechanism preventing the accumulation of truncated proteins. The DHX34 is recognized as a novel NMD factor, predominantly regulating the expression of genes involved in the cellular stress response (Longman et al., 2013). The results from the present study indicate the possible role of stress-dependent mechanisms in the TCDD-induced toxicity of the ovaries. These results also indicate the effects of TCDD (patterns A and H) that are especially evident during the early stage of its action.

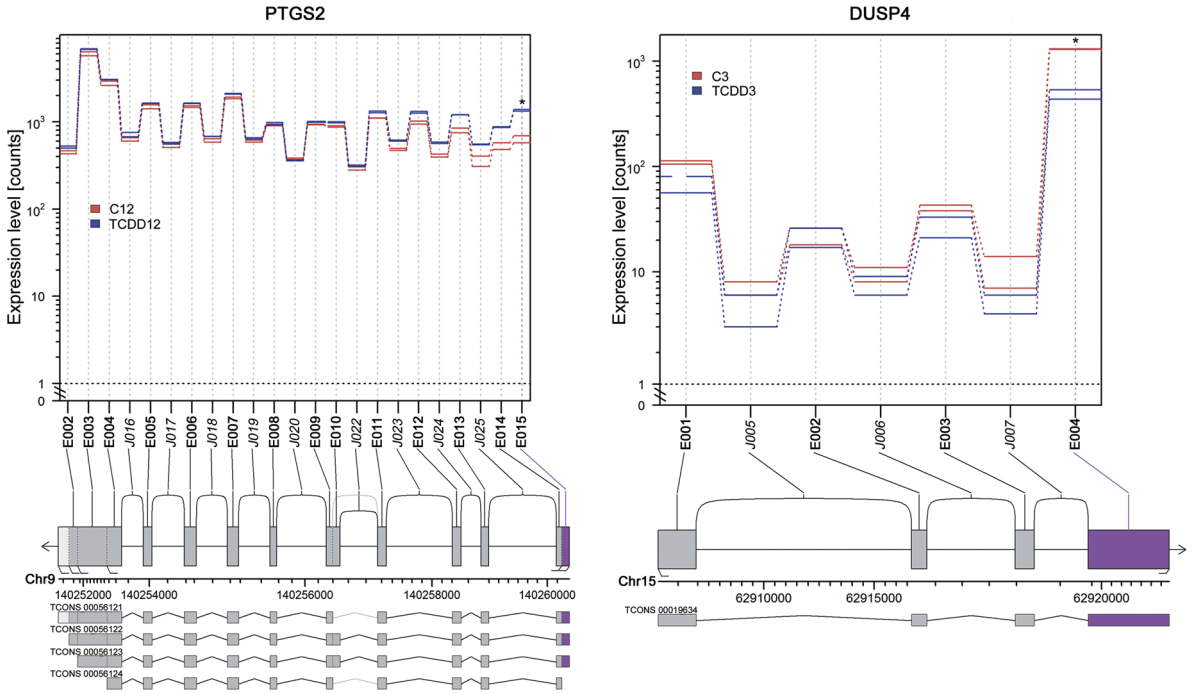

Fig. 6. Differential expression of exons (E) and the occurrence of splice junctions (J) in two exemplary DEGs (*PTGS2* and *DUSP4*) identified in granulosa cells of pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); Upper panel depicts the expression level (mean normalized read counts) of particular exons or splice junctions for DEGs identified in TCDD-treated (blue line) and control (red line) cells after 12 h (*PTGS2*) and 3 h (*DUSP4*) of TCDD treatment; Lower panel depicts the exonic regions of *PTGS2* and *DUSP4* (boxes E002-E015 and E001-E004, respectively) and known splice junctions in *PTGS2* and *DUSP4* (solid lines J016-J025 and J005-J007, respectively); Four TCONS presented in the lowest part of the left panel indicate exon-intron structures of the four *PTGS2* variants; Please note that there is only one TCONS on the right panel; Statistically significant differences in exon expression level between control and TCDD-treated cells ($P_{adjusted} < 0.05$) are presented as violet boxes (*).

5. Conclusions

To the best of our knowledge, this is the first study where multifactorial analysis was used to examine TCDD-induced temporal changes in the ovarian cell transcriptome. The results extended the knowledge concerning the TCDD effects on genes involved in the TGFβ pathway and cell proliferation (Sadowska et al., 2017) in granulosa cells. The changes over-time in the present study in the granulosal transcriptome profile after initiation of TCDD treatment indicate there is involvement of stress related molecules in the cellular response to TCDD (Melekoglu et al., 2016) and TCDD effects on the ovulation process (Petroff et al., 2001). The applied multifactorial approach used in the present study allowed for the detection of the TCDD-induced changes in gene expression of granulosa cells of pig that were time-dependent and were especially pronounced during the first 12 h of TCDD treatment.

Declarations of interest

None

Availability of data and materials

The datasets analyzed during the current study are available in the NCBI BioProject database under accession number: PRJNA429720 (https://www.ncbi.nlm.nih.gov/bioproject/?term = PRJNA429720).

Authors' contributions

A.N., R.C., S.S. and L.P. contributed to the experimental concept; A.N., R.C., M.R., S.S., A.S. and K.O. participated in creating the experimental design; A.S., M.R., A.N. and K.O. performed cell culture experiments and molecular experiments; L.P. and J.J. performed bioinformatics analysis of the data and participated in discussion and interpretation of bioinformatics data; A.N. and R.C. analyzed the data, discussed the obtained results and wrote manuscript; M.R, A.S, K.O., S.S., K.M. and T.M. participated in the interpretation of the data; A.N., L.P., T.M., K.M. and J.J. worked out the figures and tables. All authors read and approved the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.06.007.

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