Chemosensitivity and chemoresistance in endometriosis – differences for ectopic *versus* eutopic cells

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ABSTRACT

RESEARCH QUESTION

Endometriosis is a common gynecological disease defined by the presence of endometrium-like tissue outside uterus. This complex disease, often accompanied by severe pain and infertility, causes significant medical and socioeconomic burden; hence, novel strategies are sought for treatment of endometriosis. Here, we set out to explore cytotoxic effects of a panel of compounds to find toxins with different efficiency in eutopic *versus* ectopic cells, thus highlighting alterations in the corresponding molecular pathways.

DESIGN

Effect of 14 compounds on cellular viability was established in a cohort of paired eutopic and ectopic endometrial stromal cell samples from 11 patients. The biological targets covered by the panel included pro-survival enzymes, cytoskeleton proteins, proteasome, and cell repair machinery.

RESULTS

We showed that protein kinase inhibitors GSK690693, ARC-775, and sorafenib, proteasome inhibitor bortezomib, and microtubule-depolymerizing toxin MMAE, were more effective in eutopic cells. In contrast, 10 μM anthracycline toxin doxorubicin caused cellular death in ectopic cells more effectively than in eutopic cells. The large-scale sequencing of mRNA isolated from doxorubicin-treated and control cells indicated different survival strategies in eutopic *versus* ectopic endometrium.

CONCLUSIONS

Overall, our results confirm the evidence of large-scale metabolic reprogramming in endometriotic cells, which underlies the observed differences in sensitivity towards toxins. The enhanced efficiency of doxorubicin interfering with redox equilibria and/or DNA repair mechanisms pinpoints key players that can be potentially used for selective targeting of ectopic lesions in endometriosis.

INTRODUCTION

Endometriosis is an inflammatory gynaecological disease that manifests itself as a growth of endometrial stromal and epithelial cells in extra-uterine sites. The frequency of endometriosis is estimated to be 2-10% of women in their reproductive years and as there are still no effective non-surgical treatments, it has a considerable impact on the life quality of the affected women (Nnoaham et al., 2011). Endometriosis-associated symptoms such as severe pelvic pain, infertility and impaired psychological and social functioning cause socioeconomic burden because of productivity loss; furthermore, the risk to develop ovarian cancer is moderately increased in women suffering from endometriosis, being about 1.9% compared with 1.4% in the general population (Vercellini et al., 2018). Therefore, the new possibilities to treat endometriosis are actively explored.

To find potent endometriosis treatment strategies, the mechanisms behind the disease initiation should be understood. The formation of endometriotic lesions presupposes ability of endometrial cells to attach on the peritoneal surfaces, establish neo-angiogenesis, and resist apoptosis (Nasu et al., 2009). Characteristics such as high degree of inflammation, excess of iron, and increase in reactive oxygen species (ROS[i]) have also been described in endometriotic lesions (Defrere et al., 2008; Lousse et al., 2012; Scutiero et al., 2017). Furthermore, our comprehensive proteomic study has shown that extensive metabolic reprogramming (associated with downregulation of oxidative respiration), and upregulation of adhesiveness- and motility-involved proteins occur in endometriotic stromal cells (Kasvandik et al., 2016), emphasizing the similarities between endometriotic and cancer cells. Therefore, toxins affecting various molecular pathways in cancer chemotherapy could find an alternate application for research – and, potentially, therapy – of endometriosis. Some of such compounds have been briefly explored in the context of endometriosis (Celik et al., 2008), yet we are not aware of studies with focused panel of toxins that would systematically compare effect of compounds in eutopic and ectopic cells from endometriosis patients.

Here, we report quantification of cytotoxic effect of 14 compounds (Table 1) in a cohort of paired eutopic and ectopic endometrial stromal cell (euESCs and ecESCs, respectively) samples from 11 patients. The biological targets covered by this panel included pro-survival enzymes, cytoskeleton proteins, proteasome, and cell repair machinery. The rationale behind the choice of compounds took into consideration high affinity and well-defined selectivity profile of inhibitors in biochemical studies, and their applicability in cellular assays. Our goal was to find compounds demonstrating different efficiency in eutopic *versus* ectopic cells from peritoneal lesions, thus highlighting alterations in the corresponding molecular pathways, and pinpoint compounds that preferentially affect ectopic cells, thus paving the way for the possible therapeutic strategies in future.

MATERIALS AND METHODS

Chemicals and equipment

Protein kinase (PK) inhibitors were obtained from the following sources: SGI-1776 – Axon Medchem (Groningen, Netherlands); H89 – Biaffin (Kassel, Germany); sorafenib, Y-27632, HA-1077 – Cayman Chemicals (Ann Arbor, MI, USA); staurosporine – Cell Guidance Systems (Cambridge, UK); VX-689, CYC116 – Selleckchem (Houston, TX, USA); bortezomib, monomethyl auristatin E (MMAE), doxorubicin – TBD Biodiscovery (Tartu, Estonia); and GSK690693 – Tocris (Bristol, UK). ARC-775 and ARC-1859 were kindly gifted by Dr Asko Uri (University of Tartu, Tartu, Estonia). The stock solutions of compounds (5-10 mM in DMSO) were stored at -20 °C. SYTOX™ Blue Nucleic Acid Stain and NP40 lysis buffer were from Thermo Fischer Scientific (Rockford, IL, USA); cell culture grade DMSO was from AppliChem (Darmstadt, Germany); resazurin, BSA and PBS (supplemented with Ca^{2+}, Mg^{2+}; used for biochemical assays and Western blot) were from Sigma-Aldrich (St Louis, MO, USA). Other solutions, reagents and materials for SDS PAGE and Western blot were from Thermo Fischer Scientific (Carlsbad, CA, USA).

For necrosis/late apoptosis and viability assay, the initial number of cells was counted using TC-10 cell counter (Bio-Rad; Hercules, CA, USA), and the cells were seeded onto transparent 96-well clear flat bottom cell culture plates (BioLite 130188, Thermo Fischer Scientific; Rochester, NY, USA).

Fluorescence intensity and absorbance measurements were carried out using Synergy NEO, Cytation 5 (both from Biotek; Winooski, VT, USA) and PHERAstar (BMG Labtech; Ortenberg, Germany) multi-mode readers.

Patient characteristics and sample collection

The study was approved by the Research Ethics Committee of the University of Tartu (approval 276/M-13) on 18th December 2017 and informed written consent was obtained from the participants. Endometrial tissue samples and peritoneal endometriotic lesions were collected from 11 endometriosis patients (see Table 2) undergoing laparoscopy at the Tartu University Hospital Women's Clinic. Tissue samples were immediately placed into the cryopreservation medium and processed as described previously (Rekker et al., 2017). At least one endometriotic lesion sample from each patient was placed into formalin and the diagnosis was confirmed by histopathological examination of specimens. The disease severity was determined according to the American Society for Reproductive Medicine revised classification system (American Society for Reproductive Medicine, 1997). Only women who had not received any hormonal medications at least three months before surgery were enrolled in this study.

Isolation and culturing of cells

Endometriotic and endometrial tissues were treated according to the previously published protocol (Kasvandik et al., 2016). Briefly, the tissue was washed twice in 7 mL of fresh medium (1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12; Sigma-Aldrich, Steinheim, Germany) to remove any debris or excess blood cells. The biopsies were dissociated in 5 mL of DMEM (without phenol red) containing 0.5% collagenase (Sigma-Aldrich) in shaking incubator rotating at 110 rpm at 37 °C until the biopsies were digested (but not longer than 1 h). The dispersed cells were filtered through a 50 µm nylon mesh to remove undigested tissue pieces. Then, the cells were resuspended in 10 mL of culture medium in a 15 mL tube; sealed tubes were placed in an upright position for 10 min to sediment epithelial glands. The top 8 mL of medium containing stromal cells was then collected and

the tube was refilled to 10 mL with fresh medium; the sedimentation process was repeated three times and the collected fractions were pooled. The final purification of stromal cells was achieved by selective adherence of stromal cells to culture dishes for 20-30 min at 37 °C in 5% CO_2 incubator. Non-adhering epithelial cells were removed by washing the cell layer twice with 5 mL of culture medium.

The isolated ESCs were further cultured for 5-6 passages in DMEM /Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; Capricorn, Ebsdorfergrund, Germany) and a mixture of penicillin, streptomycin, and amphotericin B (Capricorn, Ebsdorfergrund, Germany) at 37 °C in 5% CO_2 incubator.

Necrosis/late apoptosis assay

euESCs and ecESCs (passage number 5-6) were seeded onto 96-well plate with the density of 4,000-6,000 cells per well in DMEM/Ham's F12 medium supplemented with FBS; euESCs and ecESCs from the same patient were thawed on the same day, and two plates were prepared for both eutopic and ectopic stromal cells. After incubation of cells for 24 hours at 37 °C in 5% CO_2 humidified incubator, medium was exchanged, and dilution series of compounds in PBS were added (Table 1); the final volume per well was 110 μL, and the concentration of DMSO in the treated wells was equal to or below 0.1% by volume. On each plate, each concentration of each compound was represented in duplicate; the controls (10% DMSO and 0.1% DMSO) were represented in sextuplicate. The cells were incubated with compounds for 22 hours at 37 °C in 5% CO_2 humidified incubator; next, the medium was removed and 1 μM Sytox Blue solution in PBS (containing Ca^{2+} and Mg^{2+}) was added. The plates were placed into multi-mode reader, incubated for 10 min at 37 °C, and fluorescence intensity was measured (excitation 430 nm, emission 480 nm, monochromator, top optics, gain 90; area scan mode 5×5, read height 2.5 mm, with lid).

Viability assay

Viability assay was performed directly after the necrosis/late apoptosis assay with the same plates. The solution of Sytox Blue was replaced with 50 μM resazurin solution in PBS (containing Ca^{2+} and

Mg^{2+}). The plates were placed into multi-mode reader, and measurement of absorbance was performed (570 nm and 600 nm, monochromator; kinetic mode with reading taken every 15 min for 2 hours, read height 8.5 mm, with lid). Next, resazurin solution was replaced with fresh sterile DMEM/Ham's F12 medium supplemented with FBS, and the cells were incubated for 24 hours at 37 °C in 5% CO_2 humidified incubator. Finally, viability assay was performed again (without the preceding necrosis/late apoptosis assay). In a pilot experiment, we confirmed that the first application of resazurin for 2 h in PBS did not cause severe cytotoxicity (data not shown).

Western blot

In case of Western blot assay, one 6-well plate was prepared for euESCs and one plate for ecESCs (passage number 5-6). When the confluency of cells was 50% or higher, dilutions of doxorubicin in PBS or DMSO in PBS (control) were added. The final volume per well was 2 mL; the final concentration of doxorubicin was 10 μM, and the final concentration of DMSO was 0.1%. On each plate, both doxorubicin and control incubations were represented in duplicate. The cells were incubated for 48 hours at 37 °C in 5% CO_2 humidified incubator.

After collection and lysis of cells on ice, the samples for SDS PAGE were prepared by adding NuPAGE sample loading buffer to supernatants and heating at 70 °C for 15 min. SDS-PAGE was performed on 10% Bis-Tris gels or 4-12% Bis-Tris gradient gel in MES buffer; samples of treated and non-treated euESCs and ecESCs from the same patients were applied on the different lanes of the same gel. Semidry transfer followed at 15 V for 60 min using methanol-activated PVDF membrane and NuPAGE transfer buffer. The membrane was then stained with primary antibody (1,000× dilution of rabbit anti-procaspase-3, #9662 Cell Signaling, RRID: AB_331439) and secondary antibody (5,000× dilution of goat anti-rabbit conjugated to alkaline phosphatase, T2191 Thermo Fischer Scientific, RRID: AB_11180336) according to the manufacturers' instructions. The same procedure was used for the subsequent staining of the same membrane with mouse anti-β-actin (4,000× dilution, A1978 Sigma-Aldrich, RRID:

AB_476692) and goat anti-mouse conjugated to alkaline phosphatase (5,000× dilution, T2192 Thermo Fischer Scientific, RRID: AB_11180852).

mRNA isolation and large-scale sequencing

euESCs (n=3) and ecESCs (n=3) were isolated and grown as described under sections Isolation and culturing of cells and Western blot, respectively; the cells were isolated from the paired eutopic and ectopic samples that were included in Western blot studies. After 24 h incubation of cells with final concentration of 2 μM doxorubicin or 0.1% DMSO (as a negative control) in growth medium, the medium was removed, the cells were rinsed with PBS and RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNase I treatment was performed using DNA-free DNA removal kit (Invitrogen). 2200 TapeStation system in conjunction with RNA ScreenTape (Agilent Technologies, Palo Alto, CA, USA) was used to determine the quality and quantity of purified RNA. For sequencing library construction, RNA from two technical replicates was pooled together. cDNA was synthesised as described previously (Teder et al., 2018), converted to next-generation sequencing library using Nextera XT Library Prep kit (Illumina, San Diego, CA, USA) and sequenced with NextSeq 500 high output 75 cycles kit (Illumina).

Quantitative real-time PCR (qRT-PCR)

The expression levels of selected genes (*HSPA2*, *PTGS2* and *PTN*) were validated by qRT-PCR using RNA from two technical replicates. cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), and real-time PCR was performed using 1× HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia). The primer sequences used were following: *HSPA2* (F: CTCCACTCGTATCCCCAAGA, R: GTCACGTCGAGTAGCAGCAG), *PTGS2* (F: CCACTTCAAGGGATTTTGGA, R: GAGAAGGCTTCCCAGCTTTT), and *PTN* (F: CAATGCCGAATGCCAGAAGACTGT, R: TCCACAGGTGACATCTTTTAATCC). As a reference gene, *ACTB* (F: TCAAGATCATTGCTCCTCC and R: ACATCTGCTGGAAGGTGGA) was used.

Statistical analysis

Data is available on request from the authors.

In case of necrosis/late apoptosis assay, the mean Sytox Blue fluorescence intensity per well was calculated; the data corresponding to the same concentration of the same compound was pooled and normalized for each plate. For normalization, signal obtained for incubation with 5 μM staurosporine was considered as 100% necrosis, and signal obtained for incubation with 0.1% DMSO as 0% necrosis.

In case of viability assay, ratio of absorbance at 570 nm and 600 nm was calculated for each well. The data obtained from one plate for the control incubations with 0.1% DMSO or 10% DMSO were pooled and plotted against time, and the linear range of the assay was established. The data corresponding to the same concentration of the same compound were pooled and normalized for each plate. For normalization, data obtained for incubation with 10% DMSO were considered as 0% viability, and data obtained for incubation with 0.1% DMSO as 100% viability.

For Western blot data analysis, the membrane was dried and scanned in. The area of bands detected with anti-procaspase-3 and anti-β-actin was assessed using ImageJ 1.51j8 software, and the ratio of two values was calculated for each lane; the data were pooled for the lanes where the identically treated samples of the same cells were applied. Next, data for lanes with samples from euESCs and ecESCs were normalized separately. For normalization, ratio obtained for incubation with 0.1% DMSO was considered as 100% to obtain results for one patient; the bottom plateau was fixed at 0%.

In case of qRT-PCR, the average values of technical replicates were used. The fold change (FC) was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

For the final comparison, results of all patients were pooled. For necrosis/late apoptosis and viability assays, the statistical significance of difference between the inhibitor/toxin-treated cells *versus* cells treated with 0.1% DMSO was established by the ordinary 1-way ANOVA using Dunnett correction for multiple comparisons. For necrosis/late apoptosis and viability assays as well as Western blot, the

statistical significance of difference between euESCs *versus* ecESCs was established by the unpaired t-test with Welch's correction. For qRT-PCR data, the statistical significance of difference between control *versus* doxorubicin treatment was established by the paired t-test, and the statistical significance of difference between euESCs *versus* ecESCs was established by the unpaired t-test. The aforementioned statistical analysis was carried out using GraphPad Prism 6.

The large-scale mRNA sequencing data was acquired from Illumina BaseSpace. The reads were quantified using Salmon 0.9.1 in quasi-mapping mode using indexed Ensemble v95 annotation. The quality control of raw sequencing data and statistics on aligned counts was performed with FastQC 0.11.5 and MultiQC 1.7. Based on QC, further data transformation was performed by trimming adapter size with Trimmomatic 0.38. Quantified transcript read counts were summarized to genes using Bioconductor packages tximport 1.10.1 and BioMart 2.38.0. Overall, 175,775 transcripts were identified from all the samples, out of which 28,796 genes with non-zero total counts were summarized. Differential RNA-seq analysis and ranking was performed with DESeq2 1.22.2. In parallel, edgeR 3.24.3 was used for comparison.

The shortlist of genes with significantly different expression in pairwise compared cell types and treatment conditions (control euESCs *vs* control ecESCs; control euESCs *vs* toxin-treated euESCs; control ecESCs *vs* toxin-treated ecESCs; and toxin-treated euESCs *vs* toxin-treated ecESCs) was generated as follows. The data for expression of each gene obtained in the same cell type and condition were averaged for 3 patients, and the binary logarithm of fold change of averages ($\log_2$FC) was found. For each pairwise comparison, the latter values were ranked and cut-off values of $\log_2 FC \leq -4$ or $\log_2 FC \geq +4$ were applied. The genes showing high variance in expression (for the same cell type and condition between different patients), or the genes for which number of counts was below 10 in all conditions were eliminated. Finally, following the individual check of the remaining candidates using the GeneCards human gene database (Weizmann Institute of Science, 2019) and g:Profiler

source (Reimand et al., 2016), the pseudogenes and the genes encoding poorly characterized proteins were excluded from the list.

RESULTS

Viability assay

To establish effect of compounds (Table 1) on viability of euESCs and ecESCs, we utilized assay that measures change in absorbance spectrum of a cell membrane-penetrating dye resazurin upon its reduction in metabolically active cells. Table 3 summarizes the results of viability assay where statistically significant reduction of viability ($P \leq 0.01$) after 22 h incubation of cells with studied compounds and after additional 24 h incubation in growth medium was observed; the full versions of the tables are presented in the Supplementary Tables S1 and S2.

Expectedly, the lowest viability after 22 h treatment was observed in case of both euESCs and ecESCs treated with a well-known apoptosis inducer staurosporine. The pan-inhibitor of PIM PKs, SGI-1776 caused significant drop of viability at 10 μM concentration in both euESCs and ecESCs ($P \leq 0.001$); it was also the only compound in the panel demonstrating large patient-dependent effect: out of 11 patients' samples, low viability of cells was evident in samples of 3 patients, whereas samples of 4 patients were practically insensitive (see supplementary Figure S1A). Other inhibitors of PKs did not cause extended amount of cell death in either euESCs or ecESCs (viability of cells remained at 75% or more relative to 0.1% DMSO control). Interestingly, after 22 h incubation of cells with ROCK inhibitor HA-1077, apparent increase of viability was observed in both in euESCs and ecESCs (*i.e.*, cells treated with 10 μM inhibitor had higher levels of resazurin reduction than cells treated with 0.1% DMSO). Similar phenomenon was evident in both in euESCs and ecESCs upon treatment with different concentrations of VX-689, and in ecESCs upon treatment with 10 μM or 2 μM ARC-1859 (see supplementary Table S1). Chemotherapeutic drugs bortezomib and MMAE were more efficient in eutopic cells, although significant drop of viability was observed in both euESCs and ecESCs ($P \leq 0.001$).

On the contrary, treatment with 10 μM and 2 μM doxorubicin was more efficient in ecESCs than in euESCs, showing similar effect across the patients (see supplementary Figure S1A).

The measurement of cell viability after subsequent 24 h incubation in growth medium demonstrated that viability of most toxin-treated euESCs and ecESCs had decreased further, whereas differences between euESCs and ecESCs became smaller (Table 3). In addition, significant decrease of viability was now observed for cells treated with MAPK pathway inhibitor sorafenib ($P \leq 0.05$), PKAc inhibitor H-89 ($P \leq 0.01$), and AURORA A inhibitor VX-689 ($P \leq 0.01$; see the full version of the table presented in the Supplementary Table S2). While sorafenib and H-89 were slightly more active in euESCs, the effect of VX-689 was more pronounced in ectopic cells. Notably, after prolonged incubation, 10 μM doxorubicin still affected ecESCs more than euESCs. The increased sensitivity of ecESCs towards high concentrations of doxorubicin was confirmed in the repeated assay with samples representing 4 patients from the same cohort (see supplementary Figure S2).

All in all, based on the results of viability assay, characteristic differences of the viability fingerprint between euESCs and ecESCs could be formulated (Figure 1).

Necrosis/late apoptosis assay

To confirm the trends observed in viability studies, we applied an additional assay by using cell membrane-impermeable Sytox Blue dye after 22 h incubation of euESCs and ecESCs with the compounds. The increase in fluorescence of Sytox Blue resulting from intercalation of dye into DNA is only possible in cells with compromised membrane structure, thus indicating elevated extent of necrosis/late apoptosis.

The results of the assay are presented in the Supplementary Table S3. The highest effect in euESCs as well as in ecESCs was observed for 5 μM staurosporine, a generic PK inhibitor, which was hence chosen as the standardizing condition setting the maximal threshold for the normalization of data. ecESCs seemed overall less prone to necrosis/late apoptosis than euESCs; however, high levels of cell death

in both euESCs and ecESCs were also observed upon treatment with 10 μM SGI-1776 (targets PIM family PKs) and 10 μM ARC-775 (targets CK2). The AKT/PKB inhibitor GSK-690693 at 10 μM concentration induced more necrosis/late apoptosis in eutopic cells; furthermore, toxins bortezomib and MMAE were more effective in euESCs *versus* ecESCs at all concentrations. Other compounds showed no effect even at the highest concentrations used (5-10 μM).

The data for doxorubicin were not included as in this case, we observed a characteristic drop of Sytox Blue signal below the value observed for the negative control (cells treated with 0.1% DMSO), which occurred in both euESCs and ecESCs from all patients. We propose that such behaviour is related to the mode of action of doxorubicin, which intercalates into DNA; in this way, doxorubicin competes with Sytox Blue for the binding sites, and necrosis or apoptosis assays based on dyes that gain fluorescence upon binding to DNA are incompatible with doxorubicin studies.

Western blot

To gain further independent evidence considering elevated efficiency of doxorubicin in ecESCs *versus* euESCs, we proceeded with an alternative assay. Due to strong autofluorescence of doxorubicin (Wang et al., 2016), most of the 'classical' techniques such as imaging or FACS utilizing immunostaining or BrdU detection can be highly prone to artefacts; therefore, we choose Western blot to quantify reduction of procaspase-3 levels in doxorubicin-treated samples of euESCs and ecESCs from 4 patients (same samples as used for the repeated viability assay, see above). The ratio of signals corresponding to procaspase-3 and β-actin was quantified for each treatment condition; the data were normalized separately for euESCs and ecESCs of each patient according to the corresponding negative control (0.1% DMSO; Figure 2A).

The results confirmed that 48 h treatment with 10 μM doxorubicin caused statistically significant ($P \leq 0.05$) difference of apoptosis in ectopic *versus* eutopic cells, with normalized procaspase-3 content reduced to 39(±8) % in ecESCs and 60(±4) % in euESCs relative to the corresponding negative controls (0.1% DMSO) (Figure 2B).

mRNA sequencing

Finally, to obtain detailed insight into signalling pathways affected by doxorubicin in euESCs and ecESCs, we performed large-scale mRNA sequencing after 24 h incubation of cells from 3 patients with 2 μM doxorubicin or 0.1% DMSO control. The concentration of doxorubicin was chosen based on results of viability assay, in order to see significant difference between euESCs and ecESCs, yet yield sufficient population of surviving cells for mRNA isolation.

The comparison of treated *versus* control cells yielded 4,009 significantly differentially expressed genes in case of euESCs, yet only 249 significantly differentially expressed genes in case of ecESCs (if base mean cut-off value of > 10 and P_{adj} cut-off value of < 0.05 are defined). For shortlisting genes that featured significantly different expression in different cells and treatment conditions (see Table 4), we sorted the sequencing data as described under section Statistical analysis. Overall, we found that several genes which were higher expressed in control euESCs relative to control ecESCs (*i.e.*, *MMP1/3/10*, *PENK*, *PTN*, *GRP*) or in control ecESCs relative to control euESCs (*i.e.*, *ESM1*, *IL33*, *PTX3*), were also higher expressed in the same cell type following treatment with doxorubicin. Furthermore, treatment with doxorubicin resulted in reduction of expression of several genes in euESCs (*e.g.*, *DUSP1/10*, *BARD1*) as well as in ecESCs (*e.g.*, *DKK1*, *HAS2*) relative to the control cells of the same type. On the other hand, while in euESCs expression of some genes (such as histone cluster 1 *H2A*/*H2B* family members) increased upon treatment with doxorubicin relative to control cells, we did not observe significant ($P_{adj} < 0.05$, $\log_2FC \leq -4$) increase of gene expression in toxin-treated ecESCs relative to the control treatment.

For technical validation of the results of large-scale mRNA sequencing, we carried out qRT-PCR analysis of *PTN* and *HSPA2* as examples of genes considerably highly expressed in eutopic cells, with PTN expression elevated in both control and toxin-treated euESCs relative to the correspondingly treated ecESCs (Table 4). In addition, we decided to validate the expression of *PTGS2*, which according to large-scale mRNA sequencing data possessed higher expression in ectopic relative to eutopic cells after

337 doxorubicin treatment, yet the statistical significance of this difference was slightly higher than the
338 classical cut-off P_{adj} value of 0.05 (supplementary Table S4).

339 The qRT-PCR confirmed the general trends observed in large-scale transcriptomic analysis, indicating
340 significantly higher expression of *PTN* in both control and doxorubicin-treated euESCs *vs*
341 corresponding ecESCs (both $P < 0.05$), and significantly higher expression of *HSPA2* in control euESCs
342 *vs* ecESCs ($P = 0.05$). In addition, doxorubicin treatment elevated the level of *PTN* and *HSPA2* in eutopic
343 and ectopic stromal cells, respectively (both $P < 0.05$). Furthermore, qRT-PCR showed significantly
344 higher expression of *PTGS2* in control ecESCs *vs* euESCs as well as doxorubicin-treated ecESCs *vs*
345 euESCs (both $P < 0.05$), confirming that *PTGS2* can indeed serve as an important target in
346 endometriosis.

347 **DISCUSSION**

348 While the molecular players behind onset and progression of endometriosis are still unclear, several
349 pathways have been closely inspected, with the special focus on inflammation processes, cell
350 migration and adhesion, abnormal proliferation and resistance to apoptosis. Here, we explored the
351 differences in cell viability of euESCs and ecESCs upon treatment with selective compounds inhibiting
352 a focused number of molecular players, as well as compounds with wide profile of biological targets.
353 Methodologically, there are two major limitations in our study: first, we focussed our attention on
354 stromal cells only, yet in the physiological milieu epithelial cells are present that may be involved in
355 the unique patterns of signalling and cellular interactions. Second, as we investigated only ESCs
356 isolated from the superficial peritoneal lesions, the observed results may not necessarily reflect the
357 effects of toxins in other types of lesions.

358 Phosphorylation of proteins serves an example of signalling mechanism that on one hand is
359 ubiquitous, yet can be dissected with high degree of precision by selective targeting of the catalysing
360 machinery – protein kinases. The human kinome includes 538 PKs, most of which have been termed
361 as potentially druggable by virtue of incorporation of a narrow solvent-hidden pocket (ATP-binding

site) that can be selectively targeted by small-molecular weight inhibitors. The panel that we utilized for screening included 11 inhibitors of PKs, 10 of which possessed focused selectivity profiles, while staurosporine was selected as a widely used apoptosis inducer (see Table 1 and supplementary Figure S3). Among PKs targeted by the selective inhibitors were enzymes for which upregulation in endometriotic cells has been reported: MAPKs (Ngô et al., 2010; Yotova et al., 2011), AKT/PKB (Cinar et al., 2009; Shoji et al., 2009), PIM1 (Hu et al., 2006; Jiménez-García et al., 2017), and CK2 (Feng et al., 2012; Llobet et al., 2008). In our study, inhibitors of MAPK (sorafenib), AKT/PKB (GSK690693) and CK2 (ARC-775) were more effective in euESCs than ecESCs, whereas PIM inhibitor (SGI-1776) showed cell type-independent effect: in patients where euESCs were affected, ecESCs were also affected (see supplementary Figure S1B and C). Overall, while overexpression of certain pro-survival PKs in cancer cells can lead to degeneration of other anti-apoptotic pathways and establishment of the so-called oncogene addiction (Ruzzene and Pinna, 2010; Sharma and Settleman, 2007), it does not seem to be the case for ectopic endometriotic cells.

Surprisingly, CK2 inhibitor ARC-1859, despite featuring structural design highly similar to ARC-775, did not reduce viability of cells. While in biochemical assays with recombinant CK2, the affinity of unmasked counterpart of ARC-775 was indeed higher than that of unmasked counterpart of ARC-1859 (Rahnel et al., 2017; Viht et al., 2015), it is hardly the only reason underlying lack of potency of ARC-1859 in assays with endometrial stromal cells. It is rather likely that a more hydrophobic ATP-site targeting fragment of ARC-1859 (tetrabromobenzimidazole moiety) contributes to accumulation of inhibitor in membranes, where it is not accessible for either esterases or the cytosolic CK2.

The effect of some compounds included in our panel had been previously explored in the context of endometriosis. A generic PK inhibitor staurosporine has been reported to demonstrate higher apoptotic effect in euESCs of patients without endometriosis than in ecESCs of patients with endometriosis (Watanabe et al., 2009). In our study, the sensitivity of eutopic *versus* ectopic cells to staurosporine depended on its concentration: while 5 μM staurosporine caused more cellular death

387 in ecESCs, 0.2 μM staurosporine was more effective in euESCs (Table 3). A proteasome-targeting
388 compound bortezomib had been shown to reduce size of endometriotic implants in rats (Celik et al.,
389 2008), yet no studies of bortezomib in euESCs of endometriosis patients have been reported; in our
390 study, treatment with bortezomib was significantly more efficient in euESCs than in ecESCs even after
391 prolonged incubation ($P \leq 0.001$).

392 The ROCK-targeting inhibitors Y-2763 and HA-1077 had been used for reduction of contractility of
393 ecESCs; while Y-27632 had demonstrated no cytotoxicity, 0.1-10 μM HA-1077 had caused significant
394 apoptosis of ecESCs – albeit after 48 h incubation (Yotova et al., 2011; Yuge et al., 2007). In our study,
395 even after prolonged incubation of euESCs and ecESCs with either Y-27632 or HA-1077, no reduction
396 in viability was observed. In principle, it is possible that the effect of ROCK-targeting inhibitors is only
397 evident in cell motility assay, although we had hoped that altered dynamics of cytoskeleton might
398 manifest itself as retarded proliferation. The latter was true for microtubule-depolymerizing
399 compound MMAE, which showed a characteristic concentration-independent profile of effect on cell
400 viability connected to the mode of action of this compound, which serves rather as anti-mitotic agent
401 than apoptosis inducer (Abdollahpour-Alitappeh et al., 2017; Chen et al., 2017).

402 Furthermore, 22 h treatment of cells with some of the chosen compounds (including inhibitors
403 targeting ROCK, AURORA family kinases, or PKAc) caused an apparent increase of viability (see
404 supplementary Table S1), which was alleviated after subsequent 24 h incubation in medium. Such
405 abnormal temporary phenomenon might be triggered by several factors. On one hand, ROCK
406 inhibitors can interfere with the apoptotic Caspase 3-ROCK signalling pathway (Song and Gao, 2011),
407 and, consequently, increase the number of viable cells. However, a more likely explanation is that as
408 a response to treatment with toxins within certain time-window, cells tend to increase metabolism,
409 which manifests itself as enhanced reduction of resazurin.

410 Overall, the compounds that significantly affected viability of cells after 22 h of treatment also caused
411 significant amount of cellular death according to the necrosis/late apoptosis assay (as illustrated by

GSK690693, ARC-775, SGI-1776, staurosporine, bortezomib, MMAE; $P \leq 0.05$). The only exception was CYC116 that did not trigger necrosis/late apoptosis yet remarkably reduced viability in euESCs at 10 μM concentration. It is possible that AURORA B-targeting CYC116 acts as an antimitotic substance and hence slows down proliferation of cells rather than triggers cellular death, yet it is not as efficient or quick as toxin MMAE with a similar mode of action.

Differently from other compounds used in the panel, doxorubicin demonstrated enhanced effect on viability in ectopic *versus* eutopic cells after 22 h as well as 22+24 h incubation at 10 μM concentration in resazurin assay (Figure 1), and after 48 h incubation in Western blot assay (Figure 2). For doxorubicin, several mechanisms of action have been reported. In cells, it accumulates into nuclei, intercalating into DNA and preventing its repair by topoisomerase-II (Thorn et al., 2011). In addition, doxorubicin can be reversibly oxidized into an unstable semiquinone metabolite, which releases ROS upon spontaneous re-formation of doxorubicin (Finn et al., 2011); the liberated ROS attack cellular components, triggering cellular death. In the context of altered redox-equilibria in ectopic *versus* eutopic endometrial cells (Kasvandik et al., 2016; Scutiero et al., 2017), enhanced efficiency of doxorubicin in ecESCs might be explained by its redox-properties.

In this way, while doxorubicin has been used in treatment of endometrial cancer (Byron et al., 2012; Chitcholtan et al., 2012), this compound might also be of remarkable interest for endometriosis studies. Unfortunately, application of anthracyclines in chemotherapy has revealed high cardiotoxicity of this class of compounds, which complicates their use in model organisms. However, several pharmacokinetic and pharmacodynamic strategies have been actively suggested for prevention of anthracycline-induced cardiotoxicity (Menna and Salvatorelli, 2017). Furthermore, specifically in the context of doxorubicin, development of novel derivatives with reduced side-effects (Shaul et al., 2013) and methods for targeted delivery (Tran et al., 2017) have been intensely pursued.

The large-scale transcriptome analysis revealed sets of genes which featured significantly higher expression in eutopic relative to ectopic ESCs or in ectopic relative to eutopic ESCs, irrespective of the

treatment conditions ($P_{adj} < 0.05$, $\log_2FC \leq -4$ or $\geq +4$; Table 4). We hypothesized that these sets might reflect variations in survival strategies of eutopic and ectopic endometrium, because it is likely that following 24 h treatment of cells with 2 μM doxorubicin, the isolated mRNA profile was characteristic of population of survivors.

Interestingly, the comparison of treated *versus* control cells yielded in excess of over ten times more significantly differentially expressed genes in case of euESCs than in ecESCs ($P_{adj} < 0.05$). Given the fact that the majority of candidate genes in control *versus* doxorubicin-treated ecESC comparison were eliminated on the basis of P_{adj} cut-off, such difference originates primarily from the large interpatient variation of gene expression in ecESC group. The latter can in turn be explained by the characteristic heterogeneity of lesions, especially taking into consideration differences in location of lesions in the three patients whose samples were used for mRNA sequencing (see Table 2).

In euESCs, among other genes, this set included genes encoding several members of matrix metalloproteinase (*MMP*) family, and a precursor for the endogenous opioid peptides, preproenkephalin (*PENK*). Another gene with significantly higher expression both in control and in doxorubicin-treated euESCs *vs* ecESCs encodes a growth factor pleiotrophin (*PTN*; $P_{adj} < 0.05$, $\log_2FC > +4$); interestingly, doxorubicin treatment further elevated the *PTN* expression in drug-treated eutopic but not in ectopic cells. Importantly, *MMPs*, *PENK* as well as *PTN* have previously been linked to endometriosis, showing significantly higher expression in eutopic endometrium of endometriosis patients relative to healthy controls or lower expression in ectopic than in eutopic tissue (Burney et al., 2007; Chung et al., 2002; Kobayashi et al., 2012), thus pointing to their possible role in initiation of peritoneal invasion. Furthermore, PTN has been reported to promote chemoresistance to doxorubicin in several cancers, including osteosarcoma and breast cancer (Huang et al., 2018; Wu et al., 2017). Therefore, we suggest that lower level of *PTN* in untreated ectopic cells is one of the factors responsible for the higher chemosensitivity of this cell type to doxorubicin – although it should be

considered that the viability of euESCs was still significantly affected by doxorubicin treatment ($P \leq 0.001$; Table 3).

The similar effect to cell viability may be mediated by *HSPA2* that was according to sequencing data more highly expressed in eutopic compared with ectopic cells. The heat shock-related 70 kDa protein 2 (HSPA2) protects cells from cytotoxic and growth inhibiting effects of doxorubicin by several mechanisms, including binding of misfolded or damaged proteins and enabling these proteins to acquire a proper folding, and by controlling the duration of the cell cycle arrest (Karlseder et al., 1996). According to qRT-PCR data, the drug-treatment enhanced the expression of *HSPA2* in ecESCs (the average FC = 4.5) suggesting the response to the toxic effect; however, as the initial expression level of *HSPA2* in untreated cells was much lower in ectopic compared with eutopic cells (the average FC = -11.8), the expression still stayed below that of the eutopic cells.

In ecESCs, the set of interest defined by the large-scale transcriptome analysis and qRT-PCR data included genes tightly connected with immune system functioning: the genes encoding interleukin 33 (*IL33*), cyclooxygenase 2 (*PTGS2*), and genes which expression is regulated by cytokines – pentraxin 3 (*PTX3*) and endothelial cell-specific molecule 1 (*ESM1*). The proteins encoded by all of the aforementioned genes have been reported to be connected with endometriosis (Cobellis et al., 2004; Fagotti et al., 2004; Kobayashi et al., 2012; Miller et al., 2017; Pelch et al., 2010), featuring correlation with endometriosis-associated inflammation and angiogenesis; inhibitors of PTGS2 have also been explored in the context of management of endometriosis-related pain (Cobellis et al., 2004). Furthermore, IL33 and PTGS2 have been shown to protect cells against doxorubicin-induced apoptosis, albeit in the context of tissues other than endometrium (Puhlmann et al., 2005; Singh et al., 2008; Yao et al., 2017). The latter observation confirms indirectly our hypothesis that the mRNA profile identified for doxorubicin-treated euESCs and ecESCs reflects the corresponding cellular survival strategies. The fact that viability of ecESCs was severely affected by doxorubicin treatment indicates that the major chemoresistance-ensuring players that contribute to survival of ectopic cells

under DNA damage and ROS-triggered stress conditions might be less efficient compared with those in eutopic tissue.

The mRNA sequencing results thus underline the interplay of factors contributing to development and sustainment of endometriosis, and necessitate application of more complex models – *e.g*., enabling presence of the epithelial cells and/or involvement of the immune system components. All in all, we believe that results of this study have pinpointed set of clues for the future research on endometriosis, both from the aspect of showing resistance of endometriotic lesions to possible therapeutic candidates, as well as providing candidate biomarkers and targets for the succeeding exploration.

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CONFLICTS OF INTEREST

The authors confirm that this article content has no conflict of interest.

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746 **TABLES**

747 Table 1. Compounds used in this study

Name	Concentrations used, μM	Major biological target	References
GSK690693	0.4, 2, 10	AKT/PKB 1, 2, 3	(Levy et al., 2009; Rhodes et al., 2008)
VX-689 (MK5108)	0.2, 1, 5	AURORA A	(Chinn et al., 2014; Shimomura et al., 2010)
CYC116	0.4, 2, 10	AURORA A, B	(Jayanthan et al., 2014; Wang et al., 2010)
ARC-775	0.4, 2, 10	CK2	(Rahnel et al., 2017)
ARC-1859	0.4, 2, 10	CK2	(Viht et al., 2015)
SGI-1776	0.4, 2, 10	PIM 1, 3	(Chen et al., 2011, 2009)
H89	0.4, 2, 10	PKAc, PKG1	(Dabizzi et al., 2003; Yoshino et al., 2003)
Y-27632	0.4, 2, 10	ROCK 1, 2	(Grewal et al., 2010; Yotova et al., 2011; Yuge et al., 2007)
HA-1077 (fasudil)	0.4, 2, 10	ROCK 2	(Tsuno et al., 2011)
sorafenib (BAY 43-9006)	0.4, 2, 10	RAF1, BRAF, KDR (VEGFR2), FLT4 (VEGFR3)	(Llobet et al., 2010; Moggio et al., 2012)
staurosporine	0.2, 1, 5	PKCα, γ, η	(Izawa et al., 2006; Watanabe et al., 2009)
bortezomib (PS-341, Velcade)	0.4, 2, 10	20S proteasome	(Kao et al., 2014)
doxorubicin (adriamycin)	0.4, 2, 10	DNA, topoisomerase-II	(Byron et al., 2012; Chitcholtan et al., 2012)
monomethyl auristatin E (MMAE)	0.04, 0.2, 1	tubulin	(Abdollahpour-Alitappeh et al., 2017; Chen et al., 2017)

748

749 Table 2. Characteristics of study participants

Patient ID	Age, years	BMI, kg/m²	Endometriosis stage	Location of lesion [a]	Study [b]
E048	29	19.8	III	Lig. sacrouterina SUP	N, V
E044	32	23.7	III	Excavatio vesicouterina SUP	N, V
E041	39	25.6	I	Fossa ovarica SUP	N, V
E205	36	22.2	I	Lig. latum SUP	N, V
E242	30	20.1	I	Lig. sacrouterina SUP	N, V
E262	40	29.8	II-III	Lig. latum SUP	N, V, V2, WB, seq
E267	25	22.1	I	Pouch of Douglas SUP	N, V, V2, WB
E270	33	21.6	III	Lig sacrouterina SUP	N, V
E278	32	20.8	I	Excavatio vesicouterina SUP	N, V, V2, WB, seq
E279	22	21.4	I	Excavatio vesicouterina SUP	N, V, V2, WB, seq
E310	24	23.5	I	Lig. sacrouterina SUP	N, V

750 [a] Abbreviations: Lig. – ligamentum, SUP – superficial. [b] Abbreviations: N – necrosis/late apoptosis assay, V – viability assay
751 with large cohort, V2 – viability assay with small cohort, WB – Western blot, seq – mRNA sequencing.

752 Table 3. Compounds inducing significant decrease in viability of euESCs and/or ecESCs after 22 h and

753 prolonged treatment (mean normalized viability ± SEM)

Compound	Concentration	Incubation time [a]	% of viability in euESCs [b]		% of viability in ecESCs [b]		Difference euESCs *vs* ecESCs [c]
GSK690693	10 μM	22 h	86 ± 2	***	94 ± 2	ns	* (euESCs)
		22 h + 24 h	78 ± 2	***	87 ± 2	***	** (euESCs)
	2 μM	22 h	89 ± 2	**	94 ± 2	ns	ns
		22 h + 24 h	85 ± 2	***	89 ± 2	***	ns
CYC116	10 μM	22 h	89 ± 2	***	103 ± 2	ns	*** (euESCs)
		22 h + 24 h	87 ± 2	***	93 ± 2	**	* (euESCs)
ARC-775	10 μM	22 h	77 ± 2	***	90 ± 2	***	*** (euESCs)
		22 h + 24 h	67 ± 2	***	70 ± 2	**	ns
	2 μM	22 h	90 ± 2	***	102 ± 2	ns	*** (euESCs)
		22 h + 24 h	92 ± 2	***	91 ± 2	***	ns
SGI-1776	10 μM	22 h	56 ± 5	***	62 ± 5	***	ns
		22 h + 24 h	48 ± 4	***	57 ± 4	***	ns
staurosporine	5 μM	22 h	15 ± 1	***	6 ± 1	***	*** (ecESCs)
		22 h + 24 h	4 ± 1	***	3 ± 1	***	ns
	1 μM	22 h	27 ± 3	***	24 ± 1	***	ns
		22 h + 24 h	16 ± 2	***	15 ± 2	***	ns
	0.2 μM	22 h	41 ± 3	***	50 ± 2	***	** (euESCs)
		22 h + 24 h	30 ± 2	***	41 ± 2	***	*** (euESCs)
bortezomib	10 μM	22 h	26 ± 2	***	40 ± 2	***	*** (euESCs)
		22 h + 24 h	5 ± 1	***	16 ± 2	***	*** (euESCs)
	2 μM	22 h	33 ± 1	***	42 ± 2	***	*** (euESCs)
		22 h + 24 h	12 ± 1	***	23 ± 2	***	*** (euESCs)
	0.4 μM	22 h	39 ± 2	***	53 ± 2	***	*** (euESCs)
		22 h + 24 h	18 ± 1	***	39 ± 2	***	*** (euESCs)
doxorubicin	10 μM	22 h	78 ± 2	***	59 ± 2	***	*** (ecESCs)
		22 h + 24 h	38 ± 2	***	22 ± 2	***	*** (ecESCs)

	2 μM	22 h	78 ± 2	***	64 ± 2	***	*** (ecESCs)
		22 h + 24 h	39 ± 2	***	37 ± 2	***	ns
	0.4 μM	22 h	85 ± 2	***	83 ± 2	***	ns
		22 h + 24 h	67 ± 3	***	68 ± 2	***	ns
MMAE	1 μM	22 h	60 ± 2	***	65 ± 1	***	* (euESCs)
		22 h + 24 h	47 ± 2	***	53 ± 2	***	* (euESCs)
	0.2 μM	22 h	60 ± 2	***	66 ± 2	***	* (euESCs)
		22 h + 24 h	49 ± 2	***	57 ± 2	***	** (euESCs)
	0.04 μM	22 h	61 ± 2	***	64 ± 1	***	ns
		22 h + 24 h	49 ± 2	***	59 ± 2	***	*** (euESCs)

[a] Incubation with inhibitors was performed for 22 h, followed by addition of growth medium for 24 h. [b] N = 11 for 22 h measurement and N = 10 for 22 h + 24 h measurement; data obtained for incubation with 10% DMSO were considered as 0% viability, and data obtained for incubation with 0.1% DMSO were considered as 100% viability. The asterisks show significance of effect difference relative to the negative control (treated with 0.1% DMSO): *** indicates $P \leq 0.001$, ** indicates $P \leq 0.01$, * indicates $P \leq 0.05$, and ns indicates $P > 0.05$. [c] The asterisks show significance of effect difference between euESCs and ecESCs; cell type with lowest viability shown in brackets; the designation for P values is the same as above.

761 Table 4. Genes featuring significantly different expression in control and toxin-treated euESCs and

762 ecESCs

Comparison [a]		**Gene names and log_2FC values** [b, c]
euESC control *vs* ecESC control	Higher expression in euESCs	*MMP12* (8.4), ***MMP10*** (8.0), ***MMP3*** (8.0), *TFAP2C* (7.4), ***RGCC*** (6.8), ***HTR2B*** (6.4), ***GRP*** (6.4), ***DIO2*** (5.7), ***MMP1*** (5.5), *RBP1* (4.9), ***CARD16*** (4.8), *LEPR* (4.8), *PRDM1* (4.7), *CTSK* (4.6), *HSPA2* (4.6), *NID1* (4.6), *GCNT4* (4.5), *PLAU* (4.5), ***PENK*** (4.5), ***PTN*** (4.4), *IFI6* (4.2), *SEMA5A* (4.1), *AREG* (4.0), *NPY1R* (4.0)
	Higher expression in ecESCs	*GIPC2* (-9.7), ***PTX3*** (-9.0), *EFEMP1* (-6.1), ***IL33*** (-6.0), *SFRP4* (-4.5), *PPP1R3C* (-4.3), ***ESM1*** (-4.0)
euESC control *vs* euESC + toxin	Higher expression in control treatment	***HTR2B*** (8.0), *CCDC107* (7.0), *ING3* (6.4), ***BARD1*** (6.2), *CARNMT1* (5.9), *KRT19* (5.8), *TUBA1A* (5.3), ***DIO2*** (5.2), *PAN3* (5.1), *DUSP1* (4.9), *PKIG* (4.9), *PBK* (4.9), *UTP18* (4.8), *CEMIP* (4.7), *SLC5A3* (4.5), *CITED2* (4.5), *CTGF* (4.4), *SASS6* (4.1), *DUSP10* (4.1), *NOP10* (4.1)
	Higher expression in toxin treatment	*HIST1H2AE* (-7.0), *INSYN2* (-6.7), *TMEFF2* (-6.0), *HIST1H2BPS2* (-5.2), *HIST1H2BK* (-5.0), *HIST2H2AA4* (-4.8), *CXCL3* (-4.7)
ecESC control *vs* ecESC + toxin	Higher expression in control treatment	*HAS2* (6.9), *MRPL14* (5.0), ***CARD16*** (4.5), *DKK1* (4.0)
euESC + toxin *vs* ecESC + toxin	Higher expression in euESCs	***GRP*** (7.3), ***MMP3*** (7.1), ***MMP10*** (6.1), ***PTN*** (5.1), ***RGCC*** (4.7), *IFITM1* (4.5), *SOX11* (4.3), ***MMP1*** (4.2), ***PENK*** (4.1)
	Higher expression in ecESCs	***ESM1*** (-6.2), *TFPI2* (-5.3), ***PTX3*** (-4.9), ***IL33*** (-4.4), ***BARD1*** (-4.1)

763 [a] Control treatment: 24 h incubation in growth medium containing 0.1% DMSO; toxin treatment: 24 h incubation in growth
764 medium containing 2 μM doxorubicin. [b] The binary logarithm of fold change of averages is shown in brackets; N = 3.
765 Negative values indicate higher expression in ectopic cells (in case of euESC *vs* ecESC comparisons) or in doxorubicin-treated
766 cells (in case of treatment comparisons). [c] Genes that are listed under more than one comparison in the table are shown in
767 bold.

FIGURES

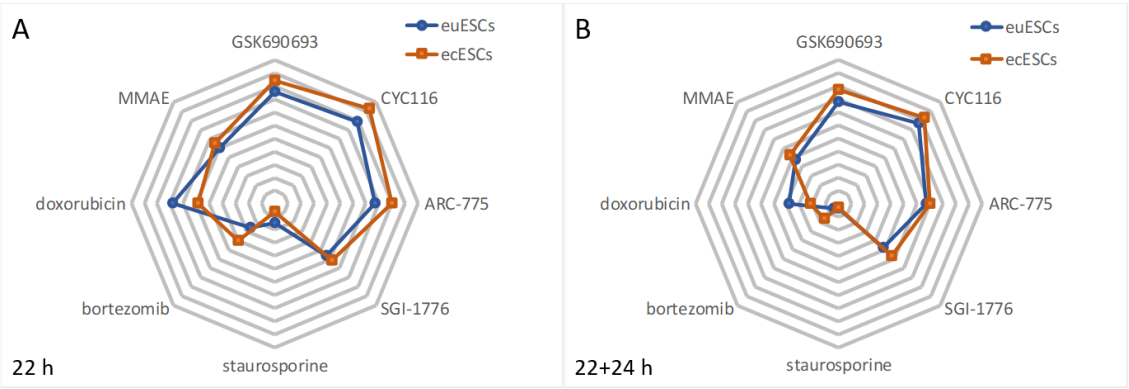

Figure 1. Viability fingerprint of euESCs *versus* ecESCs (blue and orange lines, respectively) after 22 h (A) or 22+24 h (B) of treatment with various compounds. The compounds were chosen based on Table 3. Mean data corresponding to the treatment with highest concentrations of compounds was plotted. The axis scale ranges from 0% (centre of the plot) to 110% (outer line) with grid interval of 10%.

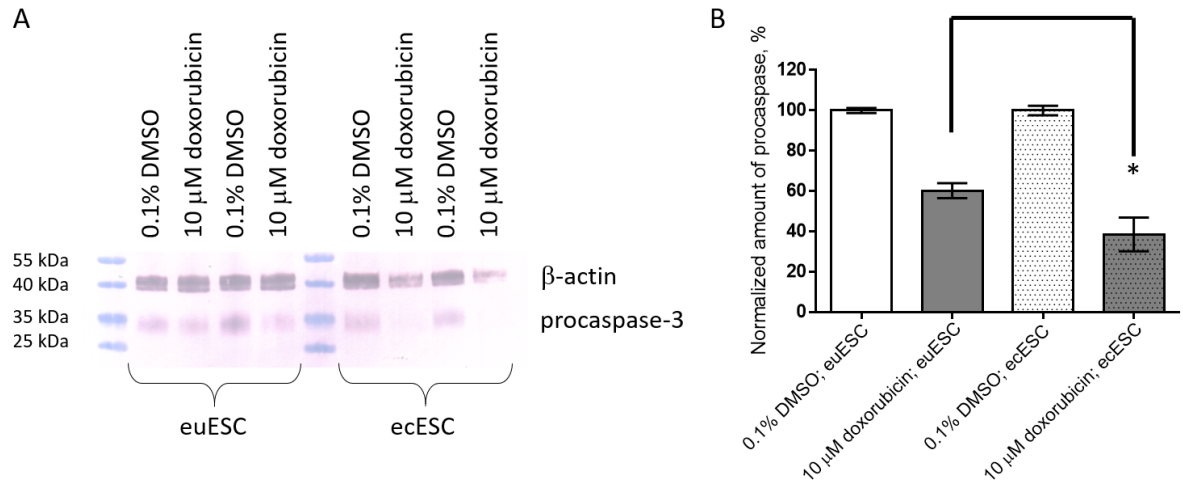

Figure 2. Effect of doxorubicin on procaspase-3 levels in euESCs and ecESCs. A, representative example of Western blot membrane with euESCs and ecESCs from one patient; different lanes represent independent incubations. B, pooled normalized Western blot data of euESCs and ecESCs from 4

779 patients (mean ± SEM). The asterisks show significance of effect difference between euESCs and

780 ecESCs: * indicates $P \leq 0.05$.

781