

Short thesis for the degree of doctor of philosophy (PhD)

**Comparative analysis of pathophysiological and
transcriptomic changes induced by estrogens in
human ovarian cell cultures**

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COMPARATIVE ANALYSIS OF PATHOPHYSIOLOGICAL AND TRANSCRIPTOMIC CHANGES INDUCED BY ESTROGENS IN HUMAN OVARIAN CELL CULTURES

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1. Introduction

Estrogens are steroid hormones that play prominent role in the regulation of the female reproductive cycle as well as other processes (e.g. nervous system, cardiovascular system, homeostasis). Therefore, in the postmenopausal years, decreased estrogen level is associated with the appearance of several age-related diseases (e.g. heart attack, memory loss). Estrogen-based hormone replacement therapy can successfully decrease these symptoms, however, it might increase the risk of cancer, such as ovarian cancer (Langer et al., 2021). The exposure to xenoestrogens – natural or synthetic molecules that mimic the effect of endogenous estrogens - might also promote the development of hormone-dependent tumors. Zearalenone (ZEA), is a mycotoxin produced by *Fusarium sp.* These filamentous fungi can infect grain (e.g. corn, oats, wheat, rice) on field or during storage thus the contamination of their metabolites in cereal products is high (Paterni et al., 2017). Bisphenol A (BPA) is a synthetic xenoestrogen that is used in the production of polycarbonate plastics and epoxy resins. BPA can leach from plastic items into foods and drinks that represents a relevant risk factor for human health (Urli et al., 2023). The carcinogenic effect of estrogens is related to their proliferative and migrative effect, which is mainly mediated by the Estrogen Receptor α (ER α). This receptor translocates to the nucleus after ligand binding and induces the transcription of several genes involved in estrogen response (Fuentes and Silveyra, 2019).

MicroRNAs (miRNAs) are considered to have prominent role in the regulation of tumor growth and metastasis formation. These are small (~18-22 nt), single-stranded, non-coding RNA molecules. They exert their effect through the post-transcriptional regulation of mRNAs. Binding to their target mRNA is led by the seed-sequence and results in the degradation of the mRNA or the inhibition of its translation (Ghini et al., 2018). Oncogenic miRNAs (oncomiRs) inhibit the formation of tumor suppressor proteins, while tumor suppressor miRNAs decrease the level of oncogenic proteins (Vishnoi

and Rani, 2016). The miRNA pattern of tumor cells differs from healthy cells that makes them promising biomarker candidates in cancer diagnostics. Furthermore, they can also be detected in body fluids that makes their application in liquid biopsy possible (Ho et al., 2022). On the other hand, increasing (e.g. via using synthetic miRNA mimics) or reducing (e.g. via using antimiR) their level in tumor cells represents a promising method in cancer therapy (Contiliani et al., 2021).

Ovarian cancer is the 7th most common cause of cancer-related death among women. The 5-year survival rate is only 47.7% that is mainly because of the late diagnosis – in stages III and IV - of the disease (Stewart et al., 2019). Rutin diagnostics mainly based on physical and imaging procedures (ultrasound, CT, MRI, PET-CT) that is supplemented with the determination of biomarkers CA125 and HE4. However, these biomarkers are not reliable enough in early stages and not suitable for screening (Doubeni et al., 2016). The application of cell free nucleic acids (e.g. circulating tumor DNA, mitochondrial DNA, miRNA) detected in body fluids might contribute to the better and faster diagnosis of ovarian cancer in the future (Szilágyi et al. 2020). MiRNAs might also support the successful therapy of ovarian cancer – that is mainly based on cisplatin, carboplatin, paclitaxel, docetaxel – since increasing tumor suppressor miRNAs or decreasing oncomiRs can decrease the growth and/or spread of tumors (Deb et al., 2018).

2. Aims

Although the role of estrogens is a well-known phenomenon in breast cancer, limited information is available about their importance in ovarian cancer. The contribution of xenoestrogens is highly understudied as well. The effect of estrogens to ovarian tumors include: i) estrogens have proliferative effect on ER+ ovarian tumor cells, ii) most of ovarian tumors express ER, iii) some ovarian tumor cells are capable of estrogen synthesis, iv) molecules that inhibit

estrogen signaling (e.g. tamoxifen) or synthesis (e.g. aromatase inhibitors) are successfully used in ovarian cancer therapy.

In our previous work, E2, ZEA and BPA in physiologically relevant doses increased the rate of cell proliferation and migration, as well as the expression of several genes and miRNAs in human ovarian cell lines, which effect depended on the presence of ER α . Here, our aim was to understand the effect of E2, ZEA and BPA on the human body in details. Thus, we performed a comparative study that included monitoring their pathophysiological effect to human ovarian cells as well as their influence to mRNA and miRNA expression. We performed the following experiments:

1. We made a comparative transcriptomic study including mRNA and miRNA expression induced by low, physiologically relevant doses of E2, ZEA and BPA by RNA sequencing.
2. We determined the effect of high dose E2, ZEA, or BPA treatments on ovarian cells, where we monitored the phenotypic response of ovarian cells (cell viability, apoptosis, cell lysis), as well as the expression of some key genes.
3. We determined the role of miR-30 family members, which have tumor suppressor function, in the estrogen response of ovarian cells. This included monitoring their expression during estrogen exposure and elucidating the role of miR-30d-5p in estrogen signaling.

3. Materials and Methods

3.1 Cell lines and cell culturing conditions

We used the PEO1 (expressing ER α ; Merck KGaA) and A2780 (not expressing ER α ; Department of Biophysics and Cell Biology, University of Debrecen) human ovarian cell lines in our experiments. These were maintained in RPMI1640 medium supplemented with 10 % (PEO1), or 5 % (A2780) FBS and 1% L-

glutamine, in a 37 °C thermostat, during 90% humidity and 5% CO₂ concentration. In the experiments, the cells were plated on 96- or 24 well plates and were cultured in the above-mentioned medium for 24 h. After this period the medium was changed to PRF-RPMI medium supplemented with 5% DCC-FBS and 1% L-glutamine. This decreased the interfering effect of phenol red present in RPMI and steroids present in conventional FBS in our experiments. Then, the cells were treated with E2, ZEA, or BPA in a final concentration of 10 nM-100 µM. In some experiments, the cells were also exposed to 10 nM MPP (methyl-piperidino-pyrazole), 100 nM AZD8835 and 1 µM tamoxifen. After the treatments, the cells were further incubated (24 h-48 h). Treated cultures were always compared to non-treated cultures.

3.2 Phenotypic studies

We examined the phenotypic response of estrogenic molecules by monitoring the rate of cell viability, apoptosis, and cell lysis. Cell viability was determined by MTT assay (Kumar et al., 2018). Apoptosis was determined by the DiIC1(5) assay that is based on the measurement of mitochondrial membrane potential (Markovics et al., 2019). The presence of cell lysis was detected by measuring lactate dehydrogenase (LDH) activity in the supernatant of cell cultures, using the CyQUANT LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific). Statistical analysis was made using untreated cells as controls by one-way ANOVA test (posthoc analysis: Dunnet test) (GraphPad Prism 7.0).

3.3 mRNA isolation and quantification

To determine gene expression, total RNA was isolated from the cells using the Quick-RNA MiniPrep Kit (Zymo Research), and cDNA transcripts were made using the Maxima first strand cDNA Synthesis kit (Thermo Fisher Scientific). The concentration of purified RNA and cDNA was measured by NanoDrop (Thermo Fisher Scientific). Gene expression was quantified by the Maxima SYBR

Green qPCR Master Mix kit (Thermo Fisher Scientific) using the LightCycler 96 (Roche). Fold changes were determined using the $2^{-\Delta\Delta C_t}$ formula, where the expression of *GAPDH* served as a reference. The degree of significance was determined by Student t-test, using the ΔC_t values (GraphPad Prism 7.0).

3.4 miRNA isolation and quantification

To determine miRNA expression, total RNA including small RNAs was isolated from the cells using the miRNeasy Kit (Qiagen), and cDNA transcripts were made using the miRCURY LNA RT Kit (Qiagen). The concentration of purified RNA and cDNA was measured by NanoDrop (Thermo Fisher Scientific). MiRNA expression was determined by the miRCURY LNA SYBR Green PCR Kit (Qiagen) with the miRCURY LNA PCR (Qiagen) primer assays using the LightCycler 96 (Roche) device. The relative expression values were determined using the $2^{-\Delta C_t}$ formula, where the expression of miR-103a-3p served as a reference. The degree of significance was determined by Student t-test, using the ΔC_t values (GraphPad Prism 7.0).

3.5 Transfection with miRNA mimics

In the functional analysis of miR-30d-5p, PEO1 and A2780 cells were transfected with miR-30d-5p mimic in 50 nM and 100 nM final concentrations using the miRIDIAN microRNA Mimic (Horizon) kit with the DharmaFECT 1 siRNA Transfection Reagent Kit (Horizon). The miRIDIAN microRNA Mimic Negative Control (Horizon) was used as a negative control.

3.6 Transcriptomic analysis by RNA sequencing

To determine the effect of E2, ZEA and BPA treatments on global mRNA and miRNA expression RNA sequencing was performed. Sequencing and the analysis of raw data was carried out by the Genomic Medicine and Bioinformatics Service Laboratory (Institute of Biochemistry and Molecular Biology, University of

Debrecen). Sequencing was performed via the Illumina NextSeq500 platform, and GRCh38.p13 was used as the reference genome. The StrandNGS software was applied for normalization with the DESeq algorithm and for the generation of FPKM values. Statistical analysis was made compared to the non-treated control samples using moderated T-test.

The bioinformatic analysis of data was based on the FPKM values, where the Log₂FC values were determined, heatmaps and MA plots were generated, and functional gene enrichment analysis was performed using the iDEP.96 software and GO_BP, KEGG, Reactome databases. Functional enrichment analysis of miRNA networks was performed by the miRNet software. Venn diagrams were created using BioVenn.

4. New scientific results

4.1 Comparative analysis of transcriptomic changes induced by E2, ZEA and BPA

In these experiments, we used the PEO1 cell line, that was treated with 10 nM E2, 10 nM ZEA, and 100 nM BPA. We detected significant response in the expression of *GREB1*, *CA12*, *DEPTOR* and *AGT* genes at these doses. Furthermore, the increase of cell proliferation and migration was also observed in our previous studies (Márton et al., 2020). During mRNA sequencing, 1847, 2019 and 901 genes responded to E2, ZEA and BPA treatment, respectively. Among these, the induction of 308, 288 and 63 genes (Log₂FC > 1), and the repression of 292, 260 and 45 genes (Log₂FC < -1) proved to be biologically relevant in response to E2, ZEA and BPA treatments, respectively. 83 genes responded to all the three treatments, that might play prominent role in the estrogen response of ovarian cells and might be promising diagnostic biomarker candidates in the determination of estrogen sensitivity of ovarian tumors in the future. It is important to mention that ZEA and E2 had comparative transcriptomic effect, which draws attention to the possibly relevant carcinogenic and

estrogen disrupting effect of ZEA. However, the effect of BPA proved to be lower, which might be caused by its lower affinity to ER α (Acconcia et al., 2015). To validate our results, we selected 17 genes, which expression was determined by qPCR. The correlation of Log₂FC values obtained from qPCR and RNA sequencing was high, the Pearson correlation coefficient was $r=0.95$, $r=0.96$ and $r=0.93$ in the case of E2, ZEA and BPA treatments, respectively.

According to the functional gene enrichment analysis pathways related to cell proliferation, amino acid- and ion transport, and RNA synthesis (non-coding, ribosomal and nuclear RNA metabolism) showed induction to estrogen treatments. This is in good agreement with our previous observation that E2, ZEA and BPA increased cell proliferation (Márton et al. 2020). The induction of ribosome biogenesis and amino acid transport might increase the protein synthetic capacity of cells that might support cell proliferation. In contrast, pathways related to the maintenance of epithelial phenotype (epithelial cell differentiation, epidermis formation) and cell adhesion (biological adhesion, cell adhesion) were repressed, which is also in good agreement with our previous observation that estrogens induced cell migration that might support metastasis formation (Márton et al. 2020).

In the case of miRNAs, the expression of 74, 47 and 73 miRNAs changed significantly in response to E2, ZEA and BPA treatments, respectively. Among these, the expression change of 13 (E2), 11 (ZEA) and 10 (BPA) miRNAs proved to be biologically relevant ($\text{Log}_2\text{FC} > 1$ or $\text{Log}_2\text{FC} < -1$). Bioinformatic analysis revealed extended networks between these miRNAs and their target mRNAs. Their functional enrichment analysis resulted in several pathways involved in the regulation of cell proliferation, cell metabolism, apoptosis, and migration. This suggests that these miRNAs might contribute to the development of cancer.

The expression of some miRNAs was also determined by qPCR. We confirmed the repression of miR-501-5p, let-7a-2-3p, miR-26a-2-3p and miR-197-5p in response to E2 treatment. However, the

repression of miR-582-3p was observed by qPCR - in contrast to our sequencing results. In response to ZEA treatment, the repression of miR-501-5p and miR-197-5p was observable by both qPCR and sequencing. We could not confirm the decrease of miR-197-5p expression in response to BPA exposure by qPCR.

4.2 Study the effect of high-dose estrogen treatments on ovarian cells

In order to get a broad picture about the effect of xenoestrogens to human cells we determined the response of ovarian cell cultures to high dose (1-100 μ M) E2, ZEA and BPA treatments. During phenotypic examination, the following parameters were monitored: (i) the degree of cell viability; (ii) the reduction of mitochondrial membrane potential, which is a marker of early apoptotic processes; and (iii) the occurrence of cell lysis, by measuring the LDH activity in the cell culture supernatant. In these experiments the PEO1 and A2780 (which does not express ER α) cell lines were applied. High-dose estrogen treatments reduced cell viability and induced cell death in both cell cultures, but the minimum inhibitory doses differed significantly. The PEO1 cell line tolerated more the high-dose E2 treatment, cell viability was decreased at 30 μ M, apoptosis was induced at 10 μ M and cell lysis was detected at 40 μ M. On the other hand, in the case of A2780, the decrease of cell viability was observed at 1 μ M E2, and intensive cell lysis was detected at 20 μ M dose. The effect of ZEA was highly comparable to the effect of E2. In the PEO1 cell line, cell viability was reduced, and apoptosis was induced at 30 μ M, and 40 μ M ZEA caused intensive cell lysis. In the case of the A2780 cell line, the decrease of cell viability was observable at 1 μ M dose of ZEA, and cell lysis was present at 30 μ M dose. BPA treatment proved to be less toxic in the case of the PEO1 cell line, the decrease of cell viability and the induction of cell lysis occurred only at 100 μ M BPA dose. A2780 tolerated worse the high-dose BPA treatments, the decrease of cell viability was observed at 1 μ M BPA. However, cell lysis was induced at 100 μ M BPA.

We selected some genes for expression analysis. Two of these were related to estrogen response (*GREB1*, *CA12*), others were involved in apoptosis (*TP53*) or autophagy (*ATG2B*, *ATG12*, *BAG3*). The induction of *GREB1* and *CA12* genes was observable in response to high dose estrogen exposure in the PEO1 cell line, which suggests the activation of ER α -mediated estrogen response in these circumstances. The dose-dependent induction of *TP53*, *ATG2B*, *ATG12* and *BAG3* genes supported the induction of apoptosis and autophagy, which were more prominent at higher doses of E2 and ZEA and did not occur in response to BPA treatment. The induction of *GREB1* and *CA12* genes were not observable in A2780, as expected. We detected here the induction of *ATG2B* and *BAG3* genes in response to E2 and ZEA exposure.

We hypothesized that the greater tolerance of the PEO1 cell line to the high-dose estrogen treatment might be - at least partly - related to the initiation of the ER α -mediated estrogen response, which might have contributed to the maintenance of cell proliferation. To prove this hypothesis, high-dose E2 treatment was also performed in the presence of 10 nM MPP (ER α specific antagonist) in the PEO1 cell line. In these experiments the induction of the *GREB1* and *CA12* genes was not observed, as expected. Furthermore, high dose E2 treatment proved to be more toxic to the PEO1 cell line in the presence of MPP: the decrease of cell viability was observed at 1 μ M E2, and cell lysis was present at 20 μ M E2 dose.

4.3 Study the role of miR-30s in the estrogen response of ovarian cells

The members of the miR-30 family are considered to be tumor suppressor miRNAs, and their function have been associated with estrogen response in other cell lines (Mao et al., 2018). Here we studied the role of miR-30a-3p, miR-30a-5p, miR-30d-5p and miR-30e-5p in the estrogen response of ovarian cells. All of these miRNAs had higher basal expression in the PEO1 cell line compared to A2780. This suggests that these miRNAs might have greater biological relevance in the ER α -expressing, estrogen-sensitive PEO1 ovarian

cell line. E2 treatment induced the expression of miR-30a-5p, miR-30d-5p and miR-30e-5p at 50 μ M and 100 μ M E2 doses in the PEO1 cell line, suggesting that these miRNAs might be important in the regulation of cell death triggered by high dose E2. Bioinformatic analysis of their targets revealed several pathways related to cell proliferation and cell death. We chose miR-30d-5p for further analysis.

Increasing the intracellular level of miR-30d-5p by miRNA mimic (50 nM and 100 nM) reduced cell viability in the PEO1 and A2780 cell lines. The expression of the previously investigated *GREB1*, *CA12*, *TP53*, *ATG2B*, *ATG12* and *BAG3* genes were also determined after mimic transfection. Only the expression of *TP53* was decreased in the transfected PEO1 cells that is in good agreement with the fact that *TP53* is a validated target of miR-30d-5p (Kumar et al., 2011). We also determined the expression of *SOX4* after transfection, which is considered to be another validated target of miR-30d-5p (Xu et al., 2021). The expression of *SOX4* was also decreased in the PEO1 cells.

In the next step we studied the effect of miR-30d-5p on the high-dose E2-induced cell death process of the PEO1 cell line. In these experiments PEO1 cells were transfected with miR-30d-5p mimic (50 nM) and were also exposed to high-dose E2 (1-100 μ M). According to our results miR-30d-5p mimic significantly reduced the tolerance of PEO1 cells. In these cultures, 1 μ M E2 dose decreased cell viability and intensive cell lysis was observed at 10 μ M E2. Furthermore, the ER α -mediated estrogen response was reduced, according to the lower induction of *GREB1* and *CA12* genes. Regarding the *SOX4* gene, this gene showed repression at 50 μ M and 100 μ M doses of E2 when E2 applied alone. When the cells were treated with both E2 and miR-30d-5p mimic, *SOX4* expression decreased at 10 μ M E2. According to previous studies miR-30d-5p affects the PI3K/Akt pathway through the regulation of *SOX4* expression (Xu et al., 2021). It is also important to mention, that the PI3K/Akt pathway is in close interaction with ER α signaling (Khatpe et al., 2021). We hypothesized

that miR-30d-5p could interfere with the ER α -mediated estrogen response via affecting the PI3K/Akt pathway, that resulted in the reduced tolerance of the transfected cultures. To prove this hypothesis, we also performed our experiments in the presence of AZD8835 that is a PI3K inhibitor. The application of AZD8835 (100 nM) had similar effect: cell viability was decreased at 1 μ M E2 dose, the induction of *GREB1* and *CA12* genes was reduced and the expression of *ATG2B* and *ATG12* genes increased. This raises the possibility of using miR-30d-5p mimic in the therapy of ovarian cancer alone or in combination with other agents. A promising candidate might be tamoxifen, that is an antiestrogen and is successfully applied in ER+ tumors (Paleari and DeCensi, 2018). In the last step, we examined the effect of such a combination therapy, we treated the PEO1 cells with a PI3k/Akt affecting molecule (miR-30d-5p or AZD8835) and tamoxifen. The application of tamoxifen (1 μ M), miR-30d-5p (50 nM), and AZD8835 (100 nM) alone reduced the proliferative effect of 10 nM E2 as well as the induction of *GREB1* and *CA12* genes. The combination of tamoxifen treatment with miR-30d-5p mimic or AZD8835 exerted a more significant inhibition on cell viability.

5. Summary

In this work, we investigated the role of E2, ZEA and BPA in the development of ovarian cancer using human ovarian cell lines. PEO1 (ER α +) cells were treated with low, physiologically relevant doses of E2 (10 nM), ZEA (10 nM) and BPA (100 nM) molecules and transcriptomic response was monitored by mRNA and miRNA sequencing. According to our analysis 308, 288 and 63 genes were induced ($\text{Log}_2\text{FC} > 1$) and 292, 260 and 45 genes were repressed ($\text{Log}_2\text{FC} < -1$) in response to E2, ZEA and BPA exposure, respectively. Furthermore, the expression of 13, 11 and 10 miRNAs altered significantly ($\text{Log}_2\text{FC} > 1$ or $\text{Log}_2\text{FC} < -1$). Functional gene enrichment analysis revealed the induction of several processes related to cell proliferation and migration that is in good agreement with our previous phenotypic results. The effect of ZEA was

comparable to E2, the expression of 407 genes altered in response to these 2 treatments. We also identified 83 genes that showed altered gene expression as a result of all the three treatments. Their expression analysis might be an applicable tool for determining the estrogen sensitivity of ovarian tumors. The downregulation of miR-501-5p, let-7a-2-3p, let-7g-3p, miR-26a-2-3p, miR-197-5p and miR-582-3p was confirmed by qPCR experiments.

In the next phase of our work, we studied the effect of high-dose (1-100 μ M) E2, ZEA and BPA treatments on the phenotype of PEO1 (ER α +) and A2780 (ER α -) ovarian cell lines. E2 and ZEA exposure decreased cell viability and induced cell death in the PEO1 cell line in 30 μ M dose, which effect was observable in 100 μ M dose in the case of BPA. According to the induction of *GREB1* and *CA12* genes, E2 and ZEA were also able to initiate ER α -mediated estrogen response in 1-10 μ M doses. Higher doses induced cell death instead where the induction of *TP53*, *ATG2B*, *ATG12* and *BAG3* genes might have played important role. In the absence of the ER α -mediated estrogen response, as in A2780, or when it was blocked by MPP (selective ER α antagonist) in PEO1, the inhibition of cell viability and induction of cell death was observed in lower, 1 μ M dose. Furthermore, the inhibition of the PI3K/Akt pathway by AZD8835 (PI3K inhibitor) or by miR-30d-5p mimic (which affects PI3K/Akt by targeting *SOX4*) sensitized PEO1 cells to cell death induced by high-dose E2 as well as to tamoxifen via interfering with ER α -mediated signaling. This suggests the application of PI3K/Akt inhibitors and estrogen response blockers in combination in the future therapy of ovarian cancer.

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8. List of publications



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PhD Publication List

Candidate: Alexandra Edit Beke-Varga

Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. Márton, É., **Beke-Varga, A. E.**, Penyige, A., Hádáné Birkó, Z., Balogh, I., Nagy, B., Szilágyi, M.:
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DOI: <http://dx.doi.org/10.3390/toxins15020140>
IF: 3.9
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Total IF of journals (all publications): 17,362

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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